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Specific adducts recognised by a monoclonal antibody against cisplatin-modified DNA

Emma L. Meczes [a], Ali Azim-Araghi [a,1], Christopher J. Ottley [b], D. Graham Pearson [b], Michael J. Tilby [a,*]

[a] *Northern Institute for Cancer Research, Paul O'Gorman Building, Medical School, University of Newcastle, Framlington Place, Newcastle Upon Tyne NE2 4HH, UK*
[b] *Arthur Holmes Isotope Geology Laboratory, Department of Earth Sciences, University of Durham, Science Laboratories, South Road, Durham City DH1 3LE, UK*

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Abstract

Numerous clinical or experimental studies have employed monoclonal antibody CP9/19 for quantification of cisplatin DNA adducts. The nature of adducts recognised by CP9/19 on polymeric DNA were defined using synthetic deoxynucleotides reacted with cisplatin. Total adduct levels were determined by atomic absorption spectrometry. The nature of adducts formed were confirmed by analysis of enzymatic hydrolysates using an established ion-exchange chromatography method combined with inductively coupled plasma mass spectrometry. Of the Pt bound to oligonucleotide A (TTTTTGGTTTTTGGTTTTTGGTTTTTGGTTTTT), 77% was recovered in a product consistent with the expected 1,2 intra-strand cross-link between GG. For oligonucleotide B (TTTTTAGTTTTTAGTTTTTAGTTTTTAGTTTTT), 62% of the bound Pt was recovered in a product consistent with the 1,2 intra-strand cross-link between AG. Of Pt bound to oligothymydylic acid, 65% was recovered in a product not previously described, small quantities of which were also formed on oligonucleotides A and B. The concentrations of adducts required to cause 50% reduction of signal in a competitive enzyme-linked immunosorbant assay (ELISA) (*K*-values) were determined. Adducts on sequences containing no guanine or only non-adjacent guanine residues, including sequences containing adenines adjacent to guanines, exhibited low or undetectable immunoreactivities (*K*-values = from 1 to >100 pmoles Pt per assay well). Adducts formed on oligodeoxynucleotides containing guanine doublets interspersed amongst thymine residues were the most immunoreactive (*K*-values: 2–7 fmoles adduct per assay well), comparable to adducts on calf-thymus DNA. The only cisplatin–DNA adducts recognised with high sensitivity by antibody CP9/19 were those involving adjacent guanine residues but immunorecognition of these was influenced by the surrounding DNA sequence.

Keywords: Cisplatin; DNA adduct; Immunoassay; Oligonucleotide; ICP-MS; Ion-exchange chromatography

1. Introduction

Platinum-based drugs, such as cisplatin and carboplatin are key agents in the management of many common human cancers. Optimisation of the use of these drugs is limited, in part, by lack of understanding of their mechanisms of action and the causes of inter-patient variation in response. These drugs exert their cytotoxic effects by covalent modification of cellular DNA. Therefore, knowledge of the levels and types of modifications in both clinical and experimental settings is important for a full understanding of how cells respond to treatment. Immunological methods have provided the necessary sensitivity for quantification of clinically relevant low levels of DNA modifications induced by anti-cancer drugs and other carcinogens [1]. A monoclonal antibody that recognizes DNA adducts formed by cisplatin and carboplatin (CP9/19) was described previously [2] and has been used to quantify DNA adducts formed in patients during therapy [3–5] as well as in various experimental investigations, e.g. [6–13]. This antibody has also been recently used for isolation of DNA fragments carrying adducts to enhance the sensitivity of subsequent PCR-based analyses [14] and is central to

* Corresponding author. Tel.: +44 191 246 4419; fax: +44 191 246 4301.
E-mail address: M.J.Tilby@ncl.ac.uk (M.J. Tilby).
[1] Present address: Cancer Biomarkers and Prevention Group, Biocentre, University of Leicester, University Road, Leicester LE1 7RH, UK.

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ongoing studies of variation in the nature of cisplatin adducts formed in different cell lines [15].

Antibody CP9/19 was raised against drug-modified polymeric DNA and unlike other antibodies, can be used to efficiently detect DNA modification by enzyme-linked immunosorbant assay (ELISA) using high molecular weight DNA [2,3,16], by immunofluorescence microscopy [8,12] and by immunoseparation of DNA fragments [14]. However, ascertaining the exact specificity of an antibody raised against modified polymeric DNA is difficult because the immunogen generally carries a variety of DNA modifications. An additional potential complication is that the interaction of an antibody with an adduct on polymeric DNA may be influenced by the sequence environment in which the adduct resides [17].

Previously available evidence indicated that CP9/19 recognised adducts formed by cisplatin but not its *trans*-isomer or a monofunctional platinum-complex. Also, adducts on native DNA were only efficiently recognised after denaturation of the DNA [2]. Since monoclonal antibody CP9/19 has been important in a range of investigations and has been made widely available, detailed knowledge of its specificity is pertinent to several past and ongoing applications. For example, the relative yields of the most frequent classes of adduct was reported to differ between cisplatin and carboplatin [18]. If an assay method is specific for just one type of the adducts, then results of assays of adduct levels following carboplatin administration may not be directly comparable to those detected after cisplatin administration. This could help explain observed lower adduct levels in patients treated with carboplatin [3].

The present report describes studies based on the use of synthetic oligodeoxynucleotides to restrict the types of adducts formed during reaction with cisplatin in order to test the hypothesis that antibody CP9/19 recognises only the intra-strand cross-links formed by cisplatin between adjacent purines. The identities of adducts on the oligodeoxynucleotides were confirmed by analysis of the products of enzymatic hydrolysis using a novel combination of ion-exchange chromatography and inductively coupled plasma mass spectrometry (ICP-MS). This revealed the presence of a new type of DNA adduct. Analysis of the oligodeoxynucleotides by competitive ELISA defined the nature of adducts recognised by antibody CP9/19.

2. Materials and methods

2.1. Preparation of cisplatin-modified DNA

Oligodeoxynucleotides were synthesised using an Applied Biosystems model 392 synthesiser, using conventional phosphoramidite chemistry. Defined sequence double-stranded polymeric DNA preparations were from Sigma and highly purified calf-thymus DNA from Merck. Each preparation of oligodeoxynucleotide, polymer or calf-thymus DNA was incubated with cisplatin (from Sigma) in 50 mM sodium phosphate (pH 7.0, 37 °C, 24 h). Each solution (2 mL) was then dialysed (7000 MW cut off) against 2× 5 L of phosphate buffer over 24 h (20 °C) to remove unreacted drug and to permit second-arm reactions to go to completion. The DNA concentrations of the resulting preparations were determined from OD_{260} measurements using estimates of extinction coefficients based on nucleotide sequence [19]. Platinum concentrations were measured by atomic absorption spectrometry.

2.2. Atomic absorption spectrometry

This was performed using a Perkin-Elmer A Analyst 600 graphite furnace spectrometer as described previously [20] using samples hydrolysed in HCl (1 M, 70 °C, 18 h).

2.3. Enzyme-linked immunosorbant assays

ELISAs were carried out as described previously [2]. In brief, mixtures of a standard dilution of antibody with varying concentrations of the nucleic acid to be tested were applied to wells of 96-well plates that had been coated with a standard quantity of cisplatin-modified DNA. The amount of antibody that became bound to the wells was determined by a fluorogenic assay. All nucleic acids samples were heated (100 °C, 5 min) and cooled in ice immediately before an assay. The quantity of adducts necessary to give 50% inhibition of antibody binding (*K*-value) were determined as described previously [21]. *K*-values are inversely proportional to adduct immunoreactivities.

2.4. Anion-exchange chromatography

Cisplatin-modified synthetic oligodeoxynucleotides and calf-thymus DNA were digested to mononucleotides according to Ref. [22] except that the ratio of enzyme to DNA was 20 μg nuclease P1 and 12 units DNAase I per 100 μg DNA. Separation of cisplatin–DNA adducts, was carried out by anion-exchange chromatography using a Mono-Q HR 5/5 column (Amersham-Pharmacia) according to the method of [22] and Azim-Araghi et al. [23]. Before injection, samples were filtered (Microcon YM-10 filter, Millipore). Fractions (500 μL) were collected and diluted in nitric acid to give a final nitric acid concentration of 3.5% (w/w).

2.5. Inductively coupled plasma mass spectrometry (ICP-MS)

The platinum concentrations in chromatography fractions were determined using ICP-MS as described by Azim-Araghi et al. [23]. In brief, samples were delivered via a standard cross-flow nebuliser and Scott-type double-pass spray chamber into a Perkin-Elmer Sciex Elan

6000 ICP-MS. RF power was generally 1150 W. Nebuliser gas flow rates varied between 0.8 and 1.0 L/min and were optimised to keep the production of CeO^+ less than or equal to 3% of the total Ce^+ signal. Minimisation of oxide formation is important in terms of reducing any potential interferences from HfO^+ species on the Pt mass spectrum. Four isotopes of platinum were monitored, 194 Pt (32.97% abundance), 195 Pt (33.83% abundance), 196 Pt (25.24% abundance) and 198 Pt (7.18% abundance) to evaluate possible isobaric interferences. Interference-free spectra should yield the correct isotope ratios after accounting for instrumental mass discrimination.

3. Results

3.1. Reaction of cisplatin with nucleic acids

The sequences of the synthetic nucleic acids reacted with cisplatin are shown in Tables 1 and 2. The molar ratio of drug to oligodeoxynucleotide in the reaction mixtures was sufficiently low so as to ensure, on average, less than one adduct per oligodeoxynucleotide molecule. Final adduct levels were determined as described in Section 2.

The nucleic acids are considered in four groups (Tables 1 and 2) containing: (a) no guanine; (b) guanine bases separated from each other by one or more pyrimidine bases; (c) guanine adjacent to adenine; (d) at least one pair of adjacent guanine bases.

Cisplatin became bound to all the oligodeoxynucleotides (Tables 1 and 2). In general, those containing no guanines, particularly those containing only thymine, showed the lowest efficiencies of platinum binding. The proportion of drug that became bound to the nucleic acids was highest when guanine and/or cytosine were present in the sequence.

3.2. Detection of adducts by anion-exchange chromatography

The nature of adducts formed by cisplatin on the oligodeoxynucleotides were investigated using the ion-exchange chromatography method developed by Fichtinger-Schepman et al. [22], except that platinum in collected fractions

Table 1
Extent of formation and immunoreactivity of cisplatin adducts on DNA molecules containing no guanine bases or guanines not adjacent to each other

DNA description	Adducts per 100 bases	Extent of binding[a] (%)	*K*-value[b] (pmoles Pt/well)	
			Mean	S.D.
No G				
AAAAAAAAAAAAAAAAAAAAAAAA	0.88	18	279	43.4
AAAAAAAAAAAAAAAAAAAAAAAA	1.3	27	>260	n.a.
AAAAAAAAAAACTAAAAAAAAAAA	1.1	23	>190	n.a.
AAAAAAAAAAACCAAAAAAAAAAA	1.6	35	8.3	0.25
AAAAAAAAAAATCAAAAAAAAAAA	1.3	28	14.5	3.52
CCCCCCCCCCCCCCCCCCCCCCCC	1.7	58	12.9	1.78
TTTTTTTTTTTTTTTTTTTTTTT	0.24	7	0.82	0.06
TTTTTTTTTTTTTTTTTTTTTTT	0.76	12	1.72	0.18
TTTTTTTTTTTTTTTTTTTTTTT	0.68	21	2.62	0.15
TTTTTTTTTTTTTTTTTTTTTTT	0.32	10	5.65	0.61
TTTTTTTTTTACCATTTTTTTTTT	0.98	29	4.62	0.83
G adjacent to C or T				
CCGCCGCCGCCGCCGCCGCCGCCGCC	2.5	81	2.58	0.32
CGCGCGCGCGCGCGCGCGCGCGCG	1.9	55	3.55	0.69
CCCCGCCCCGCCCCGCCCCGCCCC	2.1	71	7.60	1.31
TTGTTGTTGTTGTTGTTGTTGTT	1.4	46	1.36	0.34
TTTTTGCTTTTTGCTTTTTGCTTTTT	1.9	59	2.42	0.3
TGTGTGTGTGTGTGTGTGTGTG	3.3	88	3.14	0.37
TTTTGTTTTGTTTTGTTTTGTTTT	1.2	34	3.43	0.61
TTTTTGTTTTTTGTTTTTTGTTTTTT	1.5	43	7.80	2.99
Poly(dGdC)·poly(dGdC)	0.07	43	>91	n.a.
Poly(dGdC)·poly(dGdC)	0.13	38	>49	n.a.
G adjacent to A				
TTTTTAGTTTTTAGTTTTTAGTTTTT	2.3	63	3.34	0.47
TTTTTTTTTTTTAGTTTTTTTTTTTT	1.2	35	13.3	4.63
AGAGAGAGAGAGAGAGAGAGAGAG	4.0	86	0.35	0.06
AAGAAGAAGAAGAAGAAGAAGAAGAA	1.8	38	0.47	0.09
TTTTTGATTTTTGATTTTTGATTTTT	1.6	46	0.18	0.01
TTTTTTTTTTTTGATTTTTTTTTTTT	1.2	36	0.47	0.14

[a] Proportion of cisplatin that became bound to DNA.
[b] *K*-values are each the mean of at least three separate ELISA experiments.

Table 2
Extent of formation and immunoreactivity of cisplatin adducts on DNA molecules containing adjacent guanines

DNA description	Adducts per 100 bases	Extent of binding[a] (%)	*K*-value[b] (pmoles Pt/well)	
			Mean	S.D.
CCGGCCGGCCGGCCGGCCGGCCGGCC	3.6	> 95	0.020	0.007
CCCCCCCCCCCCCGGCCCCCCCCCCCC	1.5	48	0.035	0.018
AAAAAAAAAAATGGTAAAAAAAAAAA	3.7	63	0.045	0.008
AAAAAAAAAAAAGGAAAAAAAAAAAA	2.0	41	0.228	0.032
GGGGGGGGGGGGGGGGGGGGGGGG	3.0	73	0.080	0.01
GGGGGGGGGGGGGGGGGGGGGGGG	2.7	65	0.110	0.03
poly(dG)·poly(dC)	0.26	>95	0.307	0.16
poly(dG)·poly(dC)	0.45	>95	0.143	0.04
TTAGGGTTAGGGTTAGGGTTAGGG	2.7	67	0.040	0.01
TTGGTTGGTTGGTTGGTTGGTTGGTT	3.6	>95	0.012	0.003
TTTTTGGTTTTTGGTTTTTGGTTTT-TGGTTTTTGGTTTTTGGTTTTT	2.7	77	0.002	0.001
TTTTTGGTTTTTGGTTTTTGGTTTTT	2.6	77	0.006	0.002
TTTTTTTGGTTTTTTTGGTTTTTTTT	2.4	73	0.007	0.004
TTTTTGGTTTTTGGTTTTTGGTTTTT	2.2	63	0.007	0.003
TTTTTTTTTTTTGGTTTTTTTTTTTT	1.4	42	0.009	0.003
TTTTTTTTTTTTGGTTTTTTTTTTTT	2.2	45	0.037	0.009
TTTTTGGTTTTTTTTTTTTTTTTTTT	1.4	42	0.009	0.004
TTTTTTTTTTTTTTTTTTTGGTTTTT	1.4	42	0.015	0.001
TTTTTGGTTTTT	3.2	>95	0.033	0.002
Calf-thymus DNA	0.086	n.d.	0.006	0.004

[a] Proportion of cisplatin that became bound to DNA.
[b] *K*-values are each the mean of at least three separate ELISA experiments.

was determined by ICP-MS. Oligodeoxynucleotides were enzymatically digested to mono and di-nucleotides. Increasing the ratio of enzymes to oligodeoxynucleotide did not affect the results, confirming that digestion was essentially complete (data not shown). Analysis of cisplatin-modified calf-thymus DNA (Fig. 1A) gave the expected pattern of OD_{254} (i.e. normal nucleotides) and the expected four Pt-containing products with retention times and relative yields essentially the same as originally described [22]. Recovery of Pt was ≥95%. The main Pt-containing products (peaks 2 and 3, containing 20 and 67% of total recovered Pt, respectively) eluted at 2.5 and 8.0 min. These had previously been identified as the intra-strand cross-links between adjacent adenine and guanine and between adjacent guanines, respectively. The products eluting at 1.0 and 11.5 min (peaks 1 and 2, containing 1.4 and 6.5% of total recovered Pt, respectively) were previously identified as a monofunctional adduct on guanine and cross-links between non-adjacent guanines, respectively.

Fig. 1B shows typical data from analysis of a platinated-oligodeoxynucleotide, which consisted of guanine doublets separated by five thymines. The peaks in OD_{254} at 6.0 and 11.9 min confirmed the presence of TMP and GMP, respectively. The majority of the Pt (77%) was recovered in a product with a retention time of 8.1 ± S.D. = 0.3 min. This is consistent with the expectation that the major product on this type of oligodeoxynucleotide was the 1,2 intra-strand cross-link between guanines. Fig. 1C shows typical data from analysis of a platinated oligodeoxynucleotide consisting of d(pApG) pairs separated by thymines. The peaks in OD_{254} at 5.8, 6.2 and 11.9 min confirmed the presence of AMP, TMP and GMP respectively. The majority of the Pt (62%) was recovered in a product with retention time of 2.5 ± S.D. = 0.04 min. This is consistent with the expectation that the main product formed on this type of oligodeoxynucleotide was an intra-strand cross-link between adjacent A and G. Both these oligodeoxynucleotides also yielded a minor peak at 6.0 min containing 15 and 27% of the injected Pt. Analysis of oligodeoxynucleotides consisting solely of thymine residues (Fig. 1D) revealed that 62–68% of the injected platinum was recovered in a single peak also with a retention time of about 6 min.

3.3. Immunoreactivity of oligodeoxynucleotides

All nucleic acids were heat denatured immediately before the immunoassays. For comparison of oligodeoxynucleotides with and without cisplatin adducts, concentration of competing antigen must be shown as μg oligodeoxynucleotide per assay well (Figs. 2 and 3A). For platinated oligodeoxynucleotides, it is important to analyse the ELISA data in relation to concentration of platinum adducts. The concentrations of platinum adducts required to cause 50% reduction in assay signal (*K*-value) were determined (Fig. 3B; Tables 1 and 2).

Following reaction with cisplatin, oligodeoxyadenylic acid showed no significant increase in immunoreactivity above that of the unmodified oligodeoxynucleotide (Fig. 2). Cisplatin-modified oligodeoxythymidylic acid and oligodeoxycytidylic acid did exhibit slightly increased levels of immunoreactivity compared to unmodified oligodeoxynu-

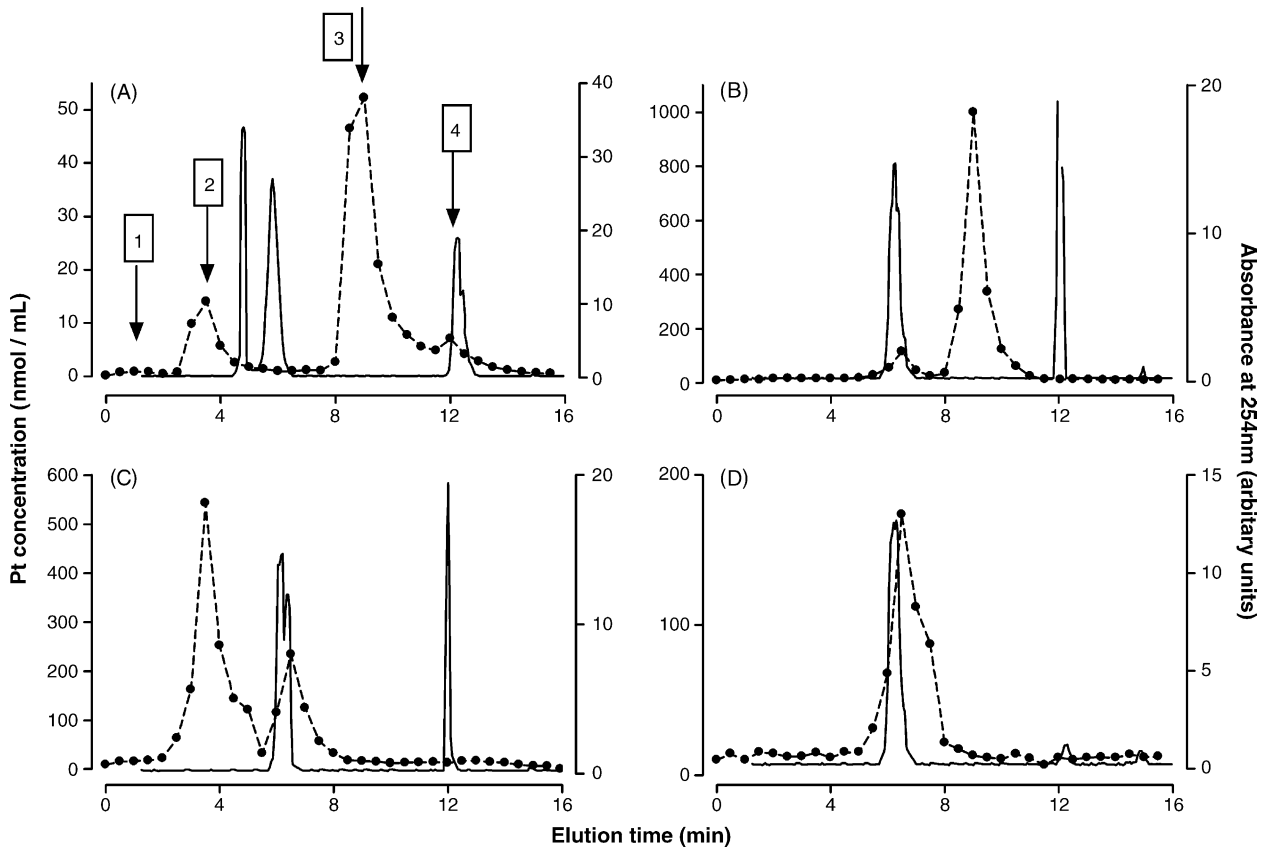

Fig. 1. Typical ion-exchange chromatography elution profiles for products formed by enzymatic hydrolysis of DNA that had been reacted with cisplatin. Elution from the MonoQ column involved a NaCl concentration gradient as described in Section 2: (A) calf-thymus DNA; (B–D) synthetic oligodeoxynucleotides of the following sequences: (B) TTTTTGGTTTTTGGTTTTTGGTTTTT; (C) TTTTTAGTTTTTAGTTTTTAGTTTTT; (D) T_{24}. OD_{254} was monitored (solid lines). Platinum concentration in each fraction was measured by ICP-MS (●). Adduct levels were 0.1, 2.1, 2.1 and 0.24 Pt per 100 bases for (A–D), respectively. For explanation of peak numbering, see text.

cleotides (Fig. 2). Adducts on oligodeoxynucleotides containing non-adjacent guanine residues exhibited immunoreactivities similar to adducts on oligodeoxynucleotides lacking guanine (Fig. 3B; Table 1). The presence of an adenine residue 5′ to a guanine did not further increase the immunoreactivity of the resulting adducts, however, adducts

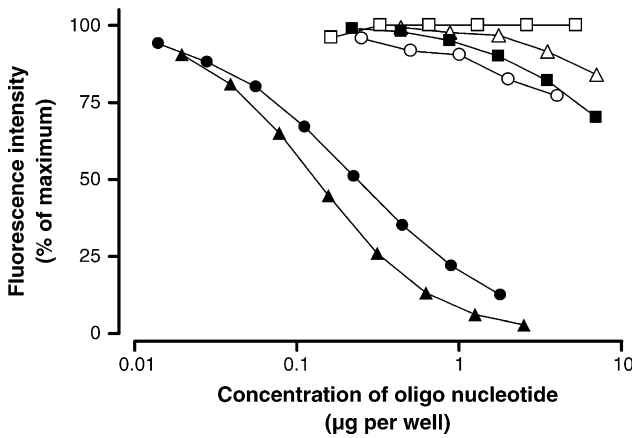

Fig. 2. Typical ELISA data for oligodeoxynucleotides lacking guanine residues with (▲, ●, ■) and without (△, ○, □) modification by cisplatin. Sequences: T_{24} (▲, △); C_{24} (●, ○); A_{24} (■, □). Each point represents the mean of four replicate assay wells. S.D.s lie within the symbols.

on oligodeoxynucleotides containing adenine residues 3′ to guanines were approximately 10-fold more immunoreactive with *K*-values in the range 200–500 fmoles adduct/well.

Non-platinated oligodeoxynucleotides containing adjacent guanines were no more immunoreactive than other non-platinated oligodeoxynucleotides (Fig. 3A), but reaction of these with cisplatin caused a much greater increase in immunoreactivity (Fig. 3A). Adducts on oligo dG showed higher immunoreactivities than adducts on oligodeoxynucleotides lacking adjacent guanines (Table 2; Fig. 3B; $K = 100$ fmoles/well). However, the most immunoreactive adducts were those formed on oligodeoxynucleotides containing guanine doublets interspersed amongst thymine residues. The immunoreactivities of these adducts were comparable to those of adducts formed on calf-thymus DNA with *K*-values down to 2 fmoles adduct/assay well.

3.4. Double-stranded polydeoxynucleotides

Double-stranded polydeoxynucleotides, poly(dG)·poly(dC) and poly(dGdC)·poly(dGdC), were each reacted with cisplatin at two different molar ratios. The adducts formed on poly(dGdC)·poly(dGdC) had a very low immu-

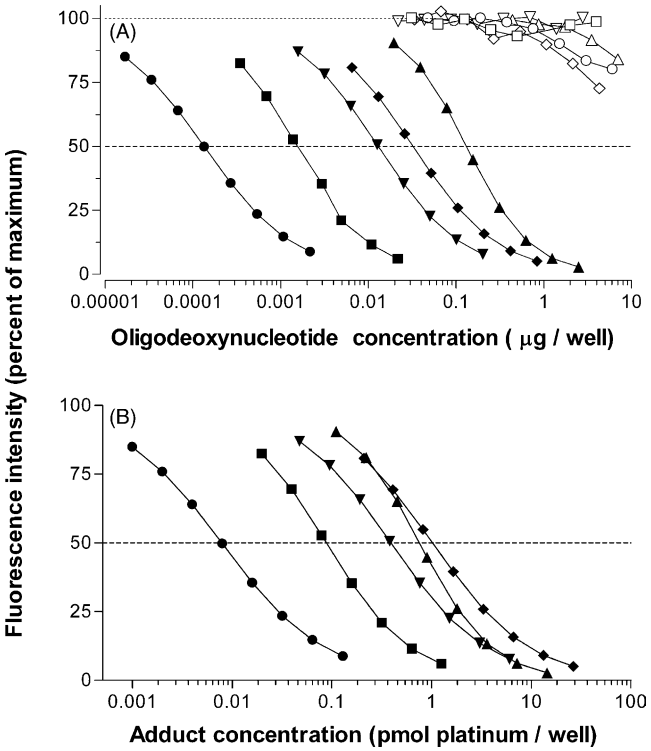

Fig. 3. (A and B) Typical ELISA data for guanine-containing oligodeoxynucleotides with (▲, ●, ■, ▼, ◆) or without (△, ○, □, ▽, ◇) modification by cisplatin. Sequences: TTTTTGGTTTTTGGTTTTGGTTTTT (●, ○); G_{24} (■, □); AAGAAGAAGAAGAAGAAGAAGAAGAA (▼, ▽); TTGTTGTTGTTGTTGTTGTTGTTGTT (◆, ◇); T_{24} (▲, △). Each point represents the mean of four replicate assay wells. S.D.s lie within the symbols.

noreactivity with *K*-values that were too high to measure (>50 pmol/well) (Table 1). The adducts formed on poly(dG)·poly(dC) exhibited an immunoreactivity (mean *K*-value = 0.22 pmoles adduct/well, Table 2) similar to that for adducts on oligo dG.

4. Discussion

Reaction of cisplatin with DNA leads to the formation of two major and two minor adducts. The major adducts are intra-strand cross-links between adjacent guanine bases and between adjacent adenine and guanine. Together, these constitute around 90% of the total adducts formed. The minor adducts result from cisplatin monofunctionally bound to guanine and from cross-links between non-adjacent guanines on the same and opposite strands [22,24]. Monoclonal antibody CP9/19 was raised against cisplatin-modified calf-thymus DNA. A rabbit anti-serum that appeared to recognize both the major adducts [25] showed preferential recognition of regions of DNA carrying particularly high adduct levels [26]. In contrast, antibody CP9/19 detected adducts present at high and low frequencies on DNA with equal sensitivity [2]. It was anticipated that this antibody recognised one or both of the major adducts, especially since they cause conformational change to DNA [27]. The nature of the recognised adduct(s) remained uncertain but was relevant to a growing number of applications.

Antibody CP9/19 recognizes adducts in high molecular weight DNA and not in enzymatic hydrolysates. Therefore, characterisation of its specificity required DNA polymers carrying single specific types of adduct. To approach this ideal, cisplatin was reacted with oligodeoxynucleotides designed to restrict the types of adducts that could form. Previous evidence indicated that cisplatin does not form adducts on thymidine [28–30] and this was consistent with the lack of thymine adducts in cisplatin-treated calf-thymus DNA reacted with cisplatin or *cis*-dichloro(ethylenediamine)platinum(II) [22,30]. The oligodeoxynucleotides used here were designed on this basis. It was necessary to use single-stranded oligodeoxynucleotides because the presence of the complimentary strands would have introduced additional reaction sites. The oligonucleotides used here were designed to achieve selective formation of particular adducts and do not necessarily reflect any specific sequences found in cellular DNA. Antibody CP9/19 was known to recognize adducts formed on denatured DNA and because of the lack of recognition of adducts in native DNA, all DNA samples have to be denatured before application of the antibody. Thus, immunoassays with single-stranded oligonucleotides were relevant to applications of CP9/19 to the analysis of adducts formed in cells. However, it seemed possible that reaction of single-stranded oligodeoxynucleotides with cisplatin might yield adducts not completely representative of adducts formed on native DNA. Combination of ICP-MS with ion-exchange chromatography permitted analysis of relatively small quantities of platinated oligodeoxynucleotides. The results obtained confirmed that 1,2 GG and AG intra-strand cross-linked products were the main adducts formed when the appropriate sequence was present in the oligodeoxynucleotides. However, in addition, a novel product was observed. This appears to involve thymine since it was formed in oligo dT molecules. When guanine dimers were included in the sequence of oligo dT, most of the cisplatin reacted with the G and only a minority of the adducts were of the proposed thymine type (Fig. 1B). This indicates that as expected, cisplatin reacts much more readily with guanine than alternative sites. Consistent with previous reports, thymine adducts were not observed in analyses of calf-thymus DNA reacted with cisplatin (Fig. 1A). The formation of this product in the oligodeoxynucleotides but not in calf-thymus DNA was probably due to the higher thymine content and/or single-stranded nature of the oligodeoxynucleotides. Ongoing studies are investigating the significance of preliminary evidence that this adduct may form to a small extent in cells.

Cisplatin adducts formed on oligodeoxyguanylic acid or poly dG·poly dC showed immunoreactivity at levels about 20-fold lower than the average immunoreactivity of adducts on calf-thymus DNA. When G-dimers were inter-

spersed in oligodeoxythymidylic acid sequences the immunoreactivity of adducts was markedly higher and approached that of adducts on calf-thymus DNA. Conformation and other properties of oligodeoxyguanylic acid presumably influence antibody affinity.

In native DNA, cross-links between adjacent adenine and guanine residues only form, where the adenine lies 5′ to the guanine (dApG) [22,31]. In oligodeoxynucleotides, the expected cross-link between d(pApG) was confirmed by chromatography and they were found to be only very weakly immunoreactive. Surprisingly, adducts on oligos containing d(pGpA) were slightly more immunoreactive than adducts on d(pApG).

The present results indicate that in the competitive ELISA situation, the only cisplatin–DNA adducts recognised with high sensitivity by antibody CP9/19 are cross-links between adjacent guanine residues. These are the most frequent type of adduct formed by cisplatin both in vitro and in vivo [32,33]. They are recognised by a number of cellular proteins and are thought to play an important role in drug action [34]. In contrast, inter-strand cross-links only constitute about 1% of the total adducts formed by cisplatin but are also thought to be important for cytotoxicity [35]. Most available evidence indicates that the various types of cisplatin–DNA adducts are formed in the same relative proportions in different cell types, e.g. [36–39]. This indicates that data from immunoassays using CP9/19 on samples removed at short times after drug exposure reflects the overall adduct level. However, differential actions of repair processes might act to modify the proportion of at least certain types of adducts [40].

The data presented also indicates that recognition of the cisplatin adducts by antibody CP9/19 is influenced by the surrounding DNA sequence. Differences in the type of adduct and/or their distribution in the genome could, therefore, give rise to differences in adduct levels detected using CP9/19 even when the total amount of platinum bound to the DNA is constant. This consideration may be relevant to understanding both the greater inter-patient variability in adduct levels when adducts were measured using immunoassay than by measurement of total DNA-bound platinum [3,5,41,42] and the atypical immunoreactivity of adducts formed in certain cell lines [15,23]. This specificity is probably reflected in the behaviour of antibody CP9/19 in non-competitive techniques, such as immunoaffinity-isolation of DNA fragments [14], where only certain DNA fragments carrying cisplatin adducts would be obtained and immunohistochemistry [12,13]. However, particularly in immunohistochemistry, the influence of factors, such as extent of local DNA denaturation and antibody access could also be significant.

An influence of neighbouring nucleotides on immunorecognition of DNA adducts, as seen with antibody CP9/19, was also observed with a monoclonal antibody that recognizes DNA modifications formed by melphalan [17]. For any antibody raised against modified polymeric DNA, the possibility should be considered that sensitivity of detection of adducts could be influenced by local DNA sequence as well as by the overall level of DNA modification. Both these factors need to be defined and taken into account when interpreting results since differences in adduct level could be either exaggerated or hidden as a result of variation in the pattern of adduct formation by different drugs or in the pattern of repair of adducts by different cell types. In addition, some observations indicate the existence of, as yet uncharacterised, differences between cell types in the relative yields of different types of adducts [15,43]. Previous studies have indicated inter-patient variation in levels of immunoreactive adducts and this was not attributable to differences in plasma pharmacokinetics [3,5]. These observations could be explained, at least in part, by the factors discussed above. Furthermore, the specific recognition of cross-links between adjacent guanines might account for the generally lower levels of immunoreactive adducts in patients treated with carboplatin compared to those treated with cisplatin [3]. For our current clinical studies we favour, where possible, the extra information that results from dual application of both the immunoassay and the sensitive quantification of total adducts by ICP-MS [44].

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References

[1] Santella RM. Immunological methods for detection of carcinogen—DNA damage in humans. Cancer Epidemiol Biomarkers Prev 1999;8:733–9.
[2] Tilby MJ, Johnson C, Knox R, Cordell J, Roberts JJ, Dean CJ. Sensitive detection of DNA modifications induced by cisplatin and carboplatin in vitro and in vivo using a monoclonal antibody. Cancer Res 1991;51:123–9.
[3] Peng B, Tilby MJ, English MW, Price L, Pearson ADJ. Boddy AV, et al. Platinum–DNA adduct formation in peripheral blood leucocytes of children in relation to pharmacokinetics following cisplatin and carboplatin therapy. Br J Cancer 1997;76:1466–73.
[4] Ghazal-Aswad S, Tilby MJ, Lind M, Baily N, Sinha D, Calvert AH, et al. Pharmacokinetically guided dose escalation of carboplatin in epithelial ovarian cancer: effect on drug-plasma AUC and peripheral blood drug–DNA adduct levels. Ann Oncol 1999;10:329–34.
[5] Veal GJ, Dias E, Price E, Parry A, Errington J, Hale J, et al. Influence of cellular factors and pharmacokinetics on the formation of platinum–DNA adducts in leucocytes of children receiving cisplatin therapy. Clin Cancer Res 2001;7:2205–12.
[6] Cross HJ, Tilby MJ, Chipman JK, Ferry DR, Gescher A. Effect of quercetin on the genotoxic potential of cisplatin. Int J Cancer 1996;66:404–8.
[7] Evans DL, Tilby MJ, Dive C. Differential sensitivity to the induction of apoptosis by cisplatin in proliferating and quiescent immature rat

thymocytes is independent of the levels of drug accumulation and DNA adduct formation. Cancer Res 1994;54:1596–603.

[8] Fichtinger-Schepman A-MJ, Welters MJP, van Dijk-Knijnenburg HCM, van der Sterre MLT, Tilby MJ, Berends F, et al. Detection of adducts formed upon treatment of DNA with cisplatin and carboplatin. In: Pinedo HM, Schornagel JH, editors, Platinum and other metal coordination compounds in cancer chemotherapy, vol. 2. Proceedings of the 7th international symposium, Amsterdam, 1995. New York and London: Plenum Press; 1996. p. 107–19.

[9] Deverman BE, Cook BL, Manson SR, Niederhoff RA, Langer EM, Rosova I, et al. Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage. Cell 2002;111:51–62.

[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: 3848–56.

[11] Mellish KJ, Barnard CF, Murrer BA, Kelland LR. DNA-binding properties of novel *cis*- and *trans* platinum-based anticancer agents in 2 human ovarian carcinoma cell lines. Int J Cancer 1995;62:717–723.

[12] Deverman BE, Cook BL, Manson SR, Niederhoff RA, Langer EM, Rosova I, et al. Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage. Cell 2002;111:51–62.

[13] Solomon DA, Cardoso MC, Knudsen ES. Dynamic targeting of the replication machinery to sites of DNA damage. J Cell Biol 2004;166: 455–63.

[14] McGurk CJ, McHugh PJ, Tilby MJ, Grimaldi KA, Hartley JA. Measurement of covalent drug–DNA interactions at the nucleotide level in cells at pharmacologically relevant doses. Methods Enzymol 2001;340:358–76.

[15] Azim-Araghi A, Ottley CJ, Pearson DG, Tilby MJ. Structures of atypical platinum–DNA adducts in certain resistant lung cancer lines. Proc Am Assoc Cancer Res 2002;43:262.

[16] Tilby MJ. Production of monoclonal antibodies for the detection of chemically modified DNA: methods in molecular biology. In: Manson M, editor. Immunochemical protocols, vol. 10. Humana Press; 1992. p. 321–8.

[17] McCartney H, Martin AM, Middleton PG, Tilby MJ. Antibody recognition of melphalan adducts characterised using immobilized DNA: enhanced alkylation of G-rich regions in cells compared to in vitro. Chem Res Toxicol 2001;14:71–81.

[18] Fichtinger-Schepman A-MJ, van Dijk-Knijnenburg HCM, van der Velde-Visser SD, Berends F, Baan RA. Cisplatin- and carboplatin–DNA adducts: is Pt-AG the cytotoxic lesion? Carcinogenesis 1995;16:2447–53.

[19] Kallansrud G, Ward B. A comparison of measured and calculated single- and double-stranded oligodeoxynucleotide extinction coefficients. Anal Biochem 1996;236:134–8.

[20] Ghazal-Aswad S, Calvert AH, Newell DR. A single-sample assay for the estimation of the area under the free carboplatin plasma concentration versus time curve. Cancer Chemother Pharmacol 1996;14:800–5.

[21] Tilby MJ, Styles JM, Dean CJ. Immunological detection of DNA damage caused by melphalan using monoclonal antibodies. Cancer Res 1987;47:1542–6.

[22] Fichtinger-Schepman A-MJ, van der Veer JL, den Hartog JHJ, Lohman PHM, Reedijk J. Adducts of the drug *cis*-diamminedichloroplatinum(II) with DNA: formation, identification and quantitation. Biochemistry 1985;24:707–13.

[23] Azim-Araghi A, Ottley CJ, Pearson DG, Tilby MJ. Combination of ICP-MS and ion exchange chromatography permits analyses of specific cisplatin DNA modifications formed in cancer cells. In: Plasma source mass spectrometry. Special Publications, Royal Society of Chemistry; 2003. p. 66–73.

[24] Fichtinger-Schepman A-MJ, Baan RA, Luiten-Schuite A, van Dijk M, Lohman PHM. Immunochemical quantitation of adducts induced in DNA by *cis*-diamminedichloroplatinum(II) and analysis of adduct-related DNA-unwinding. Chem Biol Interact 1985;55:275–88.

[25] Reed E, Gupta-Burt S, Litterst CL, Poirier MC. Characterization of the DNA damage recognised by an antiserum elicited against *cis*-diamminedichloroplatinum(II)-modified DNA. Carcinogenesis 1990;11: 2117–21.

[26] Fichtinger-Schepman A-MJ, Baan RA, Berends F. Influence of the degree of DNA modification on the immunochemical determination of cisplatin–DNA adduct levels. Carcinogenesis 1989;10:2367–9.

[27] Gelasco A, Lippard SJ. NMR solution structure of a DNA dodecamer duplex containing a *cis*-diammineplatinum(II) d(GpG) intrastrand cross-link, the major adduct of the anticancer drug cisplatin. Biochemistry 1998;37:9230–9.

[28] Mansy S, Rosenberg B, Thomson AJ. Binding of *cis*- and *trans*-dichlorodiammineplatinum(II) to nucleosides. Part I: location of the binding sites. J Am Chem Soc 1973;95:1633–40.

[29] Mansy S, Chu GYH, Duncan RE, Tobias RS. Heavy metal nucleotide interactions. Part 12: competitive reactions in systems of four nucleotides with *cis*- or *trans*-diammineplatinum(II). Raman difference spectrophotometric determination of the relative nucleophilicity of guanosine, cytidine, adenosine, and uridine monophosphates as well as the analogous bases in DNA. J Am Chem Soc 1978;100:607–16.

[30] Eastman A. Characterization of the adducts produced in DNA by *cis*-diamminedichloroplatinum(II) and *cis*-dichloro(ethylenediamine)platinum(II). Biochemistry 1983;22:3927–33.

[31] Eastman A. Re-evaluation of interaction of *cis*- dichloro(ethylenediamine)platinum(II) with DNA. Biochemistry 1986;25:3912–5.

[32] Dijt FJ, Fichtinger-Schepman A-MJ, Berends F, Reedijk J. Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair-deficient human fibroblasts. Cancer Res 1988;48:6058–62.

[33] Fichtinger-Schepman A-MJ, van der Velde-Visser SD, van Dijk-Knijnenburg HCM, van Oosterom AT, Baan RA, Berends F. Kinetics of the formation and removal of cisplatin–DNA adducts in blood and tumor tissue of cancer patients receiving chemotherapy: comparison with in vitro adduct formation. Cancer Res 1990;50:7887–94.

[34] Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov 2005;4:307–20.

[35] Knox R, Friedlos F, Lydall DA, Roberts JJ. Mechanism of cytotoxicity of anticancer platinum drugs: evidence that *cis*-diamminedichloroplatinum(II) and *cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA. Cancer Res 1986;46:1972–9.

[36] Plooy ACM, Fichtinger-Schepman A-MJ, Schutte HH, van Eijk M, Lohman PHM. The quantitative detection of various Pt-DNA–adducts in Chinese hamster ovary cells treated with cisplatin: application of immunochemical techniques. Carcinogenesis 1985;6:561–6.

[37] Fichtinger-Schepman A-MJ, van Oosterom AT, Lohman PHM, Berends F. *cis*-Diamminedichloroplatinum(II)-induced DNA adducts in peripheral leukocytes from seven cancer patients: quantitative immunochemical detection of the adduct induction and removal after a single dose of *cis*-diamminedichloroplatinum(II). Cancer Res 1987;47:3000–4.

[38] Fichtinger-Schepman A-MJ, Dijt FJ, Bedford P, van Oosterom AT, Hill BT, Berends F. Induction and removal of cisplatin–DNA adducts in human cells in vivo and in vitro as measured by immunochemical techniques. IARC 1988;1987:321–8.

[39] Hill BT, Shellard SA, Fichtinger-Schepman AM, Schmoll HJ, Harstrick A. Differential formation and enhanced removal of specific cisplatin–DNA adducts in two cisplatin-selected resistant human testicular teratoma sublines. Anticancer Drugs 1994;5:321–8.

[40] Bedford P, Fichtinger-Schepman A-MJ, Shellard SA, Walker MC, Masters JRW, Hill BT. Differential repair of platinum–DNA adducts in human bladder and testicular tumor continuous cell lines. Cancer Res 1988;48:3019–24.

[41] Schellens JHM, Ma J, Planting ASTh, van der Burg MEL, van Meerten E, de Boer-Dennert M, et al. Relationship between the exposure to

cisplatin, DNA-adduct formation in leucocytes and tumour response in patients with solid tumours. Br J Cancer 1996;73:1569–75.

[42] Bonetti A, Apostoli P, Zaninelli M, Pavanel F, Colombatti M, Cetto GL, et al. Inductively coupled plasma mass spectroscopy quantitation of platinum–DNA adducts in peripheral blood leukocytes of patients receiving cisplatin- or carboplatin-based chemotherapy. Clin Cancer Res 1996;2:1829–35.

[43] Hospers GAP, de Vries EGE, Mulder NH. The formation and removal of cisplatin (CDDP) induced DNA adducts in a CDDP sensitive and resistant human small cell lung carcinoma (HSCLC) cell line. Br J Cancer 1990;61:79–82.

[44] John T, Ottley CJ, Pearson DG, Nowell GM, Calvert AH, Tilby MJ. Determination by ICP-MS, of background and BBR 3464 induced levels of platinum bound to DNA isolated from blood cells: a comparison of the sensitivity of the Perkin-Elmer Sciex Elan 6000 and the ThermoFinnigan Neptune Instruments. In: Plasma source mass spectrometry. Special Publications, Royal Society of Chemistry; 2003. p. 82–90.