

Inhibition of double-strand break non-homologous end-joining by cisplatin adducts in human cell extracts

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ABSTRACT

The effect of *cis*-diaminedichloroplatinum(II) (cisplatin) DNA damage on the repair of double-strand breaks by non-homologous end-joining (NHEJ) was determined using cell-free extracts. NHEJ was dramatically decreased when plasmid DNA was damaged to contain multiple types of DNA adducts, along the molecule and at the termini, by incubation of DNA with cisplatin; this was a cisplatin concentration-dependent effect. We investigated the effect a single GTG cisplatin site starting 10 bp from the DNA termini would have when surrounded by the regions of AT-rich DNA which were devoid of the major adduct target sequences. Cisplatin of a substrate containing short terminal 13–15 bp AT-rich sequences reduced NHEJ to a greater extent than that of a substrate with longer (31–33 bp) AT-rich sequences. However, cisplatin at the single GTG site within the AT sequence had no significant effect on NHEJ, owing to the influence of additional minor monoadduct and dinucleotide adduct sites within the AT-rich region and owing to the influence of cisplatin at sites upstream of the AT-rich regions. We then studied the effect on NHEJ of one *cis*-[Pt(NH₃)₂{d(GpTpG)-N7(1),-N7(3)}] [abbreviated as 1,3-d(GpTpG)] cisplatin adduct in the entire DNA molecule, which is more reflective of the situation *in vivo* during concurrent chemoradiation. The presence of a single 1,3-d(GpTpG) cisplatin adduct 10 bases from each of the two DNA ends to be joined resulted in a small (30%) but significant decrease in NHEJ efficiency. This process, which was DNA-dependent protein kinase and Ku dependent, may in part explain the radiosensitizing effect of cisplatin administered during concurrent chemoradiation.

INTRODUCTION

Concurrent chemoradiation, the use of chemotherapy during a course of radiotherapy, is now the standard treatment in several cancer types and the subject of clinical trials in others. However, the molecular mechanisms that make this an effective treatment are not fully understood. *cis*-Diaminedichloroplatinum(II) (cisplatin), an agent commonly used in chemoradiation, acts by producing DNA interstrand and intrastrand adducts. *In vitro*, ~65% of the adducts are *cis*-[Pt(NH₃)₂{d(GpG)-N7(1),-N7(2)}] [abbreviated as 1,2-d(GpG)] intrastrand cross-links, 25% *cis*-[Pt(NH₃)₂{d(ApG)-N7(1),-N7(2)}] [abbreviated as 1,2-d(ApG)] intrastrand cross-links, 5–10% 1,3-d(GpNpG) intrastrand cross-links, with a small percentage of interstrand cross-links and monofunctional adducts [reviewed in (1)]. The intrastrand adducts are removed by nucleotide excision repair (NER), which involves excision of a region of ~30 nt around the adduct, followed by DNA synthesis and ligation (2). Radiation exposure can produce many types of DNA damage including base damage, single-strand breaks and double-strand breaks (DSBs). In a locally multiply damaged site where a cisplatin adduct occurs close to a single-strand break the damage may be initially repaired by NER. However, during the dual incision step a DSB, which is the most lethal type of damage a cell can sustain, could be produced (3). Alternatively, if a cisplatin adduct is close to a DSB, the DSB could physically block the cisplatin removal and repair by the NER pathway, or the cisplatin adduct could hinder the DSB repair pathways by restricting the access of the protein complexes involved. Another possibility is that the NER and the DSB repair proteins may compete for access to the locally multiply damaged site. Investigations into the efficiency of NER have shown that NER is less efficient if damaged sites [cisplatin adducts and ultraviolet (UV) lesions] are near to a DSB owing to binding of the DSB repair protein heterodimer Ku70/80 at the DNA ends (4). In addition, decreased levels of NER proteins bind to the lesion if a DSB is present (5,6).

The DSBs are repaired by the homologous recombination (HR) pathway or the non-homologous end-joining (NHEJ)

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repair pathway, the latter being more error prone. The HR pathway requires a DNA template strand, usually a sister chromatid, whereas the NHEJ pathway has no such requirement [reviewed in (7–9)]. The basic NHEJ pathway [reviewed in (7–10)] requires a number of proteins. Initially, the heterodimer Ku70/Ku80 recognizes and binds to the free DNA ends at a DSB in a sequence-independent manner. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is then recruited to the Ku heterodimer to form DNA-PK holoenzyme. The Ku heterodimer can be translocated along the DNA strand as the DNA passes through the hole in the Ku heterodimer ring-like shape (11). The DNA-PK holoenzyme has kinase activity, which recruits and co-ordinates repair proteins that may be important in phosphorylating proteins in this pathway. Finally, XRCC4 stimulates DNA ligase IV to join the broken ends. Other components thought to be involved depending on the type of DNA ends to be joined are inositol hexakisphosphate (12), Artemis (13), MRN complex (14) and DNA polymerase μ (15).

Previous work has shown that the presence of a cisplatin adduct near a DSB may decrease the rate of Ku translocation along the DNA strand and lowers the DNA-PK kinase activity (16–18). However, these investigations were performed in the presence of high levels of the purified components Ku70/80, so that multiple Ku heterodimer molecules were shown to bind and translocate along the DNA used. In addition, only the initial steps of the NHEJ pathway were examined, so that the ability to ligate two ends of DNA was not assessed. These results have been confirmed more recently (19) showing that the cisplatin adducts do not alter the ability of Ku to bind DNA ends, but do impair the translocation of Ku complex along the DNA. Using a ligation assay with recombinant Ku, XRCC4 and DNA ligase IV, the cisplatin adducts were able to dramatically inhibit DNA ligation, whereas if no Ku was present the high ligation levels were very slightly decreased in the cisplatinated DNA. The ability to perform ligation was dependent on the DNA substrate length. In the present work, we examined the effect of cisplatin adduct formation on the ability to perform NHEJ using cell-free extracts, which contain the full complement of factors required for NHEJ, rather than recombinant proteins. We also used large 3.2 kb DNA substrates rather than smaller DNA fragments, which may limit the ability to form appropriate DNA repair complexes around the DSB. DNA substrates with varying degrees of cisplatination were used ranging from substrates where the entire DNA was cisplatinated, and hence containing many different adduct types, to substrates with a single intrastrand *cis*-[Pt(NH₃)₂{d(GpTpG)-N7(1),-N7(3)}] [abbreviated as 1,3-d(GpTpG)] cisplatin adduct 10 bases from the end of the DSB. We show that substrates containing single, defined terminal cisplatin adducts are more informative than ‘globally’ cisplatinated substrates containing both terminal and internal adducts in determining the effect of cisplatin on NHEJ.

MATERIALS AND METHODS

Production of DNA substrates

Cisplatination of the linear 3.2 kb pGEM3zf+ plasmid DNA (Promega, Southampton, UK) was carried out by the

incubation of 1 μ g of the PstI restriction enzyme cut DNA with 1.1–5.3 nmol of cisplatin (Sigma, Gillingham, UK) in 3 mM NaCl, 0.5 mM Na₂HPO₄ and 0.5 mM NaH₂PO₄ at 37°C overnight. Mock-treated DNA was incubated in buffer alone. The reaction was stopped by the addition of 0.5 M NaCl, and the DNA was purified using G25 spin columns (Amersham Pharmacia, Buckinghamshire, UK). The sequence surrounding the PstI site (shown in boldface) and the space with a underline denotes the position of the restriction: 5'-ACTATAGAA-TACTCAAGCTTGCATGCCTGCA_GGTCGACACTAGT-GGATCCAAAGAATTCAAAAAGCTTCT-3'.

Cisplatination of plasmid DNA was also carried out on the substrates shortcon and shortcis, which were constructed by ligating into the EcoRI–HindIII site of pGEM3zf+ the following annealed nucleotides, respectively: 5'-AATTCATTATAATTTATC_AATTGATATAAATTATA-3' dimerized with 5'-AGCTTATAAATTTATATC_AATTGATAAATATTATATG-3' and 5'-AATTCATTGTGATATTTATC_AATTGATATAAATCACA-3' dimerized with 5'-AGCTTGTGATTTATATC_AATTGATAAATATCACAATG-3'. The region contains the MfeI restriction site (shown in boldface), which was used to linearize the substrates for end-joining reactions, and the space with a underline denotes the position of the restriction. The shortcon and shortcis differ only in that a GTG sequence is present in the shortcis substrate as shown in italics. The longer substrates (longcon and longcis) contained the following sequence ligated into the EcoRI–HindIII site of pGEM3zf+. The longcon sequence was 5'-AATTCAATA-TAATTTTATAATATTATAAATTTATC_AATTGATATAAATTATAAATTTATAAATTTATATTA-3' and the longcis sequence was 5'-AATTCAATATAAATTTTATAAATTGTGATATTTATC_AATTGATATAAATCACAATTTATAATTATTTATATTA-3'. The longcon and longcis differ only in that a GTG sequence is present in the longcis substrate as shown in italics.

DNA substrates with one cisplatin adduct in the entire molecule were produced using a 24 base oligonucleotide containing the GTG site for cisplatination shown in boldface (5'-TCTTCTTCTGTGCACTCTTCTTCT-3'). This 24mer was purified by 15% denaturing PAGE (Sequagel, National Diagnostics, Hessele, UK), identification by staining with SYBR Green I (Molecular Probes, Leiden, The Netherlands), and isolated by electroelution (Elutrap, Schleicher and Schuell, London, UK) followed by ethanol precipitation. Cisplatination was carried out as described previously and uncisplatinated and cisplatinated oligonucleotides were separated on a 15% denaturing polyacrylamide gel as described previously (20). The cisplatinated and uncisplatinated DNA were identified by UV shadowing and electroeluted. The control (con) or cisplatinated (cis) 24mer were annealed to either oligonucleotide A (5'-P-AATTAGAAGAAGAGTGCACAGAAGAAGACGCC-3') or B (5'-P-AATTAGAAGAAGAGTGCACAGAAGAAGAGGCCG-3') by heating equimolar amounts of each oligonucleotide at 60°C for 5 min and leaving to cool slowly over 2 h at room temperature. Oligonucleotides A and B are identical in sequence apart from 4 nt shown above in italics. Both oligonucleotides A and B were phosphorylated at the 5' end and were designed such that annealing of either with the 24mer (either con or cis) produced a double-stranded molecule containing a 4 nt overhang at each end, with one overhang being compatible with EcoRI digested plasmid DNA

(denoted in boldface and shown in Figure 3A) and the other overhang being compatible between A and B. The resulting annealed oligonucleotides, Acon, Bcon, Acis and Bcis, were purified by separation in a 15% native gel followed by electroelution and precipitation. Using T4 DNA ligase, the annealed oligonucleotides were separately ligated to pGEM3zf+ plasmid DNA linearized by EcoRI digestion. Experiments were initially performed with different concentrations of annealed oligonucleotides and 50-fold molar excess of oligonucleotides to plasmid DNA EcoRI ends was shown to be sufficient for >95% ligation. These four different ligated substrates of DNA (Acon, Bcon, Acis and Bcis) were then further purified by separation in a 0.6% agarose gel and purification using the QIAquick gel extraction kit (Qiagen Ltd, Crawley, UK). An initial ligation was performed with T4 DNA ligase in order to show that there was no recircularization, and hence ligation of double-stranded oligonucleotides onto the plasmid backbone had been successful. Analysis of the ^{32}P -ATP end-labelled control and cisplatinated substrate by restriction digest using ApaLI, or HindIII digestion followed by exonuclease III action confirmed the presence of the cisplatin adduct on the manufactured DNA substrate (Figure 3B).

NHEJ assay

NHEJ extracts were produced essentially as described previously (21,22). End-joining reactions (20 μl) were carried out with 40 μg protein extract from M059K cells and 20 ng DNA substrate in the presence of 50 mM HEPES, pH 8.0, 40 mM KOAc, 1 mM $\text{Mg}(\text{OAc})_2$, 1 mM ATP, 1 mM DTT, 0.1 mg/ml BSA and 1 U RNase A at 37°C. For DNA substrates with one cisplatin adduct in the entire molecule, equal amounts (10 ng) of either cisplatinated (Acis and Bcis) or control (Acon and Bcon) substrate were used. For example, 10 ng each of Acis and Bcis substrates were mixed together to provide compatible ends in the reaction mixture (in one reaction) to investigate the effect of a cisplatin adduct on either side of a DSB, while in a reaction with 10 ng each of Acis and Bcon substrates in the reaction mixture the effect of a cisplatin adduct at only one side of the DSB was examined. This was possible because the A substrate alone had incompatible 4 nt overhangs but with the addition of the B substrate, compatible overhangs would be present in the reaction. Protein was removed by incubation with proteinase K (2 mg/ml) and 0.5% (w/v) SDS for 30 min and extraction with Tris-buffered phenol/chloroform/isoamyl alcohol. Each assay was repeated at least three times. Reactions were separated on 0.7% agarose gels, and signal quantified by image analysis (Molecular Imager FX and the Quantity One version 4.1.1 software; Bio-Rad Ltd, Hertfordshire, UK), following SYBR Green I staining of the gel. To determine the dependence of DNA joining on DNA-PK or Ku, Wortmannin (10 μM ; Sigma, UK) or anti-Ku70 antibody (1:20 dilution; ab87, Abcam, UK), respectively, was included in the reaction mixture in the absence of DNA for 10 min on ice before the addition of DNA and incubation at 37°C.

RESULTS

NHEJ of cisplatinated plasmid DNA

The effect of cisplatin adducts on NHEJ was initially examined using a linear double-stranded 3.2 kb DNA substrate

with cohesive 3' four base overhangs produced by PstI digestion where the entire DNA molecule had been exposed to a range of cisplatin concentrations. Cisplatin reduced levels of NHEJ by M059K extract as measured by linear dimer, trimer and higher multimer formation (Figure 1). This effect was clearly dependent on the level of cisplatin achieved and no NHEJ was seen at the highest cisplatin

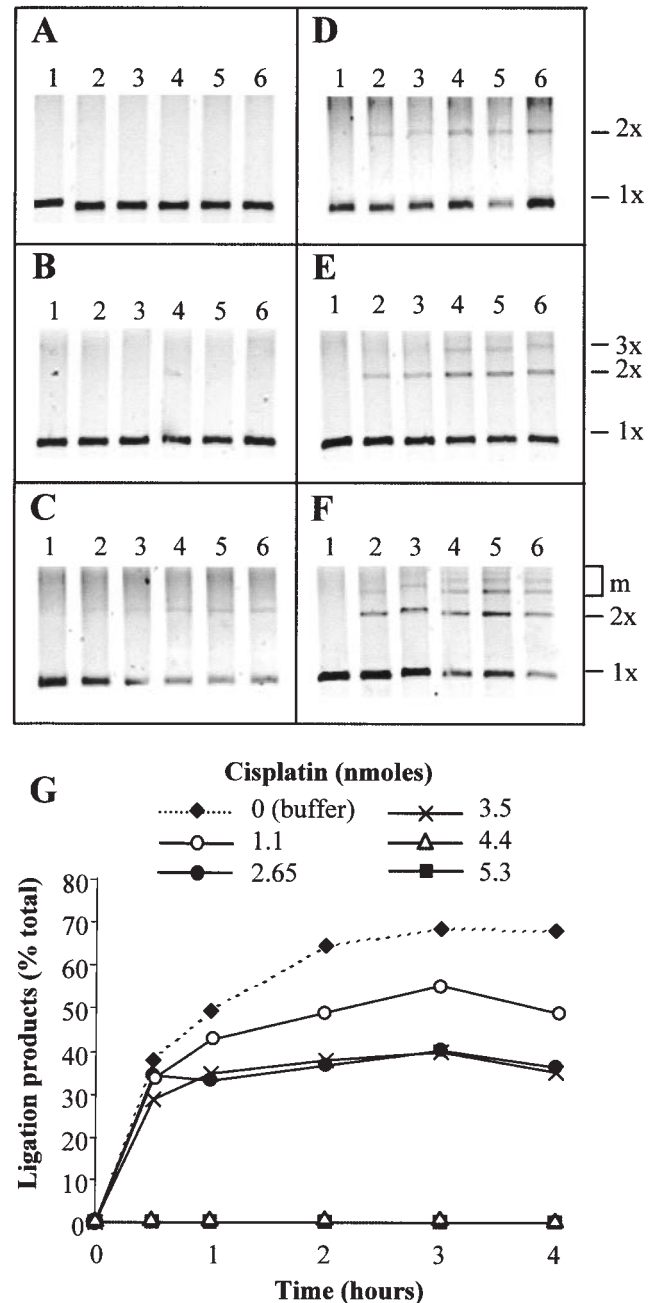


Figure 1. NHEJ of control and cisplatinated DNA substrate. End-joining performed with M059K extract and DNA substrate treated with cisplatin at the following concentration (nmol) (A) 5.3, (B) 4.4, (C) 3.5, (D) 2.65, (E) 1.1 and (F) buffer only. NHEJ was measured over a time course where DNA was incubated with extract for 0, 0.5, 1, 2, 3 and 4 h (lanes 1–6). The 3.2 kb linear monomer (1 \times) was joined to form ligated linear dimers (2 \times), trimers (3 \times) and higher multimers (m) as indicated. (G) Proportion of DNA substrate ligated against time at the indicated cisplatin concentrations.

concentration used. Examination of the DNA sequence at the PstI DNA ends showed a large number of potential sites along the molecule where the cisplatin could form an adduct (sequence in Materials and Methods). For example, both ends of this substrate contained a GpG site, which if cisplatinated could lead to fraying of the DNA ends (23), AG sites were identified at 11 and 15 bp from the DNA ends, and potential interstrand cross-links at 2/3, 6/7 and 10/11 bp on one side and 4/5 bp on the other side. Therefore, to distinguish the effect of terminal cisplatin adducts from other cisplatin-mediated effects on end-joining we constructed substrates with controlled sites for cisplatin at the DNA ends. In a preliminary experiment we generated plasmid DNA cut with a second restriction enzyme, Afl III, which did not have any G bases at the DNA ends, but did contain one GTG sequence adjacent to the 3' cohesive overhang. Once again a decrease in NHEJ was seen with increasing cisplatin (data not shown).

NHEJ of short AT-rich substrates

We reasoned that the introduction of one defined GTG cisplatin site at the termini of the substrate, which would be surrounded by DNA containing a reduced number of potential cisplatin sites, would allow us to determine the effect of a terminal cisplatin adduct on NHEJ. We engineered two new substrates (shortcon and shortcis) with fewer potential cisplatin sites as no GG, AG or GNG sequences were present in the immediate sequence surrounding the DSB owing to the insertion of a 13 and 15 bp AT-rich DNA sequence on either side of the DSB (sequence in Materials and Methods and Figure 2A). Both substrates were linearized with MfeI and the only difference between them was that the 'shortcis' substrate had a GTG sequence inserted 10–12 bases from the restriction site DSB restriction site. This GTG sequence allowed the production of a 1,3-d(GpTpG) intrastrand cisplatin adduct. This adduct was specifically selected to investigate

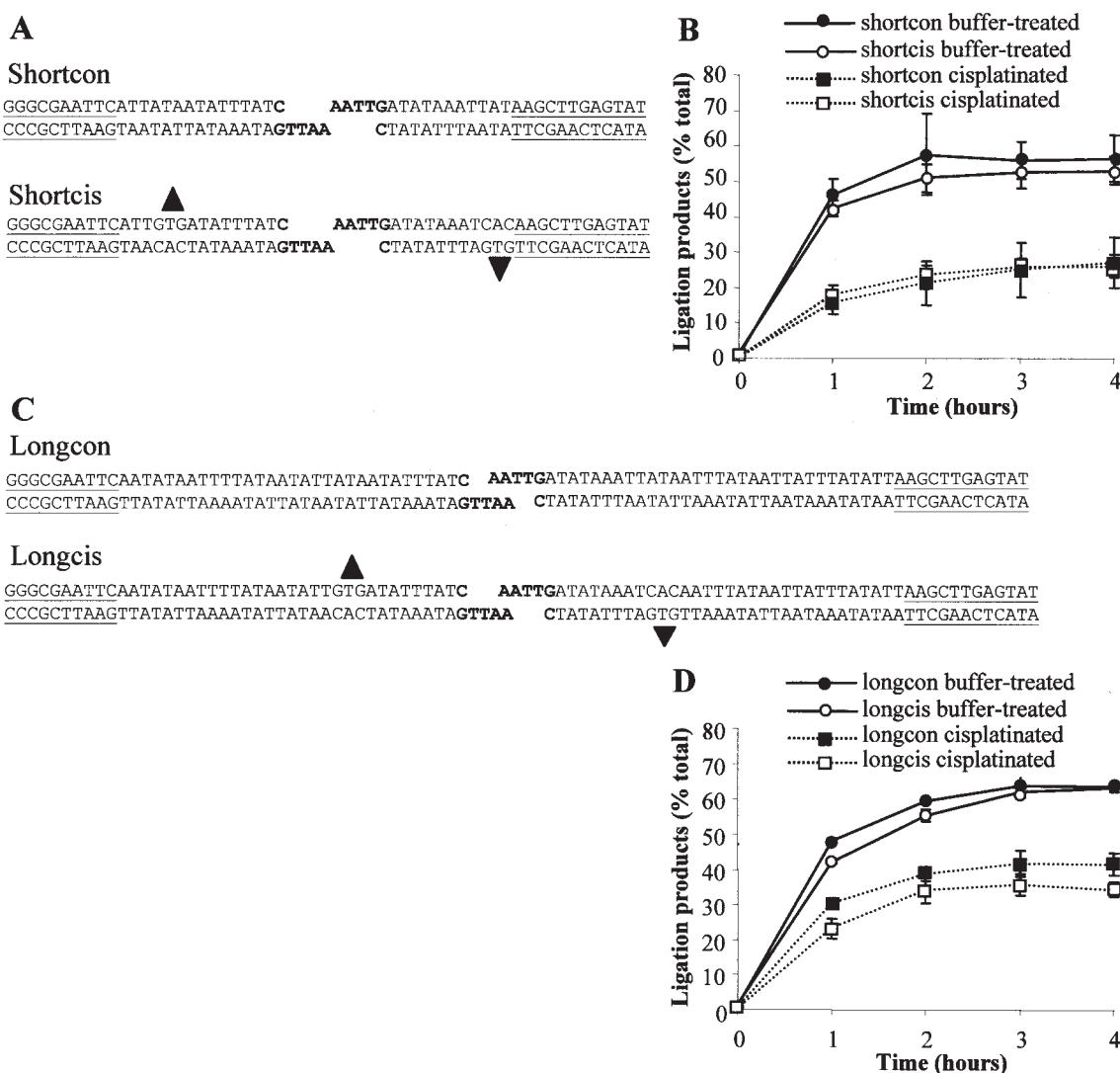


Figure 2. NHEJ of short and long AT-rich substrates. Sequence of the ends of the (A) short and (C) long AT-rich control and cisplatinated DNA substrates. Double-stranded oligonucleotides were ligated into pGEM plasmid DNA (underlined) with the MfeI restriction site shown in boldface. The inserted triangles show the position of the GTG sequence for cisplatin. Graphs (B) and (D) show the percentage of ligation products formed by MO59K extract and DNA substrates shown in (A) and (C), respectively. Substrates were either buffer treated or cisplatinated (2.65 nmol cisplatin). Experiments were performed in triplicate (\pm SD) and are representative of three independent experiments.

further as although it makes up only 5–10% of adducts from cisplatin-treated DNA, it is able to both bend and unwind the DNA; therefore, causing substantial distortion of the DNA helix [reviewed in (1)]. Once again, NHEJ by MO59K extract was much reduced with increasing cisplatin concentration (data not shown). Comparison of the effect of cisplatin on the shortcis substrate revealed a decrease in percentage ligation products from $66 \pm 11\%$ (\pm SEM) (buffer treated) to $18 \pm 5\%$ (cisplatinated) at 2 h. However, the same decrease was also observed when the shortcon substrate (in which the terminal GTG site was absent) was treated with cisplatin ($70 \pm 10\%$, buffer treated and $18 \pm 4\%$, cisplatinated) (Figure 2B). The ligation observed with both substrates was DNA-PKcs dependent, as judged by Wortmannin sensitivity, and Ku-dependent, using an anti-Ku antibody (data not shown).

NHEJ of long AT-rich substrates

The DNA substrates shortcon and shortcis had 13 and 15 bases of the AT-rich sequence on either side of the DSB, which is sufficient for a single Ku heterodimer to bind to. But following DNA-PKcs recruitment, the Ku heterodimer is predicted to translocate away from the DSB and along the DNA by a further 10 bases. This would mean that the translocated Ku heterodimer would now also cover the original plasmid DNA sequence which contained many potential cisplatin sites. To minimize the possibility that cisplatin residues other than the one 10 bases from the DSB site were inhibiting Ku translocation and hence NHEJ, two new substrates were engineered with an extra 16 and 20 bases of AT-rich sequence on either side (sequence in Materials and Methods and Figure 2C). Similar to the effect seen with the short AT-rich substrates, cisplatin of both control (longcon) and cisplatin-site containing (longcis) substrates decreased ligation product formation to the same extent. Percentage ligation for longcon substrate was 69 ± 10 (buffer treated) and $36 \pm 4\%$ (cisplatinated), while for longcis substrate was 63 ± 11 (buffer treated) and $31 \pm 2\%$ (cisplatinated). However, we did note that both the longer AT-rich substrates (longcon and longcis) were significantly less sensitive ($P < 0.05$) to the effects of cisplatin than the short AT-rich substrate; percentage ligation when cisplatinated was $36 \pm 4\%$ (longcon), $31 \pm 2\%$ (longcis), $18 \pm 4\%$ (shortcon) and $18 \pm 5\%$ (shortcis) at 2 h.

NHEJ of single 1,3-d(GpTpG) cisplatin adduct substrates

In patients receiving concurrent chemoradiation, the doses of cisplatin and radiation are such that we would not expect clusters of cisplatin adducts in the DNA nor multiple cisplatin adducts near each DSB (3). As it was possible that the additional monoadducts and the GA adduct within the AT-rich region as well as global cisplatin inhibited NHEJ irrespective of the presence or absence of a single-terminal cisplatin adduct, we investigated the joining of linear DNA substrates containing one cisplatin adduct in the entire molecule. DNA molecules (Acis and Bcis) were manufactured whereby a 1,3-d(GpTpG) cisplatin adduct was located 10 bases from each DNA end. In parallel, control substrates (Acon and Bcon) were made which were not cisplatinated. The 4 nt overhangs of the DNA substrates were self-incompatible, so that in reactions containing each substrate alone end-joining

could only occur between incompatible ends. However, substrates A and B contained 4 nt 3' overhangs that were compatible with each other such that, in the presence of both substrates A and B, compatible end-joining could take place (Figure 3A). Analysis using exonuclease III confirmed that the *cis* substrates contained the cisplatin adduct. Exonuclease III acts upon 5' overhangs on double-stranded DNA, but has no activity at 3' overhangs. Following HindIII digestion of the DNA substrates, which creates a 5' overhang, exonuclease III will digest the cisplatin adduct-containing strand starting from the HindIII site and moving towards the engineered DSB. The presence of a cisplatin adduct would impede the activity of exonuclease III and would be predicted to result in a 12 bp fragment (with a 4 base single-strand overhang) resistant to digestion. An example of an exonuclease III digestion of a DNA substrate is shown in Figure 3B. Here, the DNA containing the cisplatin adduct is digested from the HindIII-generated 5' overhang towards the 3' end of the molecule, leaving a fragment of ~ 13 base length (which migrated more slowly owing to the cisplatin adduct), whereas the non-cisplatinated control DNA produces smaller fragments (Figure 3B).

NHEJ experiments were performed with combinations of these substrates and showed that the DNA containing 1,3-d(GpTpG) cisplatin at both ends was less readily joined than the non-cisplatinated control DNA, with ligation of 26% (Acis plus Bcis) compared with 37% (Acon plus Bcon) of substrate, respectively (Figure 4); a decrease in ligation efficiency of 30%. In total, four batches of both cisplatinated and control substrate molecules were independently produced. The actual levels of joining of the control substrates were lower than those observed with the PstI cut substrate in Figure 1 and the short and long AT-rich DNA substrates (35% ligated products compared with 70 and 55–60%, respectively). This may reflect the multiple processing steps required to make the substrate. *In vivo* it is more probable that a single cisplatin adduct would occur on one side of a DSB rather than two cisplatin adducts, one on either side of a DSB; experiments were performed to mimic this situation using mixtures of control and cisplatinated substrates with only one end of the DNA containing a cisplatin adduct (i.e. Acon plus Bcis or Bcon plus Acis) (Figure 4). Here, levels of NHEJ were intermediate between the control DNA and the DNA substrates cisplatinated at both ends. This joining was DNA-PKcs dependent and Ku dependent (data not shown). We also carried out experiments with the individual substrates alone which have self-incompatible DNA ends. Again, the majority of joining was both DNA-PKcs and Ku dependent (Figure 4C). The NHEJ levels were lower than when complementary ends were present; this was as expected as incompatible end-joining is known to be a less-efficient process (24). Interestingly, the cisplatinated DNA with incompatible ends was joined with lower efficiency than the corresponding control DNA with incompatible ends (Figure 4A).

DISCUSSION

Using cell-free extracts, we have investigated the effects of cisplatin adducts on the repair of DSBs by the NHEJ pathway. This system is an advance in our understanding of this process as no other study has used long (3.2 kb) DNA substrates with

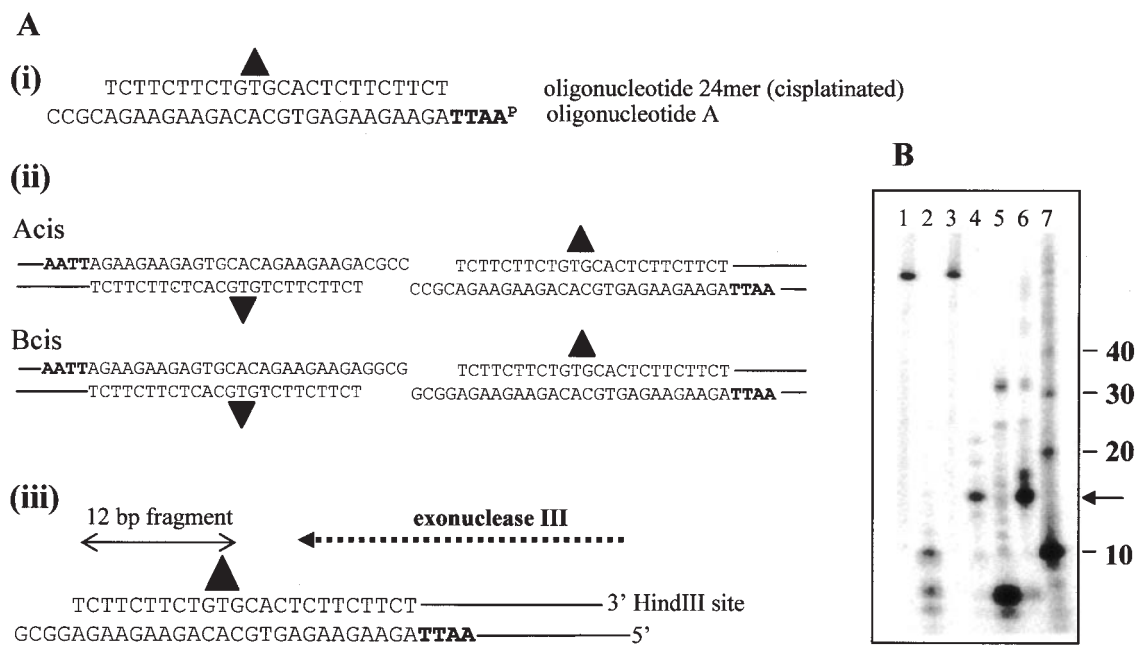


Figure 3. Manufacture of substrate with a single 1,3-d(GpTpG) cisplatin adduct. (A) Diagrammatic representation of substrate manufacture. (i) Oligonucleotides were annealed to form double-stranded molecules with A plus 24mer (as shown here) or B plus 24mer. In each case the 24mer was cisplatinated (*cis*). The GTG cisplatin site is indicated by a triangle and EcoRI compatible overhang shown in boldface. Oligonucleotides A and B were phosphorylated at the 5' end. (ii) These double-stranded molecules were then ligated onto EcoRI linearized pGEM3zf+ plasmid DNA (indicated by horizontal lines and containing no other cisplatin adducts). Acis and Bcis are shown here, Acon and Bcon were constructed identically but lacked the 1,3-d(GpTpG) cisplatin adduct at the GTG cisplatin site. The DNA substrates Acis and Bcis had self-incompatible ends but were compatible with each other. (iii) Position of HindIII site and polarity of exonuclease III digestion. The 12 base fragment resistant to digestion is indicated. (B) Exonuclease III analysis of substrates to confirm cisplatin adduct presence. ³²P-end-labelled DNA substrate was subject to restriction enzyme digestion with HindIII, subsequent exonuclease III digestion and denaturing acrylamide (15%) gel electrophoresis. Control Bcon substrate (lanes 1 and 2), cisplatinated Bcis substrate (lanes 3 and 4), oligonucleotide B annealed to control 24mer (lane 5), cisplatinated oligonucleotide B annealed to 24mer (lane 6), 10 bp molecular weight marker (lane 7). Exonuclease III digestion was for 0 h (lanes 1 and 3) and for 2 h (lanes 2 and 4–6). The arrow shows the position of the 12 base fragments (which migrated at ~13 bases owing to the presence of the cisplatin adduct) remaining owing to the blockage of nuclease action by the cisplatin adduct.

single cisplatin adducts or has used cell extracts that contain all factors required for NHEJ and, more importantly, are at the correct cellular concentration and relative molar ratios to each other; hence, our system is more relevant to the *in vivo* situation.

When treated with a high concentration of cisplatin, large numbers of different adduct types were formed throughout the entire substrate DNA, which led to a dramatic decrease in NHEJ efficiency. As the cisplatin concentration was decreased the adduct levels would decrease resulting in the reduction of NHEJ inhibition. However, the actual adduct types and levels formed were difficult to control in the individual plasmid molecules in these reactions. The reduction in end-joining could be due to multiple effects of cisplatin including the physical presence of cisplatin at or near the DNA ends so inhibiting end processing and/or ligation of ends, or to the distortion and denaturation of the DNA helix caused by the cisplatin adducts present along the DNA. Another possibility was that the recruitment of NER proteins to the cisplatin adduct may hinder NHEJ. However, preliminary data using the GM2246 cell line which is deficient in XPC, a protein known to be recruited early in the NER, suggested this last possibility was not likely (data not shown).

Therefore, we designed further experiments to help discriminate the effects of terminal cisplatin adducts from other events associated with cisplatin treatment. We investigated the use of substrates with different lengths of inserted AT-rich sequences

that did not contain the sequence GG or AG such that the most common cisplatin adducts [the intrastrand 1,2-d(GpG) and 1,2-d(ApG) adducts] would not be formed in this region of DNA near the DSB. We reasoned that these substrates could be useful intermediates between using the 'globally' cisplatinated substrate (Figure 1), the effects of which are difficult to interpret, and the single adduct substrates (Figure 4), which are more technically demanding and time-consuming to produce. Although the longer AT-rich substrates were less sensitive to the effect of cisplatin, surprisingly the presence of a single-terminal GTG nucleotide sequence as a target site for cisplatin had no significant effect on the NHEJ efficiency. It is possible that as these substrates contained purines and a GA dinucleotide near the DSB, cisplatin monoadducts and 1,2 intrastrand adducts could be formed; however, it is more likely that the distortion of the DNA structure by cisplatin at multiple sites throughout the rest of the substrate was such that the effect of a single GTG nucleotide sequence as a target site for cisplatin could not be measured. Another possibility is that the GTG sequence may not have been cisplatinated in all substrate molecules owing to the large amount of other potential sites for global cisplatin. In addition, the actual length of DNA over which the DNA-PKcs/Ku heterodimer complex interacts, or that Ku translocates on recruitment of XRCC4/ligase IV to the DNA ends (19) may be longer than the long AT-rich regions by a further 20 bp, and so would extend into the vector sequence that would contain multiple cisplatin

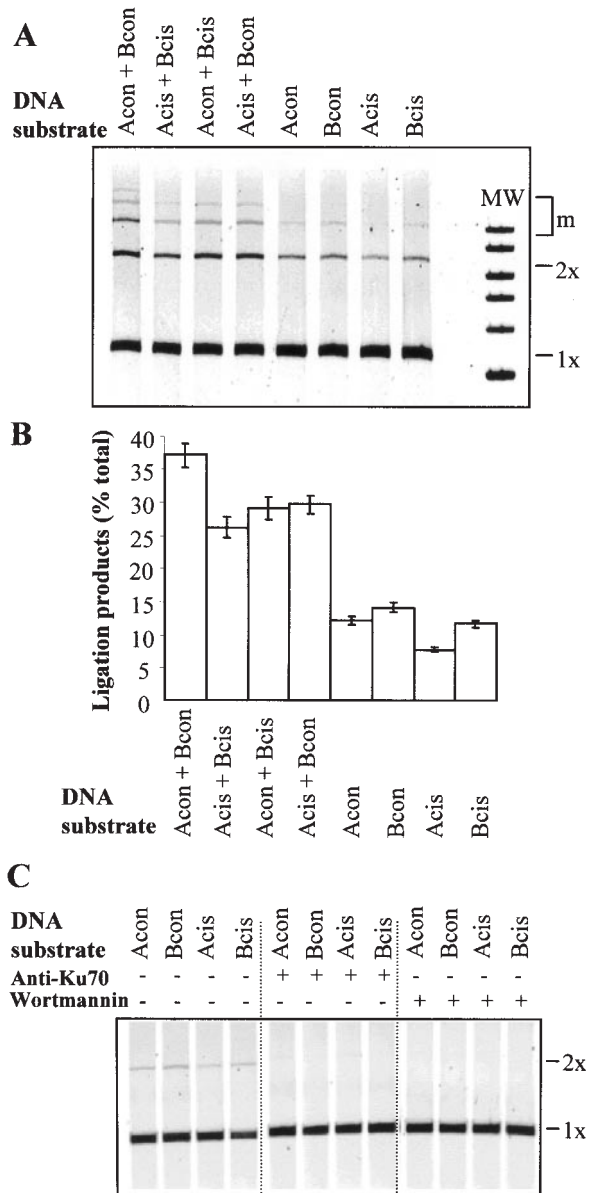


Figure 4. NHEJ efficiency in control and 1,3-d(GpTpG) cisplatinated DNA. (A) Representative NHEJ agarose gel of DNA substrates either uncisplatinated (Acon + Bcon), 1,3-d(GpTpG)-cisplatinated at both ends (Acis + Bcis), 1,3-d(GpTpG)-cisplatinated at one end (Acon + Bcis and Acis + Bcon) or single substrates alone. Reactions were incubated for 2 h at 37°C. The 3.2 kb linear monomer (1x) was joined to form ligated linear dimers (2x) and higher multimers (m) as indicated. MW indicates 1 kb molecular weight markers. (B) Corresponding graph showing the percentage ligation formation. The average of three independent experiments (\pm SEM) is shown. (C) End-joining of self-incompatible DNA substrates in the presence (+) or absence (-) of Wortmannin (10 μ M) or anti-Ku70 antibody (1:20 dilution). Linear monomer (1x) and ligated linear dimers (2x) are indicated.

adducts. Therefore, multiple internal cisplatin adducts further away from the termini, which could cause DNA distortion, have a more profound effect on NHEJ such that we were not able to measure the effect of terminal cisplatin adducts on NHEJ using these intermediate substrates. Indeed, the longer AT-rich constructs were less sensitive to the effects of global cisplatin than the shorter constructs as the extra 16–20 bp

AT sequence inserted into the longer constructs, where there was vector sequence before in the shorter constructs, again reinforced the effect of multiple cisplatin adduct sites further away from the actual DNA termini on NHEJ.

We then analysed the effect on NHEJ of a single adduct at both ends of the DSB in a molecule with no other adducts present, and the effect when only one DSB end had a cisplatin adduct. The latter situation is more likely to occur *in vivo*. NHEJ efficiency was reduced when both ends contained cisplatin adducts by \sim 30%, while a reduction of \sim 20% was found when a cisplatin adduct was present near only one DNA end. It is not possible to directly compare the percentage end-joining of substrates between experiments as substrates with different terminal polarity and sequence were used. Additionally, in experiments with substrates that had single cisplatin adducts there was a mixture of compatible and incompatible DNA ends, which were not phosphorylated. End-joining efficiency can alter depending on the polarity of DNA ends, the nucleotide sequence at the termini or the nucleotide sequence internal to the DNA ends (21,25–27); we have shown this in our work where termini differing by only two bases were joined with different efficiencies (Acis compared with Bcis; Figure 4B). This can be due to the differential activation of DNA-PK. However, the results with global platination of substrate showed that, using the highest dose of cisplatin, total NHEJ inhibition could be achieved (Figure 1) where the adducts would be both internal and at the DNA ends. The multiple cisplatin adducts situated more internally in the AT-rich substrates (Figure 2) also were able to substantially reduce NHEJ at the cisplatin concentrations used, and at higher levels of cisplatin could block NHEJ even more effectively (data not shown). This indicated that multiple internal adducts could have more influence on NHEJ than a single terminal adduct, which caused a maximal 20–30% inhibition (Figure 4). Similarly, it is likely that the multiple internal adducts in the substrate used in Figure 1 could have more influence on NHEJ than the single GG adduct site at the extreme DNA end. This reinforces the importance of our study using single defined adducts and the difficulties inherent in the interpretation of studies that use globally cisplatinated substrates. These data argue against using globally cisplatinated DNA substrates for determining the effect of terminal cisplatin adducts on the NHEJ. While this work was under review, studies by Pawelczak *et al.* (26) were published that confirmed the inhibitory effect on part of the NHEJ pathway of cisplatin adducts close to the DSB. Using purified DNA-PKs and recombinant Ku they showed that DNA-PK kinase activity, but not Ku binding, was significantly inhibited (95%) by the presence of a 1,2-d(GpG) intrastrand cisplatin adduct 6 bp from the DNA terminus, while an adduct 15 bp away caused partial inhibition (60%). In view of the different termini used and adduct types, it is not possible to directly compare levels of inhibition with this work. The authors propose a model whereby a cisplatin adduct close to the terminus prevents the DNA substrate from fully threading through the kinase such that the single-stranded ends would not be positioned in the activating cavity of DNA-PKs so preventing end-joining. Our findings agree with others in the field who suggest that cisplatin adducts can decrease NHEJ due to a decrease in the translocation rate of Ku and the consequent decrease in DNA-PK kinase activity (16–19).

However, assay systems distinct to ours were used by these authors including shorter lengths of substrate DNA, sometimes as low as 32 nt, and higher levels of Ku molecules. The relevance of having multiple Ku complexes translocating along the DNA strand *in vivo* is not known, as *in vitro* a single Ku heterodimer is sufficient to recruit DNA-PKcs (11), which could indicate that *in vivo* a functional stoichiometry is 1:1 for each DNA end. It is also unclear if DNA-PKcs is present in one assay system (19). Our assay system not only provides a measure of the entire Ku and DNA-PK dependent NHEJ process in a more physiological setting but also allowed us to study the effect of a single adduct, which is more relevant to the situation *in vivo* (3).

In mammalian cells, ionizing radiation causes ~40 DSBs per cell per gray of radiation and, following cisplatin treatment, one would expect $1-2 \times 10^4$ cisplatin adducts per cell given a total of 3.3×10^9 bp per cell [based on the data and the calculations from (3,28)]. In chemoradiation treatment, the patient is pretreated with cisplatin before the radiotherapy is administered so the assumption is made that cisplatin adducts are present before the DSB are created. Therefore after 2 Gy of radiation, Poisson statistics (3) predict there to be a 0.5–1% probability of a cell having a single cisplatin adduct within 10 bp of one end of a DSB, while the probability of a cell having a cisplatin adduct at 10 bp from both ends of the DNA involved in a DSB is infinitesimally small. We have shown that the presence of a cisplatin adduct at one end reduces the NHEJ efficiency at 2 h by ~20%. This equates to approximately one in five end-joining events being delayed/inhibited by the presence of the cisplatin adduct. Therefore in a cell population treated with chemoradiation as described, we would expect ~0.1–0.2% (one-fifth of 0.5–1%) to have DSB rejoining delayed/inhibited by the presence of a cisplatin adduct within 10 bp of one DSB end, a potentially deleterious event as only one unrepaired DSB needs to remain within the cell to initiate cell death. Repair of DSB induced by ionizing radiation is efficient, such that *in vitro* all but 10% of DSB are repaired within 2 h of radiation (29), so any reduction in efficiency of repair by the presence of a cisplatin adduct could impact on treatment outcome.

The relative importance of NHEJ and HR in the repair of DSB with a cisplatin adduct close by *in vivo* is not known. In terms of cisplatin treatment alone, cisplatin exposure has no effect on NHEJ-deficient cells, whereas HR-deficient cells are more sensitive (30,31). This is due to the decrease/stalling of replication fork progression by the presence of the cisplatin adduct, which may lead to the production of DSBs [(32,33) discussed in (34)]. This mechanism may be especially important in relation to the type of adduct formed as the most frequent adduct, GpG, is refractory to repair by NER, while the less-frequent GpNpG adduct is more efficiently repaired by NER (35). In addition, interstrand cross-links formed by cisplatin adducts require HR for their repair (36). Therefore, although we have shown that NHEJ is impeded by the presence of a nearby cisplatin adduct, thus increasing radiosensitivity, it is likely that a proportion of the radiosensitizing effect of cisplatin is related to the HR pathway. We have shown that a single cisplatin adduct, either at one or both ends of the DNA substrate, can decrease the joining of compatible DNA ends. The MO59K cell line has previously been shown to join incompatible DNA ends by NHEJ (24) and in this study we

have shown that incompatible ends are joined less efficiently if they contain a single cisplatin adduct. Our experiments with exonuclease III indicate that exonuclease movement along the DNA strand can be blocked by the presence of a single cisplatin adduct, and it is known that the presence of adducts can block DNA polymerases α , ϵ and δ (37,38), RNA polymerase II and T7 RNA polymerase (39), although DNA polymerase β can bypass adducts (38). This hinderance of nuclease and polymerase action is likely to play a significant role in the inhibition of repair processes following concurrent chemoradiation, where the ends of DSBs produced in response to irradiation are unlikely to be compatible and may need to be trimmed and filled-in prior to ligation.

In conclusion, using a cell-free extract system and an assay for NHEJ we have shown that the presence of a single cisplatin adduct near a DSB reduces NHEJ efficiency and that this occurs at cellular cisplatin adduct levels commensurate with clinical doses of concurrent chemoradiation. Therefore, one mechanism by which cisplatin may exert its radiosensitizing effect is by reducing the efficiency of NHEJ. In addition, this work demonstrates the limitations of the use of DNA substrates containing any cisplatin adducts internal to the DNA ends for determining the effect of cisplatinated substrate termini on NHEJ.

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