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Histone H2AX phosphorylation as a molecular pharmacological marker for DNA interstrand crosslink cancer chemotherapy

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ABSTRACT

The aims of this study were to investigate mechanisms of action involved in H2AX phosphorylation by DNA interstrand crosslinking (ICL) agents and determine whether γH2AX could be a suitable pharmacological marker for identifying potential ICL cellular chemosensitivity. In normal human fibroblasts, after treatment with nitrogen mustard (HN2) or cisplatin, the peak γH2AX response was detected 2–3 h after the peak of DNA ICLs measured using the comet assay, a validated method for detecting ICLs *in vitro* or in clinical samples. Detection of γH2AX foci by immunofluorescence microscopy could be routinely detected with 6–10 times lower concentrations of both drugs compared to detection of ICLs using the comet assay. A major pathway for repairing DNA ICLs is the initial unhooking of the ICL by the ERCC1-XPF endonuclease followed by homologous recombination. HN2 or cisplatin-induced γH2AX foci persisted significantly longer in both, ERCC1 or XRCC3 (homologous recombination) defective Chinese hamster cells that are highly sensitive to cell killing by ICL agents compared to wild type or ionising radiation sensitive XRCC5 cells. An advantage of using γH2AX immunofluorescence over the comet assay is that it appears to detect ICL chemosensitivity in both ERCC1 and HR defective cells. With HN2 and cisplatin, γH2AX foci also persisted in chemosensitive human ovarian cancer cells (A2780) compared to chemoresistant (A2780cisR) cells. These results show that γH2AX can act as a highly sensitive and general marker of DNA damage induced by HN2 or cisplatin and shows promise for predicting potential cellular chemosensitivity to ICL agents.

1. Introduction

There is currently much interest in the role of DNA repair proteins as predictive, prognostic and therapeutic targets in a wide variety of cancer therapies [1–3]. Chemotherapy based on interstrand crosslinking (ICL) agents such as cisplatin, carboplatin, mitomycin C (MMC) or nitrogen mustard derivatives is used extensively in the clinic and novel ICL cancer chemotherapeutics continue to be developed [4–6]. A major determinant of resistance to cell killing by ICL agents in mammalian cells is the ability to repair DNA ICLs. In dividing cells, the major DNA repair pathway for ICLs requires the ERCC1-XPF endonuclease to initiate dual incisions on either side of one arm of the crosslink, releasing the covalent linkage between the two DNA strands.

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The resected gap produced by this unhooking reaction then provides a suitable substrate for homologous recombination (HR) [7–9]. ICLs can also obstruct DNA replication fork progression in dividing cells resulting in the formation of DNA double strand breaks (DSBs). Unlike the direct DSBs induced by ionising radiation which are predominately repaired by non-homologous end joining, these ICL and replication associated DSBs require HR for their repair [8,9]. Since cells defective in ERCC1, XPF or components of HR are sensitive to cell killing by ICL chemotherapeutic agents it would appear that both unrepaired ICLs and their associated DSBs constitute the major cytotoxic lesions induced by ICL agents [9–12].

For detecting ICLs *in vitro* or in clinical samples a modified comet assay has been validated. Increased repair of DNA ICLs as measured using the comet assay contributes to clinical acquired resistance to melphalan in multiple myeloma and in ovarian tumour cells following platinum therapy [13,14]. In terms of DNA repair, it appears that the modified comet assay measures the initial rate of ICL unhooking by ERCC1-XPF since cells with defective ERCC1 or XPF show decreased unhooking and increased cellular sensitivity [9–11]. Furthermore, expression of ERCC1 can be a predictive and prognostic marker for resistance, normal tissue tolerance and patient outcome in platinum based chemotherapy [15]. However, neither the comet assay nor ERCC1 expression is able to identify ICL chemosensitive cells arising as a result of a HR repair defect. Since there is substantial evidence that defective HR contributes significantly to tumour development in a wide variety of cancers [16,17] it is important that assays relating to ICL induced DNA damage can also identify HR chemosensitivity or resistance.

In response to ionising radiation, the histone protein H2AX is phosphorylated to form γH2AX [18]. Thousands of γH2AX molecules accumulate at sites of DNA double strand breaks (DSBs) to form discrete nuclear foci, which can be visualised by immunocytochemistry [19]. Persistence of γH2AX foci is thought to be a clinical indicator of tumor cell sensitivity after radiotherapy or treatment with radiomimetic chemotherapeutics [20–22] and has been proposed as an effective target for improving radiation therapy [23]. The role of γH2AX is to recruit DNA repair and cell cycle checkpoint proteins required for the efficient processing of DNA double strand breaks. DNA damage response proteins that colocalize with γH2AX foci include the MRE11/RAD50/NBS1 (MRN) complex, BRCA1, RAD51, MDC1 and FANCD2, all major components of HR DNA repair [24,25]. DNA interstrand crosslinking induced by cisplatin, MMC and psoralen plus UV have also been reported to induce γH2AX foci [7,26–28]. This raises the possibility that persistence of γH2AX foci after treatment with ICL agents could reflect a defective HR system either as a direct inability to repair ICLs or replication associated DNA DSBs.

The role of H2AX phosphorylation in sensing ICL DNA damage or repair remains poorly understood. Based on the detection of γH2AX foci, there are conflicting reports regarding the role of ERCC1 and XPF for ICL-associated DSB induction and repair [7,27,28]. It is also unclear whether the γH2AX response is dependent on ICL-associated DSBs or whether ICLs are also important. Consequently, one aim of this study was to determine the types of DNA damage and repair pathways that contribute to the γH2AX response induced by ICL agents. A second aim was to establish if γH2AX could be a potential marker of ICL chemotherapeutic cellular sensitivity or resistance.

To assess the suitability of γH2AX as a marker of DNA damage associated with ICL agents, the γH2AX response was initially compared to interstrand crosslinking as determined using the comet assay in normal human fibroblasts after treatment with the crosslinking agents mechlorethamine (HN2) or cisplatin. In addition to ICLs, HN2 has been shown to rapidly induce ICL-associated DNA DSBs in dividing human and Chinese hamster ovary (CHO) cells while no frank DNA DSBs have been detected after treatment with cisplatin [9–11,26]. To assess the types of DNA damage or DNA repair pathways that contribute towards the formation of γH2AX foci and to establish whether it is feasible to use γH2AX as an assay for predicting chemosensitivity to ICL agents, the γH2AX response was also investigated in a panel of CHO cell lines with specific DNA repair defects. Finally, the potential of the γH2AX assay for identifying chemosensitivity or chemoresistance in tumour cells was established using a pair of cisplatin sensitive (A2780) and cisplatin resistant (A2780cisR) human ovarian cancer cell lines as a model of required resistance involving increased ICL repair [29].

2. Materials and methods

2.1. Cell culture

The normal human fibroblast cell line AGO1522B was grown in alpha minimum essential medium (α-MEM, Sigma, Poole, UK) containing 20% foetal calf serum (FCS, Helena Biosciences Europe, Sunderland, UK), 2 mM L-glutamine, 1% 100× non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (all Sigma).

CHO cell lines were grown in F-12 Ham HEPES medium supplemented with 2 mM L-glutamine and 10% FCS. AA8 and UV96 were obtained from Dr. M. Stefanini (Istituto di Genetica Biochimica et Evoluzionistics, Pavia, Italy). CHO-K1, irs1SF, and xrs5 cell lines were provided by Prof J. Thacker (MRC Radiation and Genome Stability Unit, Harwell, UK). UV96 cells are defective in the NER repair factor ERCC1, irs1SF cells are defective in the RAD51 paralogue XRCC3 involved in homologous recombination (HR) and xrs5 cells are defective in XRCC5 (Ku80) involved in non-homologous end joining (NHEJ) [9,10].

The human ovarian cancer cell line A2780 was established from tumour tissue from an untreated patient [29]. Growing A2780 cells in cisplatin and selecting for cisplatin resistance generated the stably resistant A2780cisR cell line. Both cell lines were grown in RPMI 1640 (Autogenbioclear, Wiltshire, UK) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Cell lines were maintained at 37 °C in dry incubators with 5% CO_2, harvested with trypsin EDTA 1x solution (Autogenbioclear) and shown to be free of mycoplasma.

2.2. ICL agents

Analytical grade mechlorethamine (nitrogen mustard, HN2) was obtained from Sigma. Cisplatin was obtained as a clinical 3.3 mM aqueous solution (David Bull Laboratories, Warwick,

UK). All drug dilutions were prepared in the relevant serum free medium (SFM) and all drug treatments were carried out for 1 h at 37 °C.

2.3. Cytotoxicity assay

Growth inhibition was determined using the sulforhodamine B (SRB) assay [30]. For AGO human fibroblasts 1×10^4 cells were seeded into each well of 24 well flat-bottomed microtiter plates in 1 ml of αMEM medium. For A2780/A2780cisR cells 0.75×10^4 cells were seeded into 96-well flat bottom microtiter plates in 200 μl RPMI 1640 medium. Cells were left overnight, medium aspirated and cells drug treated as required. After 1 h, drug solution was aspirated and replaced with complete medium and plates incubated for a further 96 h in 37 °C incubators. The absorbance at 540 nm was read on a standard plate reader and the growth inhibition in drug treated cells expressed as a percentage of untreated control cells. Cytotoxicity is routinely reported as the concentration of drug required for 50% growth inhibition (IC50). Cytotoxicity data for CHO cells after exposure to HN2 and cisplatin using the SRB assay have been published [9,10].

2.4. DNA interstrand crosslinking measured using a modified single cell gel electrophoresis (comet) assay

DNA interstrand crosslinking was analysed using a modified comet assay. Typically, 5×10^4 cells per well were incubated over night in 6 well plates, the medium was then aspirated and growing cells treated with 3–5 μM HN2 or 30–100 μM cisplatin in serum free medium for 1 h at 37 °C. For each cell line, the optimum drug concentration to use was the dose that achieved approximately 60–70% decrease in tail moment at the peak of crosslinking. Cells were then incubated at 37 °C in full medium as required, before being trypsinised and stored at −80°C as 1 ml aliquots in complete medium containing 10% DMSO. Prior to analysis, cells were irradiated on ice with 12 Gy of X-rays and then processed according to standard comet procedures for measuring ICLs as detailed elsewhere [9–11].

The degree of DNA interstrand cross-linking present in a drug treated sample was determined by comparing the tail moment of the irradiated drug treated samples with irradiated untreated samples and unirradiated untreated samples. The level of interstrand cross-linking is proportional to the decrease in the tail moment in the irradiated drug treated sample compared to the irradiated untreated control. The decrease in tail moment is calculated by the following formula:

$$\%\text{decrease in tail moment (DTM)} = \left\{1 - \left(\frac{\text{TMdi} - \text{TMcu}}{\text{TMci} - \text{TMcu}}\right)\right\} \times 100$$

where TMdi = mean tail moment of drug treated, irradiated sample, TMci = mean tail moment of irradiated control sample, TMcu = mean tail moment of unirradiated control sample.

2.5. γH2AX immunocytochemistry

For all cell lines, 2×10^4 cells per well were seeded in 8 well LAB-TEK® II chamber slides™ (Nalge Nunc International, Hereford, UK) and incubated overnight at 37 °C. Separate slides contained cell lines in individual chambers that were then treated with 0.5–1 μM HN2 or 5–10 μM cisplatin in serum free medium for 1 h at 37 °C. Cells were then incubated at 37 °C in full medium and at the required time point, all wells on one slide were fixed by adding 0.5 ml per well of ice cold 50% methanol: 50% acetone for 8 min at 4 °C. Cells were washed with cold PBS 3× and permeabilized with 0.5 ml per well of 0.5% Triton X-100 (Sigma) in PBS for 5 min at room temperature. Cells were blocked with blocking buffer (0.5 ml per well consisting of 0.1% Triton X-100, 0.2% skimmed dry milk in PBS) at 4 °C overnight in a humidified box.

Blocked cells were incubated with mouse monoclonal anti-γH2AX (Upstate, Hampshire, UK) at 1:10,000 dilutions in blocking buffer (100 μl per well) for 1 h at room temperature. After washing 3× with wash buffer (0.1% Triton X-100 in PBS), cells were then incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes, Paisley, UK) at a dilution of 1:2000 in blocking buffer for 1 h at 4 °C in the dark. Cells were washed with PBS and counterstained with 0.5 ml per well of propidium iodide (2 μg/ml) for 3 min. Stained cells were washed with cold PBS 3×.

Slides were mounted with Vectashield® (Vector Laboratories, Peterborough, UK), a cover slip added (25 × 80 mm, No. 1 Thickness, VWR International, Leicestershire, UK) and edges sealed with clear nail polish. Images were visualised with a Zeiss LSM510 fluorescence microscope (100× oil immersion objective), equipped with a cooled CCD camera and 2 detector channels with 488 nm Argon ion and 543 nm HeNe excitation lasers. Foci were counted in 200 cells per time point and results are expressed as mean number of foci per cell (±mean standard error) from three independent experiments for AGO human fibroblasts and CHO cells. With A2780 and A2780cisR cells, results are expressed as the % of γH2AX positive cells (±mean standard error) from three independent experiments. γH2AX positive cells are defined as having more than 10 foci per cell since untreated A2780 or A278cisR cells typically exhibited 3–7 foci per cell.

3. Results

3.1. Time dependence of ICL and γH2AX formation in normal human fibroblasts after treatment with HN2 or cisplatin

Growth inhibition assays were performed with AGO human fibroblasts to determine the concentration of drug required to inhibit growth by 50%. IC50 values for HN2 and cisplatin were estimated as 24 μM and 44 μM, respectively (Fig. 1).

Fig. 2A and B shows the time course for the induction and unhooking of ICLs following a 1 h treatment with 5 μM HN2 or 30 μM cisplatin in AGO fibroblasts. A 65% decrease in tail moment immediately after the 1 h treatment with HN2 (Fig. 2A), represents the peak of crosslinking. After 48 h, a decrease in tail moment of 25% indicated that 61.5% of HN2 induced crosslinks were unhooked over this time. With cisplatin, the peak of crosslinking was detected 12 h post-drug treatment when a 61.5% decrease in tail moment was evident. After 48 h, there was a 33% decrease in tail moment

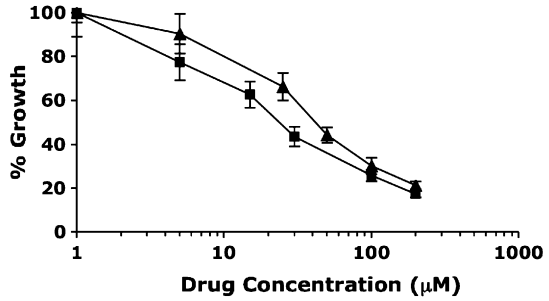

Fig. 1 – Cytotoxicity of AGO human fibroblasts after a 1 h HN2 (■) or cisplatin (▲) drug exposure and determined using the SRB assay. Results are the mean of at least three independent experiments and error bars show the standard error of the mean.

indicating that, from the peak at 12 h, 54.6% of cisplatin ICLs had been unhooked.

To determine the kinetics of γH2AX foci formation after treatment with the crosslinking agents, exponentially growing AGO fibroblasts were treated for 1 h with 0.5 μM HN2 or 5 μM cisplatin. Table 1 outlines the overall distribution of the number of γH2AX foci counted in 600 cells (200 each from 3 independent experiments) for HN2 and cisplatin treated AGO fibroblasts, respectively. In control, untreated cells no more than 5 foci per cell were detected (average 1.1 ± 0.1). Although γH2AX levels were quite variable between cells in the same culture, a significant increase in the incidence of γH2AX foci was detected after ICL drug treatment. Fig. 2C and D shows the average number of foci per cell (±mean standard error) taken from three independent experiments. After treatment with 0.5 μM HN2 for 1 h, the peak of γH2AX induction was detected after 3 h when an average of 19.5 foci per cell was counted (Fig. 2C). The number of foci gradually declined until after 48 h post drug treatment when there was an average of 2.7 foci per cell. Fig. 2D shows that the peak of γH2AX foci formation was detected 14 h post drug treatment with 5 μM cisplatin when an average of 25.3 foci per cell was counted. From this time point, the number of foci gradually decreased until after 48 h only 3.3 foci per cell were evident. Representative examples of γH2AX staining are shown in Fig. 3.

3.2. *γH2AX foci persist in ICL agent sensitive UV96 and irs1SF CHO cells after treatment with HN2 or cisplatin*

γH2AX foci formation was investigated in a panel of CHO cells with specific DNA repair defects and different levels of cytotoxicity to HN2 and cisplatin. Compared to wild type AA8/CHO-K1 or radiosensitive xrs5 (Ku80, NHEJ defective) cells, UV96 (ERCC1, NER defective) and irs1SF (XRCC3, HR

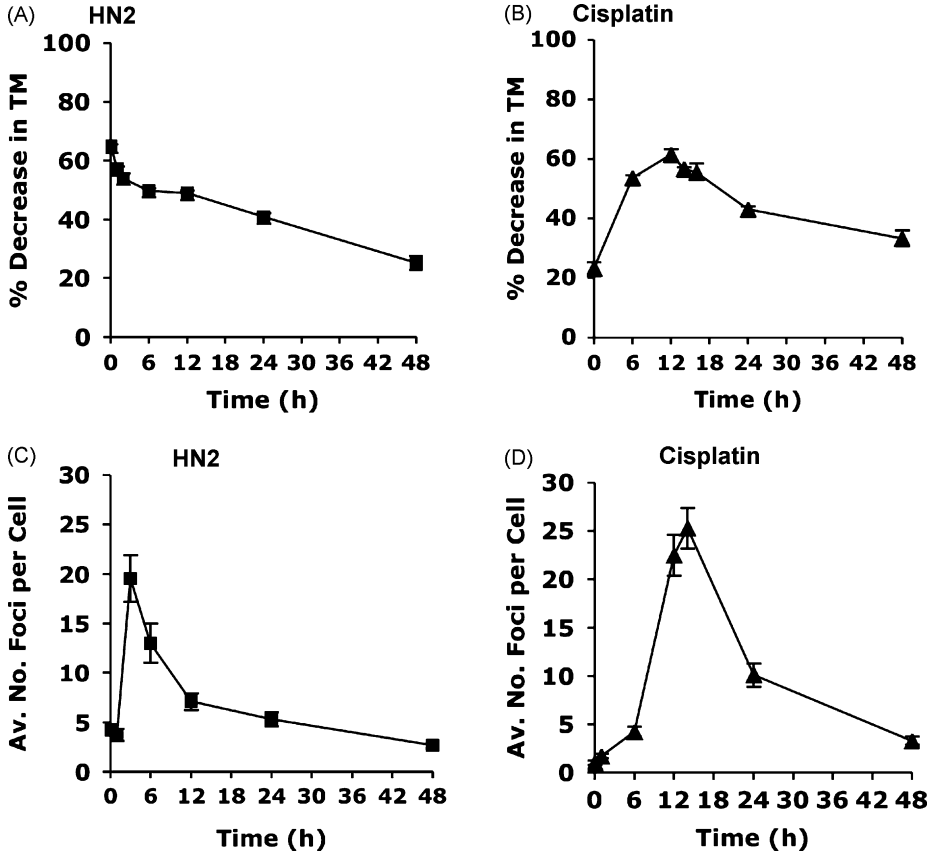

Fig. 2 – Induction and unhooking of ICLs in AGO human fibroblasts determined as a % decrease in tail moment (TM) using the modified comet assay after a 1 h exposure to (A) 5 μM HN2 and (B) 30 μM cisplatin. Formation and disappearance of γH2AX foci in AGO human fibroblasts after a 1 h exposure to (C) 0.5 μM HN2 and (D) 5 μM cisplatin. All results are the mean of three independent experiments and error bars show the standard error of the mean.

Table 1 – Distribution of the number of γH2AX foci in AGO human fibroblasts after a 1 h treatment with 0.5 μM HN2 or 5 μM cisplatin

Number of γH2AX foci	Hours after 0.5 μM HN2 treatment							
	Untreated	0	1	3	6	12	24	48
0	338	195	177	82	126	119	139	166
1–5	262	306	267	225	199	247	279	290
6-10	0	52	72	54	70	72	59	81
11–50	0	46	82	144	162	153	114	63
>50	0	1	2	95	43	9	9	0
Number of γH2AX foci	Hours after 5 μM cisplatin treatment							
	Untreated	0	3	6	12	14	24	48
0	338	404	286	142	55	2	79	122
1–5	262	163	237	218	146	97	250	284
6–10	0	20	46	67	68	64	91	80
11–50	0	13	30	162	255	343	161	108
>50	0	0	1	11	76	94	19	6

Results are for 600 cells (200 each from three independent experiments).

defective) cells are highly sensitive to the cytotoxic effects of HN2 and cisplatin [9–11]. Fig. 4A and B shows that the time dependence of γH2AX formation in CHO cells follows a similar pattern to that exhibited by AGO human fibroblasts following a 1 h drug treatment with 1 μM HN2 or 10 μM cisplatin, respectively. With HN2, 2 h post drug treatment, the average number of foci per cell was 21.6, 24.6, 25.7, 22.4 and 21.2 for AA8, CHO-K1, UV96, irs1SF and xrs5 cells, respectively. The kinetics of γH2AX foci disappearance between the cell lines was very different. Wild type AA8, CHO-K1 and xrs5 cells were almost free of foci after 24 h when levels had returned to those seen in control cells (less than 1 focus per cell). In the highly sensitive cell lines UV96 and irs1SF, 19.5 and 13 foci per cell were seen after 24 h. Significant numbers, 5.4 and 5.3 foci per cell respectively, still remained after 48 h (Fig. 4A).

With cisplatin, 15 h post drug incubation, the average number of foci per cell differed slightly between the cell lines with the lowest value of 52.3 seen in wild type AA8 cells while 73.0, 80.4, 77.0 and 88.1 foci per cell were counted for CHO-K1, irs1SF, xrs5 and UV96 cells, respectively (Fig. 4B). The persistence of γH2AX foci in the ICL agent sensitive cell lines was even more marked with cisplatin than HN2. After 48 h, the number of foci per cell in AA8, CHO-K1 and xrs5 cells was 11.6, 11.9 and 10.5 while in irs1SF and UV96 cells 50.5 and 64.5 remained. Collectively, these results support the idea that the persistence of γH2AX foci correlates with increased cytotoxicity after treatment with ICL agents. To ensure that the persistence of γH2AX foci is not inherent to irs1SF or UV96 cells the appearance and disappearance of γH2AX foci was investigated in response to 1 Gy of ionizing radiation (Fig. 4C). In these experiments only the radiosensitive xrs5 cells showed a significantly prolonged expression of γH2AX foci.

3.3. *γH2AX foci persist in chemosensitive A2780 human ovarian cancer cells compared to chemoresistant A2780cisR cells after treatment with HN2 or cisplatin*

Having established that persistence of γH2AX foci is a potential indicator of chemosensitivity to ICL agents in CHO cells the induction and loss of γH2AX was investigated in the

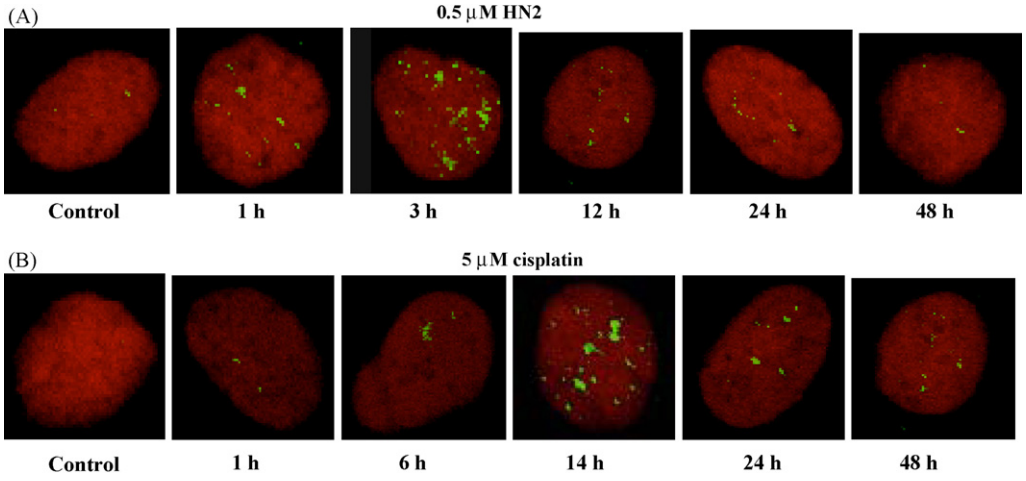

Fig. 3 – Immunocytochemistry staining for H2AX phosphorylation (green) in human AGO fibroblasts after a 1 h treatment with (A) 0.5 μM HN2 or (B) 5 μM cisplatin compared to control cells. DNA counterstaining is with propidium iodide (red).

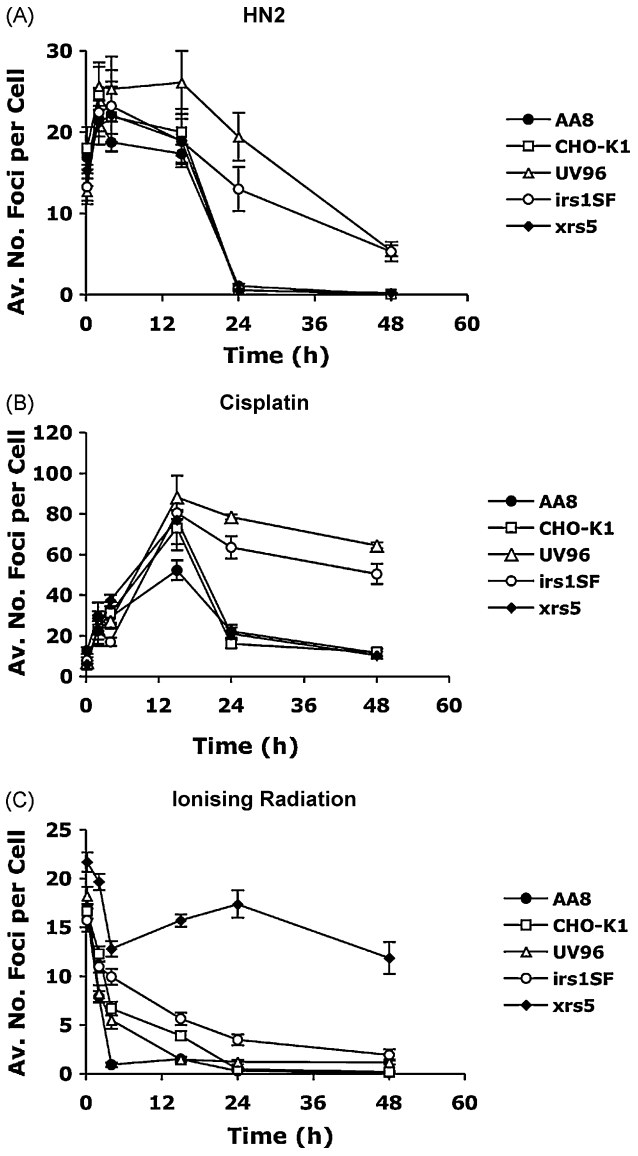

Fig. 4 – Induction and disappearance of γH2AX foci in CHO cells after exposure to: (A) 1 μM HN2, (B) 10 μM cisplatin and (C) 1 Gy of ionizing radiation. Results show the average number of foci and standard error of the mean counted in 30–100 cells for each time point for each cell line.

human ovarian cancer cell line A2780 and the cisplatin resistant cell line A2780cisR. Cytotoxicity assays established that A2780cisR cells are also cross-resistant to the ICL agent HN2 (Fig. 5A and B). IC50 values for A2780 and A2780cisR cells after 1 h drug incubations were 4.5 and 29.9 μM, respectively after treatment with cisplatin and 0.3 and 1.7 μM after treatment with HN2.

After treatment with 100 μM cisplatin for 1 h, the peak of crosslinking in A2780 and A2780cisR cells occurs 9 h post-drug incubation as detected using the modified comet assay and there is no significant difference in the overall extent of crosslinking between the two cell lines, a 62% decrease in tail moment was recorded for each cell line (Fig. 6A). Cisplatin ICLs are unhooked much more rapidly in A2780cisR cells, after 48 h an 11.5% decrease in tail moment indicates that 81.5 % of the ICLs had been unhooked. In A2780 cells a 46.2% decrease in tail moment indicate that only 25.5% of cisplatin induced ICLs had been unhooked 48 h post drug incubation. After treatment with 3 μM HN2, as expected, the peak of crosslinking occurs immediately after drug treatment when there was an 81.5% decrease in tail moment for A2780 cells and 70.2% decrease in A2780cisR cells (Fig. 6B). Again, HN2 induced ICLs are unhooked more rapidly in A2780cisR cells. After 24 h, decrease in tail moments of 4.9% and 33% indicate that 92% of ICLs had been unhooked in A2780cisR cells compared to only 46% in the chemosensitive A2780 cells. These results show that increased repair of ICLs is associated with drug resistance in A2780cisR cells.

If the level of γH2AX foci formation was to reflect chemoresistance in A2780cisR cells it would be expected that the foci would clear more rapidly than in A2780 cells. Fig. 5C and D shows the time dependant formation and clearance of γH2AX foci in these cells after a 1 h treatment with 10 μM cisplatin or 1 μM HN2. In untreated controls, approximately 3–7% of cells scored positive for γH2AX foci. In A2780 or A2780cisR cells treated with cisplatin or HN2 the number of foci per cell generally exceeded those presented for AGO or CHO cells. Since too many foci are difficult to count accurately, the % of foci positive cells (>10) was reported in Fig. 5C and D. Following the 1 h cisplatin treatment, the % of foci positive cells increased up to 16 h after drug exposure when the levels of positive cells was slightly lower in A2780cisR cells (62%, compared to 72% in A2780 cells). The clearance of cisplatin induced γH2AX foci was more rapid in A2780cisR cells, after 24 h 35% were positive and after 48 h only 12% compared to 68% and 28% in A2780 cells (Fig. 6C).

With HN2, the highest level of cells scoring positive for γH2AX occurred between 0–1 h after the 1 h, 1 μM drug exposure for both cell lines. Although there was a small decrease at subsequent time points up to 16 h there was no significant difference between the two cell lines. Between 16 and 48 h post HN2 treatments, A2780cisR cells exhibited a more rapid loss of foci with only 23% and 10 % of cells remaining positive compared to 55% and 26% in the drug sensitive A2780 cells after 24 and 48 h, respectively.

4. Discussion

γH2AX is a predictive, prognostic biomarker and a potential therapeutic target in cancer therapy in relation to DNA DSBs induced during radiotherapy [3,20–23]. γH2AX is also induced by DNA ICL agents and associates with a number of homologous recombination factors that are required for ICL repair [7,24–28]. Consequently, γH2AX may also be a potential and useful biomarker for predicting chemosensitivity during cancer therapy involving ICL drugs.

In AGO human fibroblasts treated with very low pharmacologically relevant concentrations of HN2 or cisplatin, the peak γH2AX formation was detected 2–3 h after the peak of ICLs as detected using the comet assay. These results indicate that ICL agents stimulate γH2AX signalling but that it does not coincide with the peak of ICL detection. H2AX phosphorylation

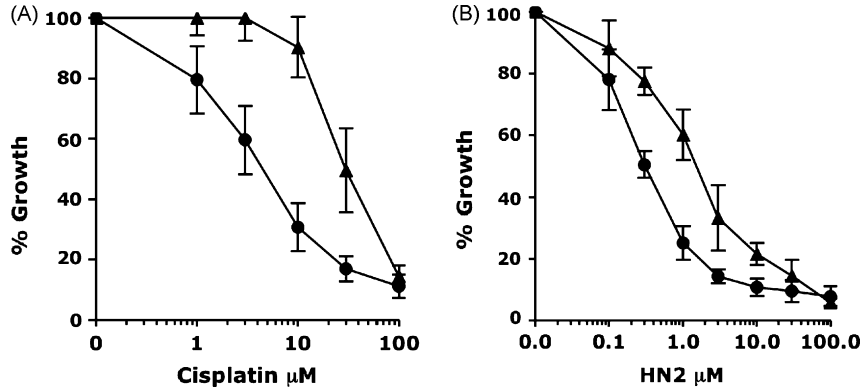

Fig. 5 – Cytotoxicity of A2780 (●) and A2780cisR (▲) cells after a 1 h exposure to (A) cisplatin or (B) HN2 and determined using the SRB assay. Results are the mean of at least three independent experiments and error bars show the standard error of the mean.

in response to ICL agents is considered to reflect the presence of ICL-associated DSBs. While DNA DSBs are readily detected immediately after exposure to HN2, no DNA DSBs have been detected in mammalian cells treated with much higher concentrations of cisplatin [9–11,26]. Consequently, it appears that γH2AX foci may be induced independently of readily detectable ICL-associated DSBs. In terms of sensitivity, γH2AX foci formation appears to be 6–10 times more sensitive than the comet assay for detecting DNA damage associated with DNA ICL agents.

To further investigate the role of ICLs or ICL-associated DNA DSBs in H2AX phosphorylation, formation of γH2AX was monitored in a panel of CHO cells with defined DNA repair defects. γH2AX foci persisted significantly longer in the ICL agent sensitive UV96 (defective in ERCC1) or irs1SF (defective in the RAD51 paralogue XRCC3 required for HR) cells after

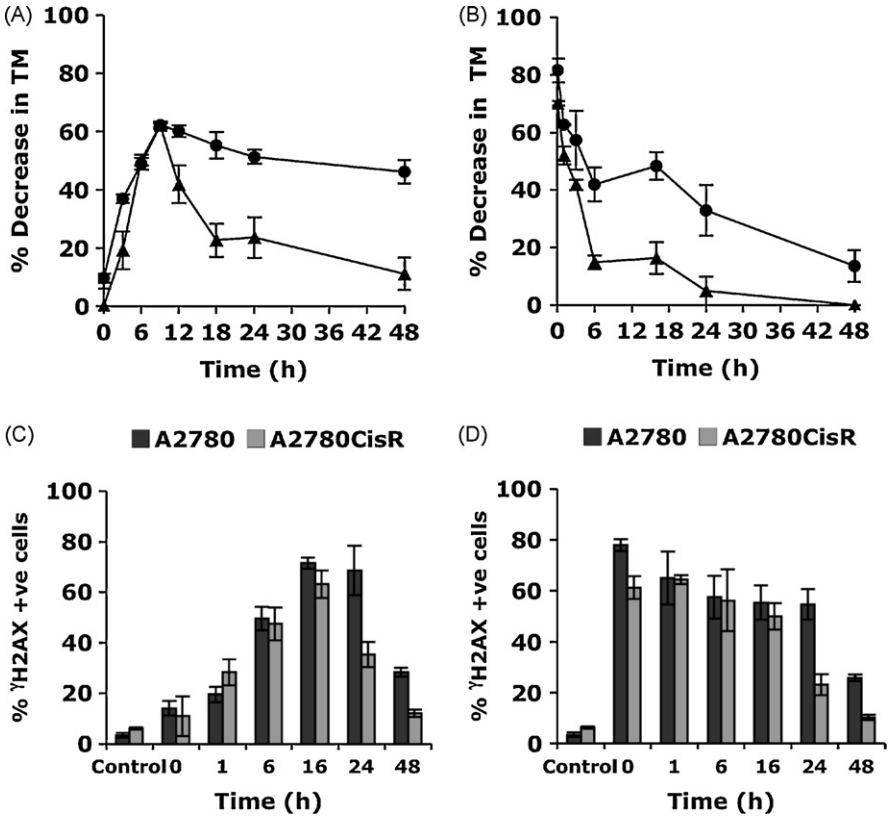

Fig. 6 – Induction and unhooking of ICLs in A2780 (●) and A2780cisR (▲) cells determined as a % decrease in tail moment (TM) using the modified comet assay after a 1 h exposure to (A) 100 μM cisplatin and (B) 3 μM HN2. The number of γH2AX foci positive cells in A2780 and A2780cisR cells after a 1 h exposure to (C) 10 μM cisplatin and (D) 1 μM HN2. All results are the mean of at least three independent experiments and error bars show the standard error of the mean.

treatment with both HN2 and cisplatin compared to wild type or NHEJ defective cells. In terms of DNA damage and repair, UV96 cells are defective in the initial incision or unhooking of ICLs induced by HN2 as detected using the comet assay but show wild type kinetics for repair of HN2-associated DNA DSBs [9]. In comparison, irs1SF cells show wild type kinetics for the unhooking of ICLs induced by HN2 but are defective in the repair of HN2-associated DNA DSBs [9]. Due to the different DNA repair defects of these cells and since no ICL associated DSBs are detected in CHO cells after treatment with cisplatin it is not possible to correlate the induction or persistence of γH2AX foci solely with the persistence of ICL-associated DNA DSBs. The data presented in this manuscript demonstrates that persistence of γH2AX may result from either the defective unhooking of ICLs or defective repair of ICL-associated DSBs. This implies that in response to ICL agents H2AX phosphorylation acts as a more general marker of DNA damage rather than being specific for DSBs. In support of γH2AX being induced in the absence of DSBs is the finding that H2AX phosphorylation within the G1 phase of the cell cycle after UV irradiation depends on nucleotide excision repair and not on DNA double strand breaks [31]. With cisplatin, the persistence of foci in irs1SF cells may be explained by the requirement of XRCC3 for the downstream processing of unhooked ICLs via homologous recombination. Defective XRCC3 would therefore contribute to incomplete ICL repair.

Prolonged expression of γH2AX in ERCC1 defective UV96 cells after treatment with HN2 or cisplatin is consistent with the persistence of γH2AX foci in mouse embryonic *ERCC1*$^{-/-}$ stem cells in response to MMC [27]. In contrast, XPF defective human fibroblasts or ERCC1 defective CHO cells are deficient in the formation of γH2AX foci after psoralen plus UVA exposure [7,28]. Since these cells are sensitive to ICL agents and since XPF and ERCC1 form a structure-specific heterodimer endonuclease, the difference in γH2AX response to different types of ICL agents highlights complexities involved in ICL processing which remain to be resolved. It has been suggested that these differences may arise from the differential processing of monoadducts produced by psoralens [27].

The γH2AX response was investigated in A2780 and A2780cisR cells to determine as proof of principle whether the persistence of γH2AX foci could distinguish between cisplatin sensitive and resistant tumour cell lines. Mechanisms of cisplatin drug resistance in A2780cisR cells that have been identified, include a mismatch DNA repair deficiency [32], elevated levels of glutathione [33] and enhanced repair of ICLs possibly through increased levels of ERCC1 [14,15]. Cytotoxic assays demonstrated that A2780cisR cells were cross-resistant to both cisplatin and HN2. The finding that increased unhooking of ICLs could contribute to the resistance of A2780cisR cells seen in cytotoxic assays was confirmed using the modified comet assay. Both cisplatin and HN2 ICLs were unhooked significantly faster in the A2780cisR cells compared to chemosensitive A2780 cells. After 24–48 h post drug incubation with either cisplatin or HN2, the number of γH2AX foci positive cells was much lower in the A2780cisR cells compared to the drug sensitive A2780 cells. These results indicate that the persistence of γH2AX following treatment with DNA ICL drugs may be able to predict cellular chemosensitivity or resistance to these agents.

These findings show that γH2AX can act as a general marker for detecting DNA damage induced by the ICL agents HN2 or cisplatin and shows potential for detecting cellular ICL chemosensitivity. One advantage of this approach over more conventional approaches, such as the comet assay, for detecting ICLs is that there appears to be a substantial increase in sensitivity for following DNA damage at the molecular level. A second advantage is that while the comet assay appears to detect the induction and initial incision or unhooking of ICLs it cannot detect defects in the downstream processing of unhooked ICLs or repair of ICL-associated DNA DSBs by homologous recombination [9–11]. γH2AX foci persisted in cells defective in both the initial unhooking of ICLs (ERCC1 defective) and homologous recombination repair (XRCC3 defective). As a potential assay for predicting chemosensitivity to ICL agents in clinical samples both these repair defects should be identified.

Clearly, further investigations are required to translate these preclinical, *in vitro* findings into clinical studies. The background level of γH2AX can vary widely between cells in different tissues, cultures or different cancer cell lines [3,21]. The extent of the γH2AX response can also vary between different cells and different ICL agents [7,27,28]. While these need to be considered, it is clear that this assay is not a quantitative method for detecting ICLs or their associated DSBs. Instead, analysis of γH2AX may reflect the level of DNA repair processes or concentration of repair proteins in the vicinity of DNA damage [26,34]. To this extent, analysis of H2AX acts as a surrogate marker of DNA damage induced by ICL agents. Further validation of the assay is also required in suitable clinical samples. To this end, we have evidence that γH2AX is a useful pharmacodynamic endpoint for detecting DNA damage (below the threshold of the comet assay) in peripheral blood lymphocytes and tumour cells from a phase 1 clinical trial involving the intravenous infusion of a novel ICL agent (manuscript in preparation). In conclusion, the results presented in this manuscript indicate that γH2AX shows potential as a sensitive and general biomarker for DNA damage associated with ICL cancer chemotherapeutics.

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REFERENCES

[1] Madhusudan S, Middleton MR. The emerging role of DNA repair proteins as predictive, prognostic and therapeutic targets in cancer. Cancer Treat Rev 2005;31(8):603–17.
[2] Ding J, Miao Z-H, Meng L-H, Geng M-Y. Emerging cancer therapeutic opportunities target DNA-repair systems. Trends Pharmacol Sci 2006;27(6):338–44.
[3] Sedelnikova OA, Bonner WM. γH2AX in cancer cells: a potential biomarker for cancer diagnostics, prediction and recurrence. Cell Cycle 2006;5(24):2909–13.
[4] Hartley JA. Alkylating agents. In: Souhami RL, Tannock I, Hohenberger P, Horoit JC, editors. Oxford textbook of

oncology. 2nd ed., Oxford University Press; 2001. p. 639–54.
[5] Kelland L. The resurgence of platinum based cancer therapy. Nat Rev Cancer 2007;7(8):573–84.
[6] Martin C, Ellis T, McGurk CJ, Jenkins TC, Hartley JA, Waring MJ, et al. Sequence-selective interaction of the minor-groove interstrand cross-linking agent SJG-136 with naked and cellular DNA: footprinting and enzyme inhibition studies. Biochemistry 2005;44(11):4135–47.
[7] Rothfuss A, Grompe M. Repair kinetics of genomic interstrand DNA cross-links: evidence for DNA double-strand break-dependent activation of the Fanconi anemia/BRCA pathway. Mol Cell Biol 2004;24(1):123–34.
[8] Dronkert ML, Kanaar R. Repair of DNA interstrand cross-links. Mutat Res 2001;486(4):217–47.
[9] De Silva IU, McHugh PJ, Clingen PH, Hartley JA. Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. Mol Cell Biol 2000;20(21):7980–90.
[10] De Silva IU, McHugh PJ, Clingen PH, Hartley JA. Defects in interstrand cross-link uncoupling do not account for the extreme sensitivity of ERCC1 and XPF cells to cisplatin. Nucleic Acids Res 2002;30(17):3848–56.
[11] Clingen PH, Arlett CF, Hartley JA, Parris CN. Chemosensitivity of primary human fibroblasts with defective unhooking of DNA interstrand cross-links. Exp Cell Res 2007;313(4):753–60.
[12] Sasaki MS, Takata M, Sonoda E, Tachibana A, Takeda S. Recombination repair pathway in the maintenance of chromosomal integrity against DNA interstrand crosslinks. Cytogenet Genome Res 2004;104(1–4):28–34.
[13] Spanswick VJ, Craddock C, Sekhar M, Mahendra P, Shankaranarayana P, Hughes RG, et al. Repair of DNA interstrand crosslinks as a mechanism of clinical resistance to melphalan in multiple myeloma. Blood 2002;100(1):224–9.
[14] Wynne P, Newton C, Ledermann JA, Olaitan A, Mould TA, Hartley JA. Enhanced repair of DNA interstrand crosslinking in ovarian cancer cells from patients following treatment with platinum-based chemotherapy. Br J Cancer 2007;97(7):927–33.
[15] Gossage L, Madhusudan S. Current status of excision repair cross complementing-group 1 (ERCC1) in cancer. Cancer Treat Rev 2007;33(6):565–77.
[16] Thompson LH, Schild D. Recombinational DNA repair and human disease. Mutat Res 2002;509(1–2):49–78.
[17] Li X, Heyer WD. Homologous recombination in DNA repair and DNA damage tolerance. Cell Res 2008;18(1):99–113.
[18] Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 2000;10(15):886–95.
[19] Rothkamm K, Löbrich M. Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. Proc Natl Acad Sci USA 2003;100(9):5057–62.
[20] Olive PL, Banáth JP. Phosphorylation of histone H2AX as a measure of radiosensitivity. Int J Radiat Oncol Biol Phys 2004;58(2):331–5.
[21] Banáth JP, Macphail SH, Olive PL. Radiation sensitivity, H2AX phosphorylation, and kinetics of repair of DNA strand breaks in irradiated cervical cancer cell lines. Cancer Res 2004;64(19):7144–9.
[22] Klokov D, MacPhail SM, Banáth JP, Byrne JP, Olive PL. Phosphorylated histone H2AX in relation to cell survival in tumor cells and xenografts exposed to single and fractionated doses of X-rays. Radiother Oncol 2006;80(2):223–9.
[23] Taneja N, Davis M, Choy JS, Beckett MA, Singh R, Kron SJ, et al. Histone H2AX phosphorylation as a predictor of radiosensitivity and target for radiotherapy. J Biol Chem 2004;279(3):2273–80.
[24] Fernandez-Capetillo O, Lee A, Nussenzweig M, Nussenzweig A. H2AX: the histone guardian of the genome. DNA Repair 2004;3(8–9):959–67.
[25] Lyakhovich A, Surrallés J. New roads to FA/BRCA pathway: H2AX. Cell Cycle 2007;6(9):1019–23.
[26] Huang X, Okafuji M, Traganos F, Luther E, Holden E, Darzynkiewicz Z. Assessment of histone H2AX phosphorylation induced by DNA topoisomerase I and II inhibitors topotecan and mitoxantrone and by the DNA cross-linking agent cisplatin. Cytometry A 2004;58(2):99–110.
[27] Niedernhofer LJ, Odijk H, Budzowska M, van Drunen E, Maas A, Theil AF, et al. The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks. Mol Cell Biol 2004;24(13):5776–87.
[28] Mogi S, Oh DH. gamma-H2AX formation in response to interstrand crosslinks requires XPF in human cells. DNA Repair 2006;5(6):731–40.
[29] Behrens BC, Hamilton TC, Masuda H, Grotzinger KR, Whang-Peng J, Louie KG, et al. Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res 1987;47(2):414–8.
[30] Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82(13):1107–12.
[31] Marti TM, Hefner E, Feeney L, Natale V, Cleaver JE. H2AX phosphorylation within the G1 phase after UV irradiation depends on nucleotide excision repair and not DNA double-strand breaks. Proc Natl Acad Sci USA 2006;103(26):9891–6.
[32] Strathdee G, MacKean MJ, Illand M, Brown R. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. Oncogene 1999;18(14):2335–41.
[33] Akiyama S-I, Chen Z-S, Sumizawa T, Furukawa T. Resistance to cisplatin. Anticancer Drug Des 1999;14(2):143–51.
[34] Celeste A, Fernandez-Capetillo O, Kruhlak MJ, Pilch DR, Staudt DW, Lee A, et al. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol 2003;5(7):675–9.