The XPF-ERCC1 endonuclease and homologous recombination contribute to the repair of minor groove DNA interstrand crosslinks in mammalian cells produced by the pyrrolo[2,1-*c*][1,4]benzodiazepine dimer SJG-136

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ABSTRACT

SJG-136, a pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) dimer, is a highly efficient interstrand crosslinking agent that reacts with guanine bases in a 5′-GATC-3′ sequence in the DNA minor groove. SJG-136 crosslinks form rapidly and persist compared to those produced by conventional crosslinking agents such as nitrogen mustard, melphalan or cisplatin which bind in the DNA major groove. A panel of Chinese hamster ovary (CHO) cells with defined defects in specific DNA repair pathways were exposed to the bi-functional agents SJG-136 and melphalan, and to their mono-functional analogues mmy-SJG and mono-functional melphalan. SJG-136 was >100 times more cytotoxic than melphalan, and the bi-functional agents were much more cytotoxic than their respective mono-functional analogues. Cellular sensitivity of both SJG-136 and melphalan was dependent on the XPF-ERCC1 heterodimer, and homologous recombination repair factors XRCC2 and XRCC3. The relative level of sensitivity of these repair mutant cell lines to SJG-136 was, however, significantly less than with major groove crosslinking agents. In contrast to melphalan, there was no clear correlation between sensitivity to SJG-136 and crosslink unhooking capacity measured using a modified comet assay. Furthermore, repair of SJG-136 crosslinks did not involve the formation of DNA double-strand breaks. SJG-136 cytotoxicity is likely to result from the poor recognition of DNA damage by repair proteins resulting in the slow repair of both mono-adducts and more importantly crosslinks in the minor groove.

INTRODUCTION

DNA interstrand crosslinks (ICLs) are critical cytotoxic lesions in dividing cells (1,2). Many commonly used anti-cancer drugs are ICL agents. These include nitrogen mustards, platinum compounds, chloroethylnitrosoureas, alkylalkanesulphonate and mitomycin C. Rational approaches continue to produce more efficient DNA interstrand crosslinking agents and novel tumour targeting strategies. Pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) is a family of anti-tumour antibiotics which include anthramycin, DC-81 and tomaymycin that are produced by various *Streptomyces* strains (3). These agents covalently bind guanine N2 in the minor groove of DNA and block transcription in a sequence-dependent manner (4). In an attempt to improve anticancer efficacy, two PBD monomers have been linked via their C8-positions to form dimers with high

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interstrand crosslinking efficiency (5,6). Second generation PBD dimers include SJG-136 (Figure 1), which is a highly efficient interstrand crosslinking agent that actively recognizes 5′-PuGATCPy-3′ sequences in the DNA minor groove (7–9). When tested *in vitro* in the National Cancer Institute (NCI) 60 human tumour cell line panel, SJG-136 exhibited a unique cytotoxicity profile when compared with other ICL agents used clinically (i.e. was COMPARE negative) (9). In human tumour cells, crosslinks form rapidly and persist compared to those produced by conventional agents, e.g. melphalan (9). SJG-136 also displayed potent activity against several human tumours *in vivo* (10), and phase I clinical trials are currently underway.

The ability to repair DNA ICLs is a critical factor determining cytotoxicity. Using Chinese hamster ovary (CHO) cells as a model system, we and others have previously shown that the increased cytotoxic effects of interstrand crosslinks induced by cisplatin and the nitrogen mustard mechlorethamine are associated with defects in the XPF-ERCC1 heterodimer component of the nucleotide excision repair (NER) pathway and in XRCC2 and XRCC3 which are Rad51 paralogues involved in the homlogous recombination repair (HRR) pathway (11–13). ICLs pose a serious problem for all cell types since genetic material is altered on both strands. The specialized role of the XPF-ERCC1 structure-specific nuclease in ICL repair has yet to be fully characterized, but several studies have indicated a role for this heterodimer during the incision of ICLs in both biochemical and cellular assays (12,14–17). Although the exact role of XRCC2 and XRCC3 has not been elucidated, recent biochemical data indicate that these Rad51 paralogues are essential for promoting Rad51-catalyzed DNA strand exchange during homologous recombination (18–21). A further complication after treatment with some crosslinking agents is the generation of DNA double-strand breaks (DSBs) (12,13). Whether or not these represent a direct ICL repair intermediate has not been fully established (22). It has been suggested that they arise when replication forks encounter ICLs. In cellular DNA, DSBs are evident after mechlorethamine treatment only in dividing cells. DNA DSBs have not, however, been detected in actively dividing cells after treatment with cisplatin (13). With the nitrogen mustard mechlorethamine, we have shown XRCC2 and XRCC3 to be required for the repair of these types of DSBs (12).

In this study, we have investigated the contribution of NER, HRR and non-homologous end-joining (NHEJ) to the cytotoxicity of SJG-136. A panel of CHO cells with defined defects in specific DNA repair pathways were exposed to the bi-functional anticancer agents SJG-136 and melphalan, and to their mono-functional analogues mmy-SJG and mono-functional melphalan (Figure 1). Melphalan is a nitrogen mustard chemotherapeutic that induces DNA ICLs in the major groove of DNA and is used clinically in the treatment of several cancers including multiple myeloma (23). The mono-functional analogues mono-melphalan (24,25) and mmy-SJG are capable of producing mono-adducts in the major and minor groove, respectively but not DNA ICLs. These agents were included in this investigation in order to understand the importance of ICLs with respect to cytotoxicity of both melphalan and SJG-136.

MATERIALS AND METHODS

Cell culture

Cell lines used in this study are listed in Table 1. AA8, UV23, UV42, UV61 and UV96 were obtained from Dr M. Stefanini (Istituto di Genetica Biochimica et Evoluzionistics, Pavia, Italy), UV135 was purchased from the American Tissue Culture Collection. V79, irs1, irs1SF, CHO-K1 and xrs5 cell lines were kindly provided by Prof J. Thacker (MRC Radiation and Genome Stability Unit, Harwell, UK). All cell lines were maintained as a monolayer in F12-Ham HEPES medium (Sigma, Poole, UK) supplemented with 2 mM glutamine and 10% fetal calf serum (Autogenbioclear, Wiltshire, UK). Cells were grown at 37°C in a 5% CO_2 incubator and harvested using trypsin EDTA 1× solution (Autogenbioclear, Wiltshire, UK).

Chemicals and drugs

Melphalan (Sigma, Poole, UK) was dissolved in ethanol with 0.5% conc. HCl. Mono-melphalan was prepared as described previously (24). SJG-136 and mmy-SJG were synthesized as described in (7,8) and stock solutions prepared in methanol.
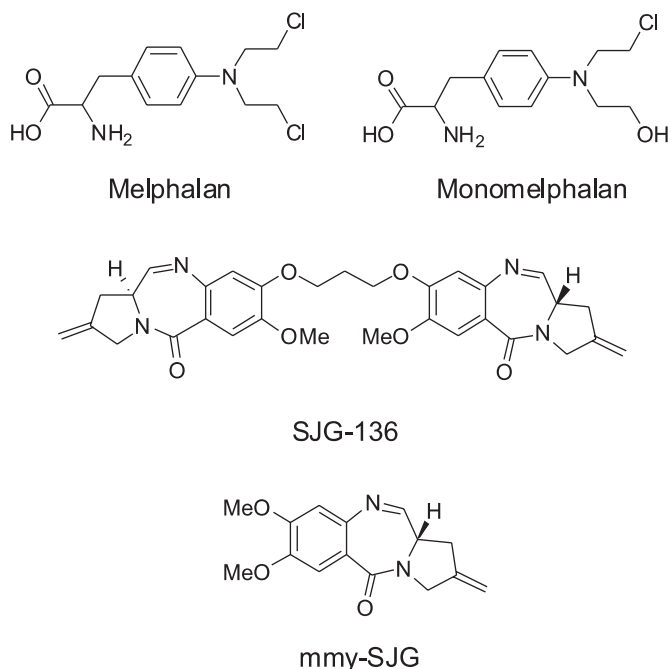

Figure 1. Structures of melphalan, mono-melphalan, SJG-136 and mmy-SJG.

Table 1. Chinese hamster ovary (CHO) cell lines

Mutant cell line	Parent cell line	Defective gene
UV23	AA8	*XPB*
UV42	AA8	*XPD*
UV47	AA8	*XPF*
UV61	AA8	*CSB*
UV96	AA8	*ERCC1*
UV135	AA8	*XPG*
irs1	V79	*XRCC2*
irs1SF	AA8	*XRCC3*
xrs5	CHO-K1	*XRCC5*

The structures of the drugs used in this study are shown in Figure 1. All other reagents were from Sigma unless otherwise stated.

Growth inhibition assay

Cytotoxicity was determined following a 1 h drug incubation using the sulforhodamine B (SRB) growth inhibition assay, described in detail previously (12,13). Growth inhibition was measured by quantifying the number of cells three days after drug exposure.

Clonogenic survival assay

A total of 200 exponentially growing CHO cells were plated in triplicate 90-mm plastic Petri dishes and incubated for 5 h to allow for cellular attachment. For clonogenic survival, attached single cells were treated with different concentrations of melphalan or SJG-136 for 1 h at 37°C in serum-free medium. Cells were washed and incubated for 7–9 days until visible colonies of more than 100 cells were obtained. Cells were then stained with 1% methylene blue and the number of colonies in each dish counted. Standard procedures were used to measure plating efficiency and the surviving fraction. For untreated cells, plating efficiencies were between 0.4 and 0.8, and the surviving fraction normalized to 1.

Induction and repair of DNA interstrand crosslinks

The level of DNA interstrand crosslinking was determined using a modification of the comet assay (26). All steps were carried out on ice and under subdued lighting. Briefly, 5×10^4 cells/well were incubated overnight in 6-well plates. Exponentially growing cells were treated with melphalan or SJG-136 in FCS-free medium for 1 h at 37°C, and then incubated in complete media as required. Cells were trypsinized and stored at −80°C as 1 ml aliquots in a complete medium containing 10% DMSO. Immediately before analysis, cells were thawed and irradiated (12.5 Gy) to introduce a fixed number of random DNA strand breaks. Cells were mixed with 2 ml of 1% Type-VII agarose and 1.5 ml spread over two agarose pre-coated microscope slides. The comet assay was performed according to established procedures (26). Slides were stained with 2.5 µg/ml propidium iodide and comets analysed using a Nikon inverted fluorescent microscope with a high pressure mercury light source, 510–560 nm excitation filter and 590 nm barrier filter at 20× magnification. Twenty-five cells were analysed per slide using Komet Assay Software (Kinetic Imaging, Liverpool, UK).

The degree of DNA interstrand crosslinking 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 (26). The level of interstrand crosslinking 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:

% Decrease in Tail Moment (DTM)

$$= 1 - (TMdi - TMcu)/(TMci - TMcu) \times 100$$

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.

Analysis of DNA double-strand breaks by pulsed field gel electrophoresis (PFGE)

Approximately 2×10^6 cells were incubated overnight in 175 cm^2 tissue culture flasks. Exponentially growing cells were treated with melphalan or SJG-136 in FCS-free medium for 1 h at 37°C and then incubated in complete media as required. Cells were harvested and PFGE plugs were prepared using the Bio-Rad Mammalian CHEF Genomic Plug Kit. PFGE was performed with 0.7% gel (Pulse Field Certified agarose, Bio-Rad) in 0.25× TBE buffer using a Biometra Rotaphor Type V apparatus. Electrophoresis runs were for 120 h at 14°C with the following parameters: interval 5000–1000 s log, angle 110–100° linear, voltage 50–45 V linear. On completion, gels were stained with 2 µg/ml ethidium bromide for 1 h, destained overnight with water and photographed. Semi-quantitative data were obtained by measuring the absolute integrated optical density of each lane using a Gel Pro Analyser (Media Cybernetics) and calculating the percentage of DNA released from the DNA plug.

RESULTS

Sensitivity of NER and recombination defective cell lines to the bi-functional ICL agents, melphalan and SJG-136

The data presented in Figure 2 show the sensitivity of wild-type, NER, HRR and NHEJ defective repair mutants to melphalan and SJG-136, using the SRB growth inhibition assay. When IC$_{50}$ values (concentration of drug to inhibit growth by 50%) were compared, the high cytotoxicity of SJG-136 (0.3 µM) is reflected by the fact that it is >100-fold more potent in wild-type AA8 cells than melphalan (33 µM). With both melphalan and SJG-136 cell lines defective in the NER genes (XPB, XPD, XPG and CSB) and the NHEJ Ku 70 subunit (XRCC5) show only a modest increase in sensitivity (<2-fold). In agreement with other studies, sensitivity to both crosslinking agents was dependent on the NER repair factors XPF-ERCC1 and the RAD51 paralogues XRCC2 and XRCC3 (12,13). After treatment with melphalan, cells defective in XPF, ERCC1, XRCC2 and XRCC3 were highly sensitive. IC$_{50}$ values were approximately 15-, 21-, 18- and 17-fold lower, respectively, when compared to their parent cell lines. In contrast, after treatment with SJG-136 these same XPF, ERCC1, XRCC2 and XRCC3 defective cell lines were approximately 7.5-, 7.5-, 7.5- and 3.5-fold more sensitive than parent cell lines. From these data, it appears that cellular sensitivity of the minor groove crosslinking agent SJG-136 is dependent on XPF-ERCC1 and homologous recombination; however, the level of sensitivity is less than with the major groove crosslinking agent melphalan.

Sensitivity of NER and recombination defective cell lines to the mono-functional agents mono-melphalan and mmy-SJG

Mono-melphalan and mmy-SJG are the mono-functional counterparts of melphalan and SJG-136, respectively (Figure 1). Neither agent can induce DNA ICLs but they
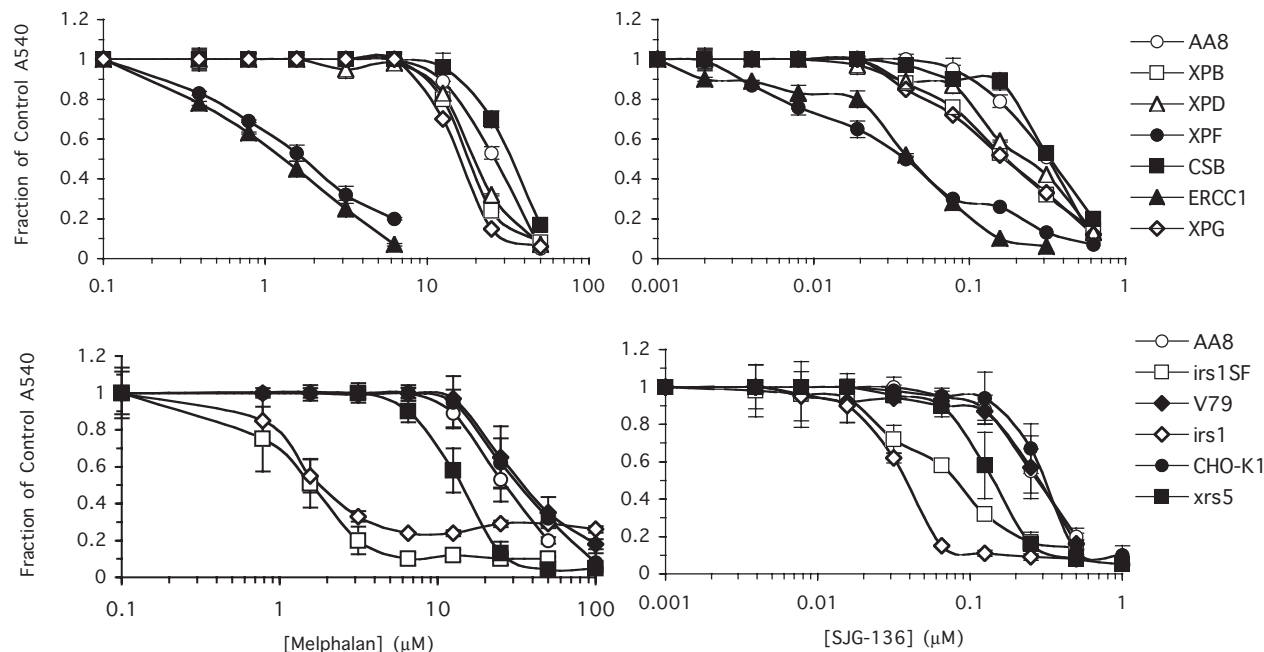
Figure 2. Melphalan and SJG-136 sensitivity in parental AA8, V79 and CHO-K1 cells, NER mutants XPB (UV23), XPD (UV42), XPF (UV47), CSB (UV61), ERCC1 (UV96) and XPG (UV135), recombination mutants XRCC2 (irs1), XRCC3 (irs1SF) and the NHEJ mutant XRCC5 (xrs5). Growth inhibition was determined using the SRB assay and the fraction of control A540 calculated as described. All results are the mean of at least three independent experiments and error bars show the standard error of the mean.
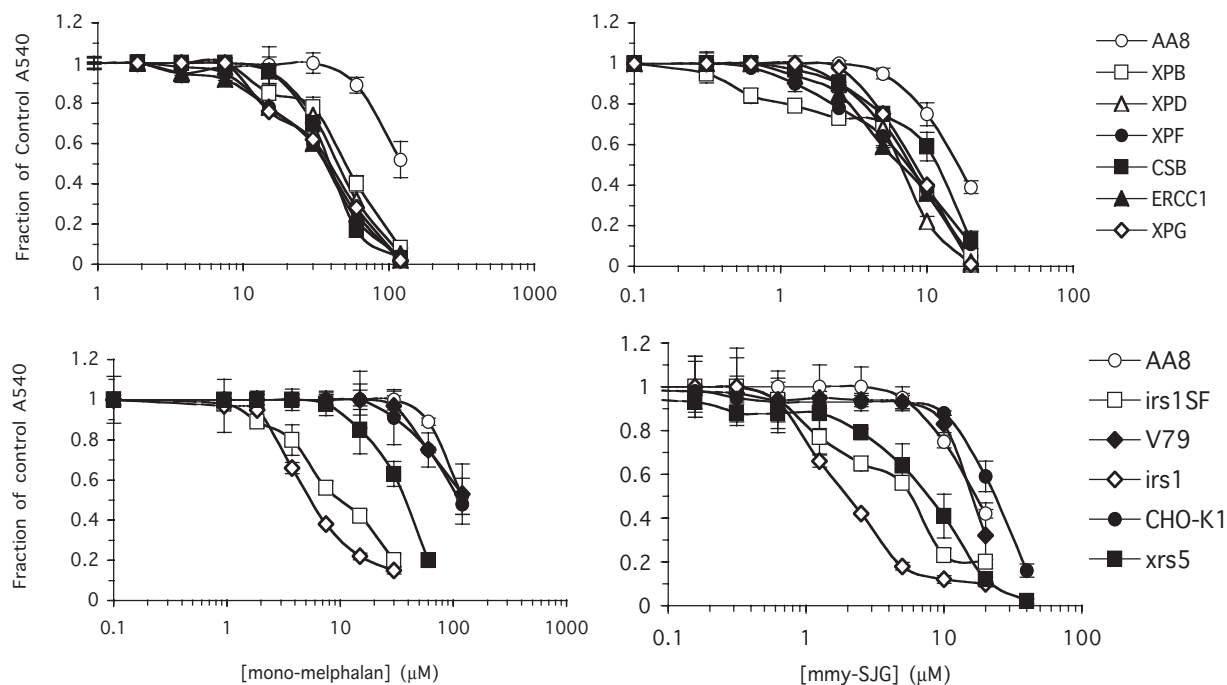

Figure 3. Mono-melphalan and mmy-SJG sensitivity of parental AA8, V79 and CHOK1 cells, NER mutants XPB (UV23), XPD (UV42), XPF (UV47), CSB (UV61), ERCC1 (UV96) and XPG (UV135), recombination mutants XRCC2 (irs1), XRCC3 (irs1SF) and the NHEJ mutant XRCC5 (xrs5). Growth inhibition was determined using the SRB assay and the fraction of control A540 calculated as described. All results are the mean of at least three independent experiments and error bars show the standard error of the mean.

can produce mono-adducts in the major and minor groove of DNA, respectively. In wild-type AA8 cells, the IC_{50} of mono-melphalan (135 μM) and mmy-SJG (18 μM) were 4- and 60-fold higher than their bi-functional counterparts (Figure 3).

This indicates, with both classes of agent, bi-functional adducts are a more cytotoxic form of DNA damage than their respective mono-functional adducts. In addition, it would suggest that mmy-SJG mono-adducts in the minor

groove are more cytotoxic than melphalan mono-adducts in the major groove.

With both mono-functional agents, NER defective CHO cells were only 2- to 3-fold more sensitive than wild-type cells (Figure 3). There was no extreme sensitivity of XPF or ERCC1 cells. The HRR mutants defective in XRCC2 and XRCC3 were 17- and 13-fold more sensitive to mono-melphalan, and 9- and 3.3-fold more sensitive to mmy-SJG. The XRCC5 mutant defective in NHEJ was ∼2-fold more sensitive than wild-type cells to the mono-functional agents. The pattern of sensitivity of the HRR and NHEJ mutants was, therefore, similar between the respective mono-functional and bi-functional agents.

Clonogenic survival of NER and recombination defective cells following melphalan and SJG-136 treatment

SRB assays identified a difference in the relative growth inhibition of XPF, ERCC1, XRCC2 and XRCC3 defective cell lines to melphalan and SJG-136. Clonogenic assays were undertaken to establish if these differences were also observed at the level of cell killing. Clonogenic survival of AA8, and representative defective cells UVB, ERCC1 and XRCC3 was determined after treatment with melphalan or SJG-136. The hypersensitivity of ERCC1 and XRCC3 defective cells was confirmed (Figure 4). A comparison of cell killing also

confirms that the level of sensitivity of the ERCC1 and XRCC3 mutants is less after treatment with SJG-136 than with melphalan.

SJG-136 is a highly efficient crosslinking agent

We have previously demonstrated that the sensitivity of XPF and ERCC1 defective cell lines to mechlorethamine correlates with an inability to initiate repair by failing to unhook ICLs (12). Using the modified comet assay, the induction and unhooking of DNA ICLs has been investigated after treatment with melphalan and SJG-136. Figure 5 shows the percentage decrease in tail moment with increasing concentrations of melphalan or SJG-136 for the wild-type AA8 cell line. A decrease in tail moment reflects increased DNA interstrand crosslinking (26). SJG-136 is a highly efficient ICL agent. Concentrations of melphalan and SJG-136 required to introduce a 50% decrease in tail moment following a 1 h exposure are ∼40 and 0.5 μM, respectively (Figure 5). Mono-melphalan and mmy-SJG did not produce ICLs (data not shown).

The peak of melphalan crosslinking occurs 16–24 h after drug treatment (9). More than 50% of the melphalan crosslinks had been incised or 'unhooked' after 48 h with almost complete unhooking evident after 72 h in AA8 and the NER defective mutants XPB and XPG (Figure 6A). In agreement with other studies using the nitrogen mustard mechlorethamine (12), there was little or no unhooking of melphalan ICLs in
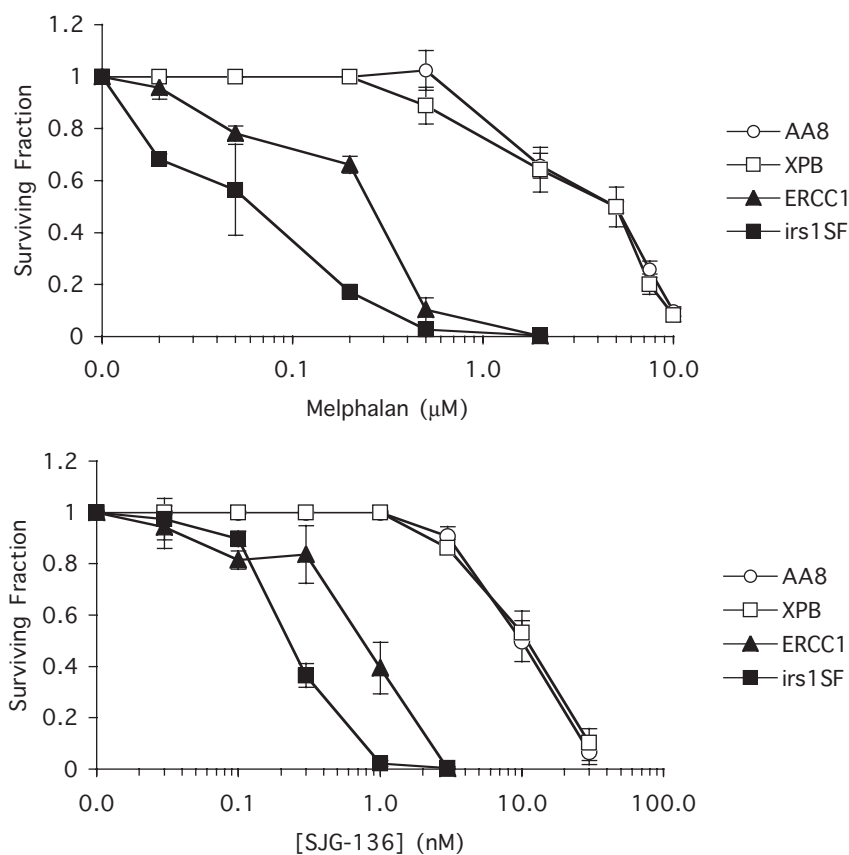

Figure 4. Clonogenic Survival assays for parental AA8, NER mutants XPB (UV23), ERCC1 (UV96) and the recombination mutant XRCC3 (irs1SF) cells after treatment with melphalan or SJG-136. The surviving fraction was calculated as described. All results are the mean of two independent experiments and error bars show the standard error of the mean.
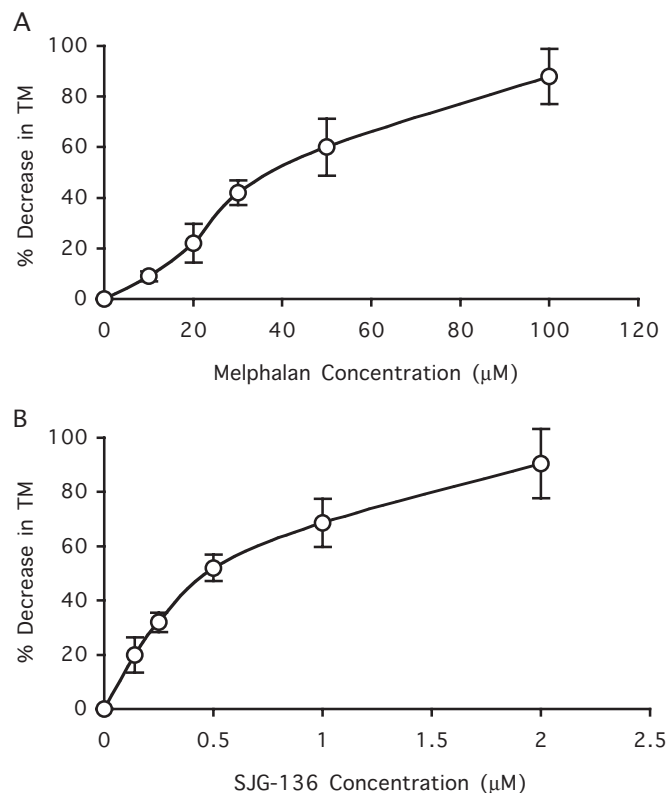
Figure 5. Decrease in comet tail moment (TM) with increasing concentration of (**A**) melphalan and (**B**) SJG-136 in AA8 cells. AA8 cells were treated with each compound for 1 h and analysed immediately. For each drug concentration, the TM of 50 cells was measured and the mean values calculated. The percentage decrease in TM in comparison with untreated samples was calculated. Results are the mean of at least three independent experiments and error bars show the standard error of the mean.

XPF or ERCC1 mutants. In contrast, SJG-136 ICLs were unhooked very slowly. With wild-type AA8 cells, >50% of the crosslinks persisted after 72 h. There was little evidence of repair in XPB and XPF cells, and no detectable unhooking in the XPG and ERCC1 cells (Figure 6B). The lack of a clear correlation between sensitivity to SJG-136 and ICL repair capacity in the different NER mutants implies that the differential sensitivities of the NER mutants to SJG-136 is not due to their ability to initiate repair of ICLs induced by this drug. A comparable level of ICLs was observed in all cell lines immediately after SJG-136 treatment demonstrating that the differences are not due to differential induction of ICLs.

SJG-136 does not induce DNA double-strand breaks

The induction of DSBs following melphalan and SJG-136 treatment was examined in exponentially growing AA8 cells using PFGE. There was a dose-dependent increase in lower molecular weight DNA, representing DSB formation, immediately after a 1 h treatment with different concentrations of melphalan between 10 and 50 µM (Figure 7). No DSBs were observed following treatment with SJG-136 concentrations up to 1.5 µM (five times the IC_{50}) (Figure 7). Furthermore, no DSBs were observed at any time up to 24 h after 1.5 µM SJG-136 treatment (data not shown). In agreement with results generated with mechlorethamine (12), cells defective

in homologous recombination (XRCC3) are defective in the repair of DSBs associated with melphalan ICLs (Figure 8). Repair of these DSBs is similar to the wild-type cells in the sensitive ERCC1 mutant. A dose of 30 µM melphalan was used to investigate the repair of ICL associated DNA DSBs. With an equimolar dose of mono-melphalan no DNA DSBs were observed, indicating that these DSBs are dependent on the presence of ICLs (data not shown).

DISCUSSION

These results confirm previous reports that bi-functional agents are more cytotoxic than their mono-functional analogues (27). The increased cytotoxicity and clinical efficacy of bi-functional agents is associated with their ability to produce DNA interstrand crosslinks. The minor groove binding bi-functional agent SJG-136 exhibits potent cytotoxicity and is a highly efficient crosslinking agent. Its mono-functional analogue mmy-SJG is also highly cytotoxic compared to the major groove binding melphalan or mono-melphalan. Similarly, other minor groove mono-functional alkylating agents such as tallimustine and CC-1065 have shown higher cytotoxicity than more conventional major groove binding agents (11).

With bi-functional ICL agents, the XPF-ERCC1-specific nuclease has been demonstrated to play a critical role leading to the repair of ICLs both *in vivo* and *in vitro* (1,12,13,15). A possible model for ICL repair in mammalian cells is that the XPF-ERCC1-specific nuclease initiates repair by making dual incisions flanking the crosslink that 'unhook' or release the lesion in the form of an oligonucleotide attached to the complementary DNA strand (12,28). Re-section of the gap produced by ICL incision in a 5′–3′ direction would then produce a suitable substrate for homologous recombination. This model is consistent with the results presented here in that XPF-ERCC1 mutants do not show extreme sensitivity to mono-melphalan or mmy-SJG compared to their respective bi-functional analogues. However, the XRCC2 and XRCC3 HRR defective mutants show a similar, increased sensitivity to both the mono-functional and bi-functional paired agents used in this study. It would appear that HRR is required for the downstream repair of unhooked and re-sectioned ICLs and for the repair of their equivalent mono-adducts.

Cytotoxicity data indicate that SJG-136 minor groove crosslinks are recognized and repaired in a manner similar to that of melphalan (this study) and other major groove crosslinking agents (1,12,13) in that a combination of XPF-ERCC1 and HRR is required. However, the extent of sensitivity of XPF, ERCC1, XRCC2 and XRCC3 mutants to SJG-136 is significantly less than for melphalan or to other major groove crosslinking agents such as mechlorethamine and cisplatin (12,13). The XPF, ERCC1, XRCC2 and XRCC3 defective mutants were 7.5, 7.5, 7.5 and 3.5 times more sensitive than parent cell lines to SJG-136. With melphalan, mechlorethamine or cisplatin, all mutants were at least 15 times more sensitive and in some instances >40 times more sensitive than parent cell lines (12,13). This indicates that SJG-136 cytotoxicity is less dependent on XPF-ERCC1 and HRR factors than melphalan or other more conventional major groove crosslinking agents.

A possible explanation for these differences is that minor groove alkylating agents do not cause as much DNA helix
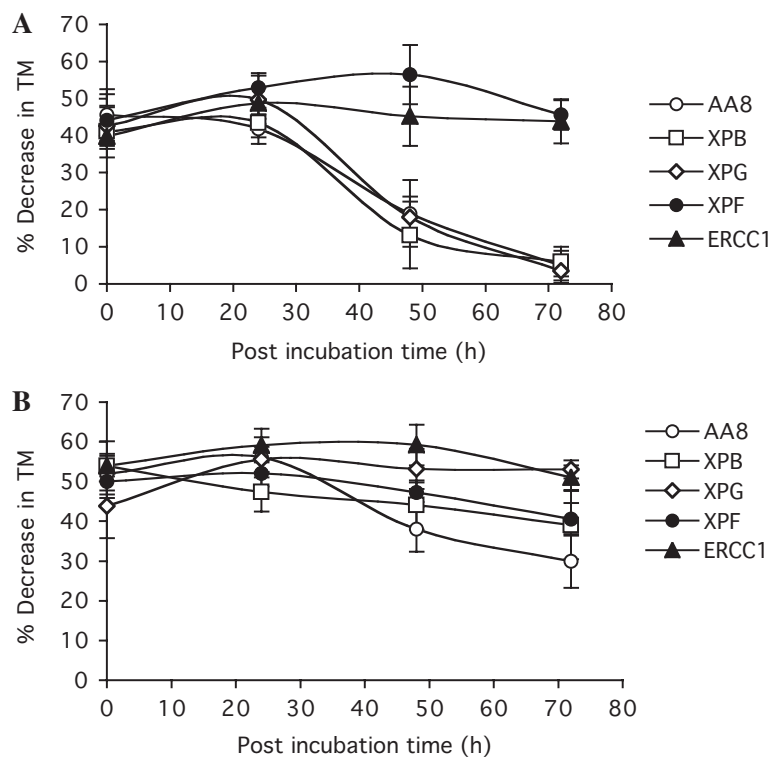
Figure 6. Kinetics of unhooking of (**A**) melphalan and (**B**) SJG-136 induced ICLs in parent (AA8) and NER mutant cell lines; XPB (UV23), XPG (UV135), XPF (UV47) and ERCC1 (9UV96). Cells were treated with 30 μM melphalan or 0.3 μM SJG-136 for 1 h, incubated in fresh medium and the level of ICLs measured at specific time points using the modified comet assay. Results are the mean of three independent experiments and error bars show the standard error of the mean.
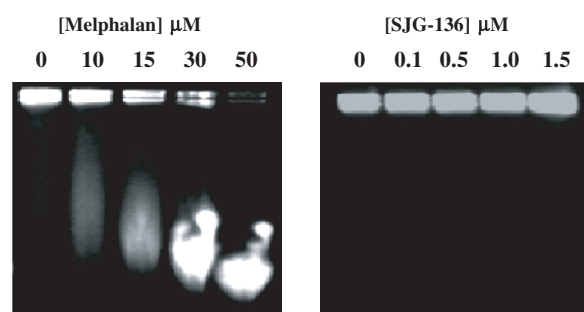

Figure 7. Induction of DNA DSBs following treatment with melphalan or SJG-136. Exponentially growing AA8 cells were treated with increasing doses of either compound for 1 h, and DSBs analysed immediately by PFGE.

Figure 8. Repair kinetics of melphalan-induced DNA DSBs in parent AA8 cells, the NER mutant ERCC1 (UV96) and the recombination mutant XRCC3 (irs1SF). Cells were treated with 30 μM melphalan for 1 h and the level of DSBs assessed immediately, and 4, 8 and 24 h after drug treatment. Semi-quantitative analysis of the percentage of DNA released from the plugs from a typical experiment are shown. Results show the mean of two independent experiments and error bars show the standard error of the mean.

distortion as major groove binders. Major groove crosslinking agents such as cisplatin induce a high degree of helical distortion (29). Molecular modelling of an SJG-136 crosslink in the minor groove of DNA shows very little helical distortion (30,31). Since helical distortion or changes in chromatin structure are major DNA motifs recognized by many DNA repair proteins (32,33), minor groove DNA damage and crosslinks would be less well recognized and repaired less readily. It might be expected that poor recognition of minor groove DNA damage would result in less dependency for repair processes and result in greater cytotoxicity. Not only is SJG-136 a highly cytotoxic and efficient crosslinking agent, repair of SJG-136 ICLs in the minor groove is much slower than for those formed in the major groove by melphalan.
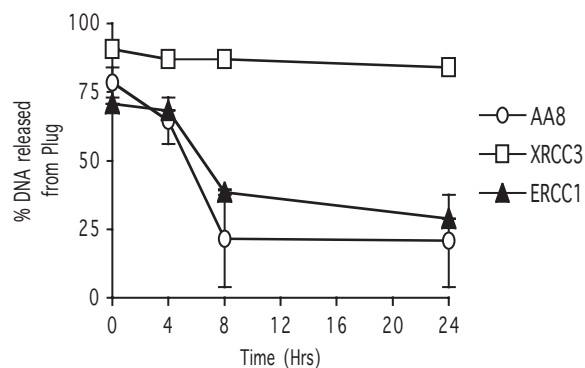
With melphalan, there is a direct correlation between XPF and ERCC1 mutant sensitivity and the ability to unhook melphalan ICLs. The mechanism of incision of melphalan ICLs appears to be the same as that for mechlorethamine (12). However, with SJG-136 there is no clear correlation between cellular sensitivity and the ability to unhook ICLs. Despite showing only modest increased sensitivity, there is little unhooking of SJG-136 ICLs by XPB or XPG mutants. The same situation has been reported in NER-defective CHO cells

treated with cisplatin (13). Cisplatin ICLs are, however, incised efficiently in wild-type cells, and this requires the full NER apparatus (13). In contrast, SJG-136 ICLs are not incised to any great extent over a 72 h period in wild-type or NER-defective cells. The increased sensitivity of XPF-ERCC1 mutants to SJG-136 would therefore appear to suggest a role in a HR-dependent repair pathway. Our data and that of other workers (12,14–17) would suggest that the role of the ERCC1-XPF endonuclease complex in ICL repair is to unhook the crosslink and catalyse subsequent resectioning reactions to provide a suitable repair intermediate which can be processed further by HRR.

The significance of DNA DSB formation following treatment with ICL agents is unknown. Nitrogen mustards including melphalan induce DNA DSBs (12). The maximum yield of DNA DSBs is immediately after drug treatment and these DSBs are repaired rapidly in a HRR-dependent manner. This is in contrast to the relatively slow repair of melphalan ICLs. Consideration of the differential induction and repair rates of melphalan-induced DSBs and ICLs make it unlikely that the DSBs are arising as ICL repair intermediates. It is more likely that in cells, melphalan-induced DSBs are formed as a result of stalled replication forks in the vicinity of melphalan ICLs as suggested by studies with different cross-linking agents (12). It is intriguing why no ICL associated DNA DSBs are detected by PFGE after treatment with SJG-136 (this study) or cisplatin (13). Although it remains to be resolved, a possible explanation for the lack of DSB induction by SJG-136 is that, due to the lack of helical distortion, SJG-136 ICLs could be good substrates for replication bypass. Mechanisms for the replication bypass of ICLs have been reported [(34) and references within].

Collectively, these studies indicate that there are differences in the processing of ICLs produced by different drugs. In the case of SJG-136, the high level of cytotoxicity is likely to result from the poor recognition of DNA damage by repair proteins leading to the slow repair of both mono-adducts and more importantly ICLs in the minor groove. While HRR clearly contributes to the repair of SJG-136 ICLs and mono-adducts, of particular note is the differential sensitivity between the XRCC2 and XRCC3 mutants. Only with the minor groove binding agents SJG-136 and mmy-SJG did the XRCC3 mutants exhibit significantly lower levels of cytotoxicity than XRCC2 mutants. During homologous recombination, at least two stable complexes have been identified: a dimeric complex composed of XRCC3 and Rad51C and a larger complex composed of XRCC2, Rad51B, Rad51C and Rad51D (35,36). While the exact roles of these separate complexes remain unclear, it may be that the repair of minor groove DNA damage is more dependent on the complex containing XRCC2.

In conclusion, homologous recombination repair plays an essential role in the protection against both major groove and minor groove DNA interstrand crosslinking agents. There are a number of cancer-prone genetic disorders involving proteins required for or associated with homologous recombination. These include ataxia telangiectasia, Nijmegen breakage syndrome, Blooms syndrome and Fanconi anemia (37). Genes involved in homologous recombination are tumour suppressor genes and mutations in BRCA1, BRCA2 and Rad51 have been identified and associated with specific cancers (37).

Collectively, this suggests that specific tumours are likely to be defective in HRR and that targeting the HRR pathway with specific chemotherapeutics may result in selective tumour cytotoxicity.

REFERENCES

1. Dronkert,M.L. and Kanaar,R. (2001) Repair of DNA interstrand cross-links. *Mutat. Res.*, **486**, 217–247.
2. Lawley,P.D. and Phillips,D.H. (1996) DNA adducts from chemotherapeutic agents. *Mutat. Res.*, **355**, 13–40.
3. Thurston,D.E. (1993) Advances in the study of Pyrrolo[2,1 *c*][1,4]benzodiazepine (PBD) antitumour antibiotics. In: Neidle,S. and Waring,M.J. (eds), *Topics in Molecular and Structural Biology: Molecular Aspects of Anticancer Drug–DNA Interactions*. The Macmillan Press Ltd, London, pp. 54–88.
4. Puvvada,M.S., Forrow,S.A., Hartley,J.A., Stephenson,P., Gibson,I., Jenkins,T.C. and Thurston,D.E. (1997) Inhibition of bacteriophage T7 RNA polymerase *in vitro* transcription by DNA-binding pyrrolo [2,1 *c*][1,4]benzodiazepines. *Biochemistry*, **36**, 2478–2484.
5. Thurston,D.E., Bose,D.S., Thompson,A.S., Howard,P.W., Leoni,A., Croker,S.J., Jenkins,T.C., Neidle,S., Hartley,J.A. and Hurley,L.H. (1996) Synthesis of sequence-selective C8-linked pyrrolo[2,1-*c*][1,4]benzo-diazepine DNA interstrand crosslinking agents. *J. Org. Chem.*, **61**, 8141–8147.
6. Smellie,M., Kelland,L.R., Thurston,D.E., Souhami,R.L. and Hartley,J.A. (1994) Cellular pharmacology of novel C8-linked anthramycin-based sequence-selective DNA minor-groove cross-linking agents. *Br. J Cancer*, **70**, 48–53.
7. Gregson,S.J., Howard,P.W., Jenkins,T.C., Kelland,L.R. and Thurston,D.E. (1999) Synthesis of a novel C2/C2'-*exo* unsaturated pyrrolobenzodiazepine cross-linking agent with remarkable DNA binding affinity and cytotoxicity. *J. Chem. Soc. Chem. Commun.*, **9**, 797–798.
8. Gregson,S.J., Howard,P.W., Hartley,J.A., Brooks,N.A., Adams,L.J., Jenkins,T.C., Kelland,L.R. and Thurston,D.E. (2001) Design, synthesis and evaluation of a novel pyrrolobenzodiazepine DNA-interactive agent with highly efficient cross-linking ability and potent cytotoxicity. *J. Med. Chem.*, **44**, 737–748.
9. Hartley,J.A., Spanswick,V.J., Brooks,N., Clingen,P.H., McHugh,P.J., Hochhauser,D., Pedley,B.R., Kelland,L.R., Alley,L.C., Schultz,R. *et al.* (2004) SJG-136 (NSC 694501) A novel rationally designed DNA minor groove interstrand crosslinking agent with potent and broad spectrum antitumour activity. *Cancer Res.*, **64**, 6693–6699.
10. Alley,M.C., Hollingshead,M.G., Pacula-Cox,C.M., Waud,W.R., Hartley,J.A., Howard,P.W., Gregson,S.J., Thurston,D.E. and Sausville,E.A. (2004) SJG-136 (NSC 694501) a novel rationally designed DNA minor groove interstrand crosslinking agent with potent and broad spectrum antitumor activity. Part 2: Efficacy evaluations. *Cancer Res.*, **64**, 6700–6706.
11. Damia,G., Imperatori,L., Stefanini,M. and D'Incalci,M. (1996) Sensitivity of CHO mutant cell lines with specific defects in nucleotide excision repair to different anti-cancer agents. *Int. J. Cancer*, **66**, 779–783.

12. De Silva,I.U., McHugh,P.J., Clingen,P.H. and Hartley,J.A. (2000) Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. *Mol. Cell. Biol.*, **20**, 7980–7990.

13. De Silva,I.U., McHugh,P.J., Clingen,P.H. and Hartley,J.A. (2002) Defects in interstrand cross-link uncoupling do not account for the extreme sensitivity of ERCC1 and XPF cells to cisplatin. *Nucleic Acids Res.*, **30**, 3848–3856.

14. Mu,D., Bessho,T., Nechev,L.V., Chen,D.J., Harris,T.M., Hearst,J.E. and Sancar,A. (2000) DNA interstrand cross-links induce futile repair synthesis in mammalian cell extracts. *Mol. Cell. Biol.*, **20**, 2446–2454.

15. Kuraoka,I., Kobertz,W.B., Ariza,R.R., Biggerstaff,M., Essigmann,J.M. and Wood,R.D. (2000) Repair of an interstrand DNA cross-link initiated by ERCC1-XPF repair/recombination nuclease. *J. Biol. Chem.*, **275**, 26632–26636.

16. Adair,G.M., Rolig,R.L., Moore-Faver,D., Zabelshansky,M., Wilson,J.H. and Nairn,R.S. (2000) Role of ERCC1 in removal of long non-homologous tails during targeted homologous recombination. *EMBO J.*, **19**, 5552–5561.

17. McHugh,P.J., Spanswick,V.J. and Hartley,J,A. (2001) Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *Lancet Oncol.*, **2**, 483–490.

18. Cartwright,R., Tambini,C.E., Simpson,P.J. and Thacker,J. (1998) The XRCC2 DNA repair gene from human and mouse encodes a novel member of the recA/RAD51 family. *Nucleic Acids Res.*, **26**, 3084–3089.

19. Liu,N., Lamerdin,J.E., Tebbs,R.S., Schild,D., Tucker,J.D., Shen,M.R., Brookman,K.W., Siciliano,M.J., Walter,C.A., Fan,W. *et al.* (1998) XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. *Mol. Cell.*, **1**, 783–793.

20. Yamada,N.A., Hinz,J.M., Kopf,V.L., Segalle,K.D. and Thompson,L.H. (2004) XRCC3 ATPase activity is required for normal XRCC3-Rad51C complex dynamics and homologous recombination. *J. Biol. Chem.*, **279**, 23250–23254.

21. Yokoyama,H., Sarai,N., Kagawa,W., Enomoto,R., Shibata,T., Kurumizaka,H. and Yokoyama,S. (2004) Preferential binding to branched DNA strands and strand-annealing activity of the human Rad51B, Rad51C, Rad51D and Xrcc2 protein complex. *Nucleic Acids Res.*, **32**, 2556–2565.

22. Niedernhofer,L.J., Odijk,H., Budzowska,M., van Drunen,E., Maas,A., Theil,A.F., de Wit,J., Jaspers,N.G., Beverloo,H.B., Hoeijmakers,J.H. and Kanaar,R. (2004) The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks. *Mol. Cell. Biol.*, **24**, 5776–5787.

23. Anagnostopoulos,A., Aleman,A., Ayers,G., Donato,M., Champlin,R., Weber,D., Alexanian,R. and Giralt,S. (2004) Comparison of high-dose melphalan with a more intensive regimen of thiotepa, busulfan and cyclophosphamide for patients with multiple myeloma. *Cancer*, **100**, 2607–2612.

24. Tilby,M.J., McCartney,H., Gould,K.A., O'Hare,C.C., Hartley,J.A., Hall,A.G., Golding,B.T. and Lawley,P.D. (1998) A monofunctional derivative of melphalan: preparation, DNA alkylation products and determination of the specificity of monoclonal antibodies that recognize melphalan-DNA adducts. *Chem. Res. Toxicol.*, **11**, 1162–1168.

25. Gould,K.A., Nixon,C. and Tilby,M.J. (2004) P53 elevation in relation to levels and cytotoxicity of mono- and bi-functional melphalan DNA adducts. *Mol. Pharmacol.*, **66**, 1301–1309.

26. Spanswick,V.J., Hartley,J.M., Ward,T.H. and Hartley,J.A. (1999) Measurement of drug-induced interstrand crosslinking using single-cell gel electrophoresis (Comet) assay. In Brown,R. and Boger-Brown,U. (eds), *Methods in Molecular Medicine: Cytotoxic Drug Resistance Mechanisms*. Humana Press, Totowa NJ, Vol. 28, pp. 143–154.

27. Hartley,J.A. (2001) Alkylating agents. In Souhami,R.L., Tannock,I., Hohenberger,P. and Horiot,J.C. (eds), *Oxford Textbook of Oncology; 2nd edn*. Oxford University Press, pp. 639–654.

28. Sasaki,M.S., Takata,M., Sonoda,E., Tachibana,A. and Takeda,S. (2004) Recombination repair pathway in the maintenance of chromosomal integrity against DNA interstrand crosslinks. *Cytogenet. Genome Res.*, **104**, 28–34.

29. Brabec,V., Sip,M. and Leng,M. (1993) DNA conformational change produced by the site-specific interstrand cross-link of trans-diamminedichloroplatinum(II). *Biochemistry*, **32**, 11676–11681.

30. Mountzouris,J.A., Wang,J.-J., Thurston,D.E. and Hurley,L.H. (1994) Comparison of a DSB-120 DNA interstrand cross-linked adduct with the corresponding bis-tomaymycin adduct: an example of a successful template-directed approach to drug design based upon the monoalkylating compound tomaymycin. *J. Med. Chem.*, **37**, 3132–3140.

31. Jenkins,T.C., Hurley,L.H., Neidle,S. and Thurston,D.E. (1994) Structure of a covalent DNA minor groove adduct with a pyrrolobenzodiazepine dimer: evidence for sequence-specific interstrand cross-linking. *J. Med. Chem.*, **37**, 4529–4537.

32. Isaacs,R.J. and Spielmann,H.P. (2004) A model for initial DNA lesion recognition by NER and MMR based on local conformational flexibility. *DNA Repair (Amst.)*, **3**, 455–464.

33. Lisby,M. and Rothstein,R. (2004) DNA damage checkpoint and repair centers. *Curr. Opin. Cell Biol.*, **16**, 328–334.

34. Dronkert,M.L.G. and Kanaar,R. (2001) Repair of DNA interstrand crosslinks. *Mutat. Res.*, **486**, 217–247.

35. Masson,J.Y., Tarsounas,M.C., Stasiak,A.Z., Stasiak,A., Shah,R., McIlwraith,M.J., Benson,F.E. and West,S.C. (2001) Identification and purification of two distinct complexes containing the five RAD51 paralogs. *Genes Dev.*, **15**, 3296–3307.

36. Liu,N., Schild,D., Thelen,M.P. and Thompson,L.H. (2002) Involvement of Rad51C in two distinct protein complexes of Rad51 paralogs in human cells. *Nucleic Acids Res.*, **30**, 1009–1015.

37. Thompson,L.H. and Schild,D. (2002) Recombinational DNA repair and human disease. *Mutat. Res.*, **509**, 9–78.