The Use of Taq DNA Polymerase to Determine the Sequence Specificity of DNA Damage Caused by cis-Diamminedichloroplatinum(II), Acridine-tethered Platinum(II) Diammine Complexes or Two Analogues*

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cis-Diamminedichloroplatinum(II) (cisplatin) forms adducts with DNA. The sequence specificity of formation of cisplatin adducts with plasmid DNA was investigated using Taq DNA polymerase. This procedure involved the extension of an oligonucleotide primer by Taq DNA polymerase up to the cisplatin adduct. Using thermal cycling, this process is repeated many times in order to amplify the signal. The products of this linear amplification process would then be examined on DNA sequencing gels, and the sequence specificity of cisplatin adduct formation can be determined to the exact base pair. In the pUC8 plasmid, the sequences that produced the most intense damage sites (as determined by densitometry) were runs of two or more Gs. Adducts could also be detected at GA, AG, and GC dinucleotides. Four other cisplatin analogues were also tested in the system. Two of these analogues contained an attached intercalating chromophore, and the strong damage with these compounds was similar to that found for cisplatin, but the medium and weak damage tended to be different. Weak damage was also detected with trans-diamminedichloroplatinum(II). With this compound, a large number of the damage sites were at the CG dinucleotide. This technique represents a simple, accurate, and quick method for determining the sequence specificity of damage for a cisplatin analogue in any DNA sequence.

The abbreviations and trivial name used are: cisplatin, cis-Diamminedichloroplatinum(I1); transplatin, trans-Diamminedichloroplatinum(I1); dichloro(ethylenediammine)platinum(II); trans-Pt, trans-Diamminedichloroplatinum(II); PtCl₂, dichloro(ethylenediammine)platinum(II); 2AcC₃PtenCl₄, N-[3-N-(ethylenediamino)propyl]acridine-2-carboxamididichloroplatinum(II); 4AcC₃PtenCl₄, N-[3-N-(ethylenediamino)propyl]acridine-4-carboxamididichloroplatinum(II).

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activity against solid tumors. Its mechanism of action is at present uncertain but is thought to involve covalent adducts to DNA which result in cross-links between 2 residues. Intrastrand DNA cross-links are the most prevalent, although interstrand DNA cross-links and DNA-protein cross-links also occur. These cross-links would be expected to inhibit or stop DNA replication and RNA transcription.

The nature of the Pt adducts formed with purified DNA has been investigated after hydrolysis to nucleotides (2, 3, 4 and references therein). These studies have shown that the site of adduct formation is a cross-link involving the N² of purines. The most common lesion is an intrastrand cross-link at the dinucleotides GG (60-65%), AG (20-25%), and GNG (3-7%). Interstrand cross-links between G bases (~1%) are also present as well as monoadducts (~2%).

There are several methods to elucidate the DNA sequence specificity of cisplatin adduct formation using DNA sequencing gels. Exonuclease III (5) and DNA polymerase I (6, 7) have been used to obtain adduct sequence specificity with plasmid DNA. An indirect method, which detects distortions in DNA caused by cisplatin adducts, has also been utilized (8). DNAase I footprinting experiments have not been successful because of the size of the adduct.

The linear amplification method provides a simpler system for obtaining information on the DNA sequence specificity of cisplatin adducts. The linear amplification procedure was originally developed for genomic sequencing (9), but we have adapted it for the cisplatin experiments. The method uses Taq DNA polymerase and thermal cycling to amplify the products. Taq DNA polymerase extends from a radioactively labeled primer oligonucleotide up to the cisplatin adduct. Polymerization terminates at the adduct which results in a specific bond on a sequencing gel. By running appropriate dideoxy sequencing reactions on the same sequencing gel, the sequence specificity of cisplatin can be determined to the exact base pair.

In this paper we examine the sequence specificity of cisplatin and four analogues (see Fig. 1 for structures): trans-diamminedichloroplatinum(II) (transPt), dichloro(ethylene diammine)platinum(II) (PtenCl₂), N-[3-N-(ethylenediamino)propyl]acridine-2-carboxamididichloroplatinum(II) (2AcC₃PtenCl₄), N-[3-N-(ethylenediamino)propyl]acridine-4-carboxamididichloroplatinum(II) (4AcC₃PtenCl₄). The latter two compounds contain an attached intercalating chromophore (10). Recently, Ponti et al. (11) have used a similar method to examine the sequence specificity of cisplatin and
carboplatin adducts in plasmid DNA. This method can also be used to examine the sequence specificity of other DNA-damaging agents.

**EXPERIMENTAL PROCEDURES**

**Materials**—Established procedures were used to synthesize cisplatin and transPt (12) and PtenCl (13). Likewise, 2AcC3PtenCl and PtenC12 were synthesized as described previously (10). All complexes were dissolved to give a 1 mM stock solution in dimethylformamide. The [γ-32P]ATP was from Amersham. AmpliTaq DNA polymerase was purchased from Perkin-Elmer Cetus.

**Cisplatin Treatment**—In a total volume of 40 μl, there were 2 μg of pUC8 plasmid (14) (prepared by the alkaline lysis method) in 25 mM Tris-HCl, pH 7.8, and 0.5-50 μM cisplatin or analogue. This was incubated at 37 °C for 18 h. After adjustment of the solution to 0.3 M sodium acetate, the DNA was ethanol-precipitated and resuspended in 10 μl of 10 mM Tris-HCl, pH 8.8.

**Linear Amplification Method**—One pmol of primer oligonucleotide (the 15-base forward sequencing primer (Bethesda Research Laboratories (BRL)) 5' TCCCCAGTCACGACGT 3' (bp 363-377 in pUC8) or the 16-base reverse sequencing primer (BRL) 5' AACAGCTAT-GACCATG 3' (bp 455-440 in pUC8)] was 5'-labeled in a final volume of 10 μl with 60 mM Tris-HCl, pH 7.5, 9 mM MgCl2, 10 mM dithiothreitol, 2 units of polynucleotide kinase, and 5 μCi of [γ-32P]ATP at 37 °C for 30 min.

The linear amplification procedure was an adaption of the methods of Saluz and Just (9) and Murray (15). Two μl of damaged DNA and controls were added to (final concentrations) 16.6 mM NH4(SO4)2, 67 mM Tris-HCl, pH 8.8, 6.7 mM MgCl2, 0.2 mg/ml bovine serum albumin, 300 μM each of dATP, dGTP, dCTP, dTTP, 0.05 pmol of 5'-32P-labeled oligonucleotide, 0.5 unit of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) in a final volume of 5 μl and overlaid with 50 μl of mineral oil. In the same experiment dideoxy double-stranded DNA sequencing was performed using the same plasmid and primer oligonucleotide as DNA sequence standards (15). Linear amplification by thermal cycling was carried out at 95 °C for 30 s (time at the temperature), 50 °C for 20 s, 72 °C for 20 s for 25 cycles in a Perkin-Elmer Cetus DNA Thermal Cycler 480. Two μl of the reaction was loaded onto a 6% polyacrylamide-urea DNA sequencing gel. The gel was dried down on 3MM paper before autoradiography.

**Densitometry**—A Molecular Dynamics Computing Densitometer with ImageQuant software was used to quantify the intensity of the bands on the sequencing gel. For the drug at 5 μM, an area containing the band of interest was defined and the intensity “volume” was calculated. The intensity “volume” for the same area in the duplicate blank lanes (no cisplatin) was also calculated and subtracted from the cisplatin (or analogue) lanes. The relative intensity of damage was determined relative to an average of the intensity at bp 390, 396, 425, 456, 475, and 487 for the forward sequencing primer and bp 430, 420, 412, 347, 336, and 315 for the reverse primer. A densitometer-derived relative intensity of damage of greater than 0.7 was classified as strong, 0.3-0.7 as medium, and less than 0.3 as weak. At 5 μM transPt no damage was detected, but at 15 and 50 μM weak damage was detectable. The transPt experiments were not analyzed by densitometer because of the low intensity of the bands.

**RESULTS**

**Cisplatin Experiments**—The cisplatin-damaged pUC8 (and controls) were subjected to the linear amplification procedure using either the forward sequencing primer or the reverse primer. The products of these reactions were run on DNA sequencing gels along with dideoxy sequencing reactions which used the same 5'-labeled primer and plasmid. The results of typical experiments are shown in Figs. 2 and 3.

Figs. 2 and 3 show damage to pUC8 with the forward and reverse (respectively) sequencing primers. In lanes 1, 2, and 3 there was no cisplatin, and these lanes indicate the acceptably low background level. For lanes 4 (0.5 μM cisplatin), 9 (1 μM), and 14 (5 μM), a number of bands can be seen which correspond to cisplatin adducts, especially in lane 14. These bands are significantly above background. For lanes 4, 9, and 14 there was an increasing level of cisplatin, and the intensity of damage appears to increase across these lanes. Other experiments have indicated that cisplatin adducts can be detected at 0.1 μM, and the intensity of damage increases up to 5 μM, but at higher concentrations the damage intensity decreases.

Several plasmid/oligonucleotide combinations were tested to give the lowest background in the lanes lacking cisplatin. The plasmid pUC8 gave the lowest background, and the forward sequencing primer gave a slightly lower background than the reverse primer.

The position of the bands on the gel were mapped relative to the bands produced by the dideoxy sequencing reactions. The position of the damage bands can be determined to the nearest base pair. The sequence specificity of these damage

![Fig. 1. The chemical structures of cisplatin and four analogues.](image-url)

![Fig. 2. Autoradiograph of a DNA sequencing gel containing the linear amplification products of pUC8 DNA treated with cisplatin and four analogues using the forward sequencing primer. Lanes 1-3 are untreated controls, and lane 1 was not incubated at 37 °C or ethanol-precipitated. The DNA was treated with cisplatin in lane 4 (0.5 μM), lane 9 (1 μM), and lane 14 (5 μM); with transPt in lane 5 (0.5 μM), lane 10 (1 μM), and lane 15 (5 μM); with PtenCl in lane 6 (0.5 μM), lane 11 (1 μM), and lane 16 (5 μM); with 2AcC3PtenCl in lane 7 (0.5 μM), lane 12 (1 μM), and lane 17 (5 μM); with 4AcC3PtenCl in lane 8 (0.5 μM), lane 13 (1 μM), and lane 18 (5 μM). Note that the dideoxy sequencing lanes labeled as G, A, T, and C give the sequence on the template strand.)
bands is shown relative to the pUC8 DNA sequence in Fig. 4. The damage sites were classified into strong, medium, and weak using densitometer scanning of the gel autoradiographs, and this is indicated in Fig. 4. Most sites did not produce a single discrete band, and these extra bands are also indicated in Fig. 4.

In Table I the damage sites are ordered in decreasing order of intensity. It is clear from Table I that for cisplatin, runs of two or more Gs are the strongest damage sites with 5 and 4 Gs giving the sites of most intense damage. Adduct formation always appears to be associated with at least a single G since GA, AG, and GC sites are found.

The damage sites were classified into strong, medium, and weak using densitometer scanning of the gel autoradiographs, and this is indicated in Fig. 4. Most sites did not produce a single discrete band, and these extra bands are also indicated in Fig. 4. The numbers represent the position in the pUC8 sequence.

**Fig. 3. Autoradiograph of a DNA sequencing gel containing the linear amplification products of pUC8 DNA treated with cisplatin and four analogues using the reverse sequencing primer.** See legend to Fig. 2.

**Fig. 4. The sequence specificity of adduct formation with cisplatin and four analogues on pUC8.** The analogues were present at 5 µM except transPt which was at 15 µM. The damage bands for the reverse sequencing primer are above the double-stranded pUC8 sequence, while the forward sequencing primer is below the sequence. Strong damage sites are depicted by an S, medium by M, and weak by w. These letters are at the band of peak intensity, and other associated bands are indicated by dots. The numbers represent the position in the pUC8 sequence.

Control experiments were also carried out at different temperatures. DNA samples were denatured at 95 °C and then extended by Taq DNA polymerase with a single incubation at 25, 37, 50, or 72 °C. These experiments were performed in parallel with the thermal cycling conditions described under "Experimental Procedures." All the cisplatin damage sites observed at 25, 37, 50, or 72 °C were also observed with the standard thermal cycling conditions. (The thermal cycling method is superior because a larger signal is observed with a lower background.) Thus these control experiments confirm that the thermal cycling method is a reliable indicator of the sequence specificity of cisplatin damage and that no significant lesions are being bypassed during the thermal cycling process.

**Comparison of Cisplatin Damage with That Caused by Other Analogues—** There were two types of differences in observed damage between the analogues: differences in position of peak of damage and differences in intensity of damage. For PtenClz the position and intensity was very similar to that of cisplatin. Differences in intensity occurred at bp 390, 396, 487, and 503 with the forward primer and an alteration in the position of peak of damage at bp 358 with the reverse primer. There were a total of 36 cisplatin damage sites.

In general, analogues 2AcC3PtenCl2 and 4AcC3PtenCl2 had a higher degree of low intensity bands across the whole sequence, whereas cisplatin and PtenClz had more discrete damage sites. With 2AcC3PtenCl2 there were 14 differences in the intensity of damage and 6 in the position of the peak of damage compared to that of cisplatin. For 4AcC3PtenCl2 there were 15 differences in the intensity of damage and 13 in the position of peak of damage compared to that of cisplatin. A comparison between 2AcC3PtenCl2 and 4AcC3PtenCl2 revealed 19 differences in the intensity of damage and 18 in the position of the peak of damage.

The weak transPt damage was generally at sites similar to cisplatin but GC and CG sites were common. In fact 9 of the 31 sites were of the type CG. In this site the Taq DNA polymerase stops after the presumed site of adduct formation at the N7 of guanine.

It proved difficult to obtain an even intense banding pattern for 4AcC3PtenCl2 and transPt. This was because at lower concentrations low intensity bands were seen. At higher concentrations more intense bands were confined to regions close to the primer and were faint at sequences further from the primer. This drawback probably occurs with compounds that are inefficient at stopping Taq DNA polymerase but strongly react with DNA and prevent the primer oligonucleotide from hybridizing with the template DNA.
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TABLE I
Sites of cisplatin damage in decreasing order of intensity

The sequences are written 5' to 3'. The damage sites with the largest intensity are at the top. Above the line are the strong sites; between the lines are medium; and below the line are weak. The bases in capital letters are the presumed site of adduct formation. The underlined base indicates the position of peak intensity at the damage site.

<table>
<thead>
<tr>
<th>Reverse sequencing primer</th>
<th>Cisplatin</th>
<th>PtenCl2</th>
<th>2AcC3PtenCl2</th>
<th>4AcC3PtenCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>320 aggGGGa</td>
<td>320 aggGGGa</td>
<td>430 cGGGa</td>
<td>430 cGGGa</td>
<td></td>
</tr>
<tr>
<td>430 CGGGa</td>
<td>430 CGGGa</td>
<td>320 nGGGa</td>
<td>412 caGtc</td>
<td></td>
</tr>
<tr>
<td>412 caGtc</td>
<td>358 caGGt</td>
<td>412 caGtc</td>
<td>420 acGGat</td>
<td></td>
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<tr>
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<td>358 caGGt</td>
<td>358 caGGt</td>
<td>407 ttGGct</td>
<td></td>
</tr>
<tr>
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<td>412 caGtc</td>
<td>336 aaGGg</td>
<td>391 acGGgc</td>
<td></td>
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<tr>
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<td>336 aaGGg</td>
<td>420 caGGat</td>
<td>388 caGGGt</td>
<td></td>
</tr>
<tr>
<td>420 caGGat</td>
<td>311 ggGtg</td>
<td>407 ttGgt</td>
<td>388 ggGGg</td>
<td></td>
</tr>
<tr>
<td>315 ggGga</td>
<td>315 ggGga</td>
<td>347 ttGGt</td>
<td>356 gttGca</td>
<td></td>
</tr>
<tr>
<td>311 ggGcg</td>
<td>420 caGGat</td>
<td>315 gcaGaa</td>
<td>320 aGGGGa</td>
<td></td>
</tr>
<tr>
<td>391 acGGgc</td>
<td>391 acGGgc</td>
<td>311 eGCGc</td>
<td>347 ttGGGt</td>
<td></td>
</tr>
<tr>
<td>407 ttGGc</td>
<td>407 ttGGc</td>
<td>391 acGGgc</td>
<td>375 acAGgc</td>
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</tr>
<tr>
<td>369 ccAGgc</td>
<td>393 caAGtc</td>
<td>375 acAGgc</td>
<td>383 ccAGtg</td>
<td></td>
</tr>
<tr>
<td>393 ccAGtc</td>
<td>369 caAGtc</td>
<td>386 acAGc</td>
<td>315 gcGAaa</td>
<td></td>
</tr>
<tr>
<td>375 acAGc</td>
<td>375 acAGc</td>
<td>396 tgGca</td>
<td>386 acGAcg</td>
<td></td>
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<tr>
<td>386 acAGc</td>
<td>367 acAGg</td>
<td>393 ccAGtg</td>
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</tr>
<tr>
<td>367 ccAGtc</td>
<td>396 gcGca</td>
<td>369 ccAGtc</td>
<td>369 ccAGtc</td>
<td></td>
</tr>
<tr>
<td>396 gcGca</td>
<td>386 acAGc</td>
<td>367 ccAGtc</td>
<td>367 ccAGtc</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

In this study we have demonstrated that *Taq* DNA polymerase can detect cisplatin-induced lesions in double-stranded DNA. The sequence specificity of cisplatin damage to the pUC8 plasmid was determined on both strands. The most intense sites of cisplatin damage was at runs of four and five Gs. The cisplatin results were consistent with those previously published that runs of two or more Gs were found to be the major site of damage (5–7, 11). However, in our study damage at GA, AG, and possibly GC dinucleotides was also detected. The weak adduct formation at this latter dinucleotide was probably due to an interstrand cross-link. The major adduct formation at runs of Gs is most likely to be intrastrand cross-links. Damage at GA and GC dinucleotides has not been reported before. This is probably because previous methods are not as sensitive as the linear amplification procedure. No damage could be unequivocally assigned to GNG sequences.

The use of *Taq* DNA polymerase to detect cisplatin adducts raises the question of whether this polymerase is halted by a subset of adducts; for instance why are only cross-linked adducts detected and not single covalent adducts? This idea could be investigated by use of a monofunctional cisplatin analogue that forms only single adducts.

The position of peak intensity at the damage site can be variable. For instance at a GG site, the position of peak intensity can be at the base before the dinucleotide, or at a position that is not involved in base pairing interactions. Hence, it is probable that the initial fast reaction produces a double G. AA dinucleotides are not significantly damaged at these sites by the distortion induced in DNA by these adducts. It is likely that single adducts do not strongly halt DNA polymerase, but cross-linked adducts are much more efficient at stopping the polymerase.

Damage always appeared to be associated with at least a single G. AA dinucleotides are not significantly damaged which could indicate that the initial fast reaction produces a monofunctional adduct at G and in the cross-linking second stage both As and Gs can react (16, 17).

Four analogues were also tested. Although *transPt* differs only in the relative position of the chlorine and amine substituents (see Fig. 1), it is inactive as an antitumor agent despite forming adducts *in vitro* with DNA at a similar level to cisplatin. The results from this study suggest that *transPt* may be inactive as an antitumor agent because it is much less efficient than cisplatin at inhibiting DNA polymerase.

At higher concentrations of *transPt*, weak damage could be
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detected with a sequence specificity similar to that of cisplatin, but with extra bands at GC and especially CG dinucleotides. These latter sites are interesting because the Taq DNA polymerase stops after the presumed site of adduct formation at the N7 of guanine. These sites are presumed to be interstrand cross-links. Alternatively, intrastrand cross-links could be forming with the N-3 of cytosine. Using single-stranded DNA and DNA polymerase I (containing a 3' to 5' exonuclease activity), Pinto and Lippard (6) found that transPt had a preference for GNG sites, an observation not confirmed in the present study.

PtenCl2 (Fig. 1) is similar to cisplatin except that the nonleaving amines are linked by an aliphatic chain. This compound shows significant antitumor activity. The other two analogues (Fig. 1) are derivatives of PtenCl2 and contain an attached intercalating chromophore. They differ in the position to which the platinum linker arm is attached to the acridine chromophore. The analogue 4AcC3PtenC12 has significant antitumor activity, while 2AcC3PtenCl2 is inactive (10). Thus three active compounds and two inactive compounds have been tested. The latter two compounds were designed as DNA-directed analogues of cisplatin (10); the rationale being that the DNA-intercalating acridine chromophore would target the reactive platinum to the DNA, allowing a more efficient reaction with the nucleophilic sites on the polymer.

Experiments with supercoiled plasmid DNA in vitro (10) have shown that the acridine-linked compounds do interact strongly with DNA, with intercalation occurring adjacent to the covalent attachment site. However, despite the presence of the intercalating chromophore and the corresponding increase in binding site size, the present work indicates that the sequence selectivity of these intercalating analogues is broadly similar to that of cisplatin and PtenCl2, since the damage sites of greatest intensity were runs of two or more Gs. However, there were a number of significant differences. In general analogues 4AcC3PtenCl2 and 2AcC3PtenCl2 showed a higher degree of low intensity bands across the whole sequence, whereas cisplatin and PtenCl2 had more discrete damage sites. This may be attributable to the enhanced DNA association of these compounds.

The two DNA-targeted analogues were also different from each other in their sequence-specific pattern of adducts as well as being different to that of cisplatin and PtenCl2 (the latter two compounds were very similar to one another). The analogue 2AcC3PtenCl2 showed the largest number of medium damage sites (nine) at GA and other non-GG sites. Whereas 4AcC3PtenCl2 was the most different from the untargeted cisplatin and PtenCl2 in the position of peak intensity at the damage site. These data suggest that the reported kinetic differences (10) between the binding of the longer chain homologues 2AcC3PtenCl2 and 4AcC3PtenCl2 has its origins in different sequence specificities.

In summary, this study has shown that the Taq DNA polymerase technique can provide detailed information about the sequence selectivity of DNA adduct formation by cisplatin and analogues. The intrinsic selectivity of cisplatin is for runs of two or more Gs, and this selectivity is not significantly altered in DNA-targeted platinum analogues.

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REFERENCES