The interaction of DNA-targeted platinum phenanthridinium complexes with DNA

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Received May 27, 1998; Revised and Accepted July 21, 1998

ABSTRACT

Cisplatin analogues were synthesised that consisted of platinum(II) diamine complexes tethered via a polymethylene chain \((n = 3, 5, 8\) and \(10\)) to a phenanthridinium cation. Both chloro and iodo leaving groups were examined. DNA adduct formation was quantitatively analysed using a linear amplification system with the plasmid pGEM-3Zf(+). This system utilised Taq DNA polymerase to extend from an oligonucleotide primer to the damage site. This damage site inhibited the extension of the DNA polymerase. The products were electrophoresed on a DNA sequencing gel enabling adduct formation to be determined at base pair resolution. The damage intensity at each site was determined by densitometry. The platinum phenanthridinium complexes were shown to damage DNA at shorter incubation times than cisplatin. To produce similar levels of damage, an 18 h incubation was required for cisplatin compared to 30 min for the \(n = 3\) platinum phenanthridinium complexes; this indicates that the intercalating chromophore causes a large increase in the rate of platination. A reaction mechanism involving direct displacement of the chloride by the N-7 of guanine may account for the rate increase. These results indicate that further development of these compounds could lead to more effective cancer chemotherapeutic agents.

INTRODUCTION

Cisplatin \([\text{cis-diaminedichloroplatinum(II)}]\) is widely used as an anti-tumour drug especially against testicular and ovarian cancer (1). It forms covalent adducts with DNA resulting in both intra- and inter-strand crosslinks (2,3). The main site of adduct formation is at the N-7 of guanine with the GG intra-strand crosslink being the most common adduct. Cisplatin–DNA adducts are expected to inhibit DNA replication and RNA transcription.

The sequence selectivity of cisplatin damage to DNA has been determined in plasmid (4–8) and in cells (9,10) and found to be mainly at runs of consecutive guanines with lower levels of damage at GA, AG and GC dinucleotides. The sequence specificity can be determined using a linear amplification assay. In this technique a 32P-labelled oligonucleotide is extended by Taq DNA polymerase up to the damage site. Linear amplification of the extension products is accomplished by repeated cycles of denaturation, annealing and extension. These products are run on a DNA sequencing gel along with dideoxy sequencing reactions to determine the precise sites of damage. A GG intra-strand crosslink inhibits the passage of Taq DNA polymerase in 97% of cases (11).

Since the introduction of cisplatin into the clinic, there has been a concerted effort to produce more effective cisplatin analogues (12–14). This has resulted in the introduction of one cisplatin analogue, carboplatin, into the clinic. One way to develop cisplatin analogues with an improved spectrum of activity is to target a platinum moiety to DNA by its attachment to a suitable carrier (15). Compared with untargeted analogues, such compounds may show an enhanced rate of platinum binding to DNA. Moreover, targeting could also serve to minimise exposure to inactivating agents such as thiols. This may prove to be highly desirable since overexposure to thiols and thiol-transferring enzymes is a recognised mechanism of cellular resistance to cisplatin (16,17). DNA-targeted platinum drugs that show improved selectivity with respect to cisplatin-resistant cell lines have included analogues which utilise anthracyclines (18), 9-anilino–acridines (19), 9-aminoacridine (20) and acridine carboxamides (21) as the targeting elements.

In this report we describe the results of our studies on a series of platinum complexes, \(3a, 3b, 3c, 3d, 4a, 4b, 4c\) and \(4d\) (Fig. 1), in which diamine coordinated platinum moieties are tethered by a polymethylene chain \((n = 3, 5, 8\) and \(10\)) to the cationic phenanthridinium chromophore. Both chloro \((4a–d)\) and iodo \((3a–d)\) leaving groups were examined. As shown in Figure 1, the attachment of an intercalating moiety places the platinum in close proximity to its proposed biological target, DNA. The complexes are positively charged and this will increase the interaction with...
Figure 1. Synthesis of the platinum phenanthridinium complexes. See Materials and Methods section for further details. Also shown are the structures of cisplatin and PtenCl₂. The expected mode of interaction of the platinum phenanthridinium complexes with DNA is depicted at the bottom right of the diagram. The phenanthridinium moiety is shown intercalated into double-stranded DNA with the flexible alkyl linker chain permitting the platinum group to react with DNA.

DNA by electrostatic attraction with the phosphate groups in DNA.

MATERIALS AND METHODS

Chemical synthesis

Cisplatin and dichloro(ethylenediammine)platinum (II) (PtenCl₂) were prepared as previously described (8). The platinum phenanthridinium complexes were synthesised by conventional methods (21). An example is given using the \( n = 3 \) compound (4c): 8-bromooctylphenanthridinium bromide (1c) (22) was reacted with 1,2-diaminoethane to form \( N_\text{3} \)-\( N_\text{3} \' \)-oct-8-ylphenanthridiniumchloride-1,2-diaminoethane dihydrochloride (2c); reaction with K₂PtI₄ gave \( N_\text{3} \)-\( N_\text{3} \' \)-oct-8-ylphenanthridiniumiodide-1,2-diaminoethaneplatinum(II)dichloride (3c) which was then converted to \( N_\text{3} \)-\( N_\text{3} \' \)-oct-8-ylphenanthridiniumchloride-1,2-diaminoethaneplatinum(II)dichloride (4c). Similarly starting with 1a, 2a and 4a gave rise to 3a, 3b, 3d, 4a, 4b and 4d. The complexes were characterised and purity established using \(^1\)H and \(^{13}\)C NMR spectroscopy and FAB mass spectroscopy.

Cisplatin, PtenCl₂ and the \( n \)-phenanthridinium platinum iodides were dissolved in dimethylformamide to give stock solutions of 5 mM while the \( n \)-phenanthridinium platinum chlorides were prepared as 1 mM stock solutions in dimethylformamide.

Determination of DNA sequence specificity

The plasmid pGEM-3Zf(+) was prepared by a heat-alkali method (23). Sequencing primers utilised were (SEQ) 5′-TCCCAGTCAGACGAG-3′ as the forward sequencing primer and (REV) 5′-AACAGCTATGACC-3′ as the reverse sequencing
primer. The oligonucleotides were 5'-labelled as previously described (24) with polynucleotide kinase (Pharmacia) and [γ-32P]ATP (Amersham).

pGEM-3Zf(+) plasmid DNA (5 μg) was treated with 0.01, 0.05, 0.1, 0.5, 1 and 5 μM of compound (final concentration) for varying times: 0, 1, 3, 10 and 30 min, 1, 3, 6 and 18 h. All damage reactions were maintained in darkness at 37°C in 2 mM HEPES, pH 7.8, 10 mM NaCl, 10 mM EDTA, in a final volume of 40 μl. After ethanol precipitation (4.4 μl 3 M Na acetate, 88.8 μl ethanol) and two ethanol washes, the DNA was dissolved in 20 μl of 10 mM Tris–HCl, pH 8.5, 100 mM EDTA.

The linear amplification reaction comprised a mix of 16.6 mM (NH4)2SO4, 67 mM Tris–HCl, pH 8.8, 6.7 mM MgCl2, 300 μM each of dATP, dGTP, dCTP, dTTP, 0.05 pmol 32P-labelled oligonucleotide and 0.25 U AmpliTaq DNA polymerase (Perkin Elmer Cetus) (final concentrations). DNA (2 μl) was added to this mix producing a final volume of 5 μl which was overlaid with 50 μl of mineral oil. Dideoxy double-stranded DNA sequencing was performed using the same sequencing primers to accurately pinpoint the precise site of damage. The linear amplification procedure subjected the reaction mix to 95°C for 30 s (time at temperature), 50°C for 60 s and 72°C for 90 s for 20 cycles in a Perkin Elmer Cetus DNA thermal Cycler 480. The reaction mix (2 μl) was then electrophoresed on a 6% polyacrylamide–urea DNA sequencing gel. The gel was dried and analysed by a Molecular Dynamics PhosphorImager after exposure to a phosphor storage screen.

Densitometry was performed using Molecular Dynamics ImageQuant software. The intensity at each site was determined by calculating the peak area and the background was subtracted. For each compound the DNA damage sites were ordered with respect to the damage intensity (Table 1).

RESULTS

The plasmid pGEM-3Zf(+) was damaged with cisplatin and the platinum phenanthridinium complexes at various concentrations. The damaged DNA was then subjected to the linear amplification procedure and electrophoresed on a DNA sequencing gel as shown in Figure 2 for the chloro complexes (4a-d) and in Figure 3 for the iodo complexes (3a–d). The level of damage in the no drug control lanes (lanes 1 and 2) was negligible compared to damage in the drug-treated lanes.

Sequence specificity

The sequence specificity of the adducts formed by the platinum complexes was determined by reference to the dideoxy sequencing lanes. The intensity of damage at each of the damage sites was quantified using densitometry. The sequence present at the 10 most intense sites of damage is shown in Table 1. It can be seen that runs of consecutive guanines are the main damage sites for all the compounds tested.

The main difference in the pattern of DNA damage between cisplatin and the platinum phenanthridinium complexes was the precise point at which the peak intensity occurred at the damage site. The platinum phenanthridinium complexes had a strong preference for production of peaks closer to oligonucleotide primer i.e. at the 3'-end of the damage site on the template. This can be clearly seen at bp 419–421 and 435–437, where the phenanthridinium complexes damaged at the proximal end of the damage site (with respect to oligonucleotide primer), compared to cisplatin which mainly damaged at the distal end. The sites that were affected were 5’-aagGgGgat-3’ (319–321 bp), gttGgGtaa (346–348), ccaGgGtt (357–359), ataGgGcga (419–421), ctcGgTacc (435–437) and ccGgGatc (443–445); the peak of damage is depicted as a capital letter. It can be seen that the difference in peak position is usually 2 bp and occurred in runs of consecutive guanines. The sites of damage can also be viewed on the other strand by use of the SEQ primer (data not shown). The same pattern emerged with the phenanthridinium complexes damaging at the proximal end of the damage site (with respect to oligonucleotide primer) compared to cisplatin that mainly damaged at the distal end.

Linker chain length

The influence of linker chain length was investigated for alkyl lengths n = 3, 5, 8 and 10. The pattern of damage was different as the linker chain length was varied. The damage sites became less distinct as the linker chain length was increased. With the chloro series the sequence selectivity was relatively similar (Table 1). Larger changes were seen with the iodo series; in particular, as the linker chain length increased, relative DNA damage intensity decreased at bp 445 and 468 while it increased at bp 321.

Figure 2. A DNA sequencing gel showing DNA damage caused by cisplatin and four platinum phenanthridinium chloro complexes in pGEM-3Zf(+) using the REV primer. Lanes 1–4 are untreated control lanes. Lanes 5–10 are from DNA treated with 0.01, 0.05, 0.1, 0.5, 1 and 5 μM cisplatin, respectively; lanes 11–16: 0.01, 0.05, 0.1, 0.5, 1 and 5 μM n = 3 platinum phenanthridinium chloro complex (4a), respectively; lanes 17–22: 0.01, 0.05, 0.1, 0.5, 1 and 5 μM n = 5 platinum phenanthridinium chloro complex (4b), respectively; lanes 23–28: 0.01, 0.05, 0.1, 0.5, 1 and 5 μM n = 5 platinum phenanthridinium chloro complex (4c), respectively; lanes 29–34: 0.01, 0.05, 0.1, 0.5, 1 and 5 μM n = 10 platinum phenanthridinium chloro complex (4d), respectively. The dideoxy DNA sequencing was carried out on pGEM-3Zf(+) (lanes G, A, T and C) and give the sequence on the template strand.

Figure 3. A DNA sequencing gel showing DNA damage caused by cisplatin and four platinum phenanthridinium iodo complexes in pGEM-3Zf(+) using the SEQ primer. Lanes 1–4 are untreated control lanes. Lanes 5–10 are from DNA treated with 0.01, 0.05, 0.1, 0.5, 1 and 5 μM cisplatin, respectively; lanes 17–22: 0.01, 0.05, 0.1, 0.5, 1 and 5 μM n = 3 platinum phenanthridinium chloro complex (3a), respectively; lanes 23–28: 0.01, 0.05, 0.1, 0.5, 1 and 5 μM n = 5 platinum phenanthridinium chloro complex (3b), respectively; lanes 29–34: 0.01, 0.05, 0.1, 0.5, 1 and 5 μM n = 5 platinum phenanthridinium chloro complex (3c), respectively; and lanes 35–40: 0.01, 0.05, 0.1, 0.5, 1 and 5 μM n = 10 platinum phenanthridinium chloro complex (3d), respectively. The dideoxy DNA sequencing was carried out on pGEM-3Zf(+) (lanes G, A, T and C) and give the sequence on the template strand.
addition, negative controls were included where the drug was added after ethanol was added to the reaction (lanes 1–5).

It can be seen that the platinum phenanthridinium chloro and iodo complexes were able to damage DNA at significantly shorter times than cisplatin or PtenCl₂. The platinum phenanthridinium chloro and iodo complexes showed detectable damage after 10 min. At 30 min the level of damage was similar to that of cisplatin after 18 h. A low level of damage could be detected for cisplatin and PtenCl₂ at 6 h.

DISCUSSION

The interaction of a series of platinum phenanthridinium complexes with DNA was investigated using a plasmid system. The DNA sequence specificity of adduct formation was examined using a linear amplification and DNA sequencing techniques. Two types of information can be extracted from this system; the precise sites of adduct formation and the degree of damage at each site. The extent of reaction under different conditions can also be determined.

Sequence specificity

The sequence specificity of the reaction of the platinum phenanthridinium complexes with DNA was found to be very similar to that of cisplatin with runs of consecutive guanines being preferentially damaged. However, the precise position of the peak intensity at the damage sites differed for the phenanthridinium complexes. The platinum phenanthridinium complexes had a strong tendency to produce peak intensity at the proximal end of a damage site (with respect to oligonucleotide primer) while cisplatin damaged at the distal end. For example, the damage site at bp 319–321 with template sequence 5'-aagGgGat-3', the peak for cisplatin was at the G (capital letter) nearer the 5'-end while for the platinum phenanthridinium complexes it was at the G (capital letter) nearer the 3'-end. Note that the Taq DNA polymerase approaches from the right of the above template sequence. The difference in the position of peak intensity was usually 2 bp. It predominantly occurred in runs of consecutive guanines. Damage sites were examined on both strands and the phenanthridinium complexes always had a preference for peak intensity at the 3'-end of a damage site (with respect to oligonucleotide primer), relative to cisplatin. There are several possible explanations for this: (i) the platinum phenanthridinium complexes produce bulkier lesions that cause the Taq DNA polymerase to halt two bases before the cisplatin-induced lesion; (ii) the platinum phenanthridinium complexes damage different sequences. The fact that the peak intensity is always biased towards the oligonucleotide primer suggests that the former mechanism is operating. If mechanism (i) is operating, it implies that the platinum phenanthridinium complexes are producing bulkier or more distorted lesions on DNA compared to cisplatin and are causing the Taq DNA polymerase to be inhibited further from the damage site. If mechanism (ii) is operating, it indicates that cisplatin prefers to damage the 5'-end of a run of guanines while the platinum phenanthridinium complexes prefer the 3'-end.

The Taq DNA polymerase assay is capable of distinguishing between monofunctional cisplatin adducts and crosslinks (25). Compounds that were only capable of forming monofunctional cisplatin adducts had a displaced damage position (towards the

**Figure 3.** A DNA sequencing gel showing DNA damage caused by cisplatin and four platinum phenanthridinium iodo complexes in pGEM-3Zf(+) using the REV primer. Lanes 1–4 are untreated control lanes. Lanes 5–10 are from DNA treated with 0.01, 0.05, 0.1, 0.5 and 1 µM cisplatin, respectively; lanes 11–16: 0.01, 0.05, 0.1, 0.5 and 1 µM n = 3 platinum phenanthridinium iodo complex (3a), respectively; lanes 17–22: 0.01, 0.05, 0.1, 0.5 and 1 µM n = 5 platinum phenanthridinium iodo complex (3b), respectively; lanes 23–28: 0.01, 0.05, 0.1, 0.5 and 1 µM n = 8 platinum phenanthridinium iodo complex (3c), respectively; lanes 29–34: 0.01, 0.05, 0.1, 0.5 and 1 µM n = 10 platinum phenanthridinium iodo complex (3d), respectively. The dideoxy DNA sequencing was carried out on pGEM-3Zf(+) (lanes G, A, T and C) and give the sequence on the template strand.
Table 1. The 10 most intense sites of cisplatin, platinum phenanthridium chloride and iodide complexes’ damage in the plasmid pGEN-3Zf(+ ) arranged in decreasing order of intensity

<table>
<thead>
<tr>
<th>Complex</th>
<th>4a</th>
<th>4b</th>
<th>4c</th>
<th>4d</th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
</tr>
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<tbody>
<tr>
<td>PtenCl2</td>
<td>gggG (445)</td>
<td>gggG (321)</td>
<td>gggG (321)</td>
<td>gggG (321)</td>
<td>gggG (445)</td>
<td>gggG (445)</td>
<td>gggG (321)</td>
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The sequences are written 5′ to 3′ with a capital letter indicating the peak of damage intensity. The number in brackets denotes the position in the plasmid DNA sequence. Note that the Taq DNA polymerase approaches from the right of the indicated sequence.

5′-end) (25). This effect was not found with the phenanthridinium Pt compounds. This suggests that monofunctional platinum adducts are formed at low levels in the interaction of the phenanthridinium complexes with DNA and the adducts detected are due to bifunctional binding of platinum.

There are several factors that might influence the binding of cisplatin analogues to DNA. Intercalation could affect the nucleophilicity of neighbouring guanines. This effect was explored by the addition of equimolar ethidium bromide to the cisplatin reaction (data not shown) but no significant effect was observed. Several variables were examined for the platinum phenanthridinium complexes including a time course, concentration of the complex, alkyl linker arm length and the influence of the leaving group (chloro versus iodo complexes).

**Time course of interaction with DNA**

Since the biological site of action of cisplatin is thought to be DNA, the influence of DNA-targeting on the time course of platinum adduct formation by the phenanthridinium Pt complexes was investigated in some detail. In these experiments we observed that the platinum phenanthridinium complexes produced the same level of damage in 0.5 h as cisplatin and PtenCl2 in 18 h. This large increase in the rate of platinum binding suggests that there are mechanistic differences between the targeted complexes and cisplatin (and PtenCl2) in their mode of interaction with DNA.

A detailed mechanistic study of the interaction of cisplatin with DNA using 195Pt NMR (26) has revealed that the main reason for the slow reaction of cisplatin (and by analogy PtenCl2) with oligonucleotides have also been studied in detail and again reaction with DNA proceeds through an aquated intermediate, cis-[Pt(NH3)2NH2C6H11]Cl(OH2)]+ (28). In contrast to the binding of cisplatin, intercalation of simple phenanthridines is a rapid event which is usually complete within the millisecond time scale (29). Thus for the targeted complexes one possibility is that intercalation of the phenanthridinium cation rapidly localises the platinum dichloro centre on DNA where it can undergo rapid and direct substitution by the N-7 of guanine to form a bifunctional adduct (Fig. 5). In this way, the slow first hydrolysis step, which characterises the cisplatin or PtenCl2 interaction with DNA, would be circumvented. Our observation that monofunctional lesions do not form in detectable levels also suggests that, for these complexes, closure to form bifunctional complexes is a rapid process.

A second mechanistic possibility is that the rate of aquation of the dichloro species is greatly increased in the vicinity of the DNA helix, and that this species then undergoes rapid base substitution. However, this prospect contrasts with the observation that hydrolysis of monofunctionally bound cisplatin, in which the second chloride ion is lost, is still a slow process (t1/2 = 2.1 h, 37°C) (26).

Other studies have examined the kinetics of interaction of cisplatin analogues with DNA and found increases in the rate of reaction compared to cisplatin (30,31). The mode of interaction of ethidium-tethered platinum complexes with DNA has been investigated (32). Under our experimental conditions, the DNA-targeted phenanthridinium complexes react very rapidly with DNA when compared to cisplatin and PtenCl2. To our knowledge this is the first time this effect has been demonstrated with intercalator-tethered platinum complexes and further investigation of the mechanistic basis of these observations is underway.
Figure 4. A DNA sequencing gel depicting a time course of DNA damage caused by platinum phenanthridinium complexes, cisplatin and PtenCl₂ with pGEM-3Zf(+) using the REV primer. Lanes 1, 6, 11, 16 and 21 are untreated control lanes. Lanes 2, 7, 12, 17 and 22 are from DNA treated with cisplatin; lanes 3, 8, 13, 18 and 23: n = 3 platinum phenanthridinium iodo complex (3a); lanes 5, 10, 15, 20 and 25: n = 3 platinum phenanthridinium chloro complex (4a); lane 26 contained DNA treated with ethidium bromide. All compounds were present at 0.1 µM. In lanes 1–5 the compounds were added to a combined reaction mix and sodium acetate/ethanol mix and placed at –70 °C. In lanes 6–10 for the zero time control, the compounds were added to the reaction mix, immediately combined with the ethanol precipitation mix and placed at –70 °C—this operation took ∼10–15 s. Lanes 11–15 were incubated at 37 °C for 10 min; lanes 16–20: 30 min; lanes 21–26: 18 h. The dideoxy DNA sequencing was carried out on pGEM-3Zf(+) (lanes G, A, T and C) and give the sequence on the template strand.

Concentration

Both cisplatin, PtenCl₂ and the phenanthridinium complexes showed no significant differences in input concentrations needed to cause the same level of DNA damage after an 18 h incubation period. Thus, although the platinum phenanthridinium complexes are DNA targeted, this does not influence the total extent of DNA damage at equilibrium in these solution studies. This contrasts with the behaviour of DNA targeted alkylating agents (33) where, under analogous circumstances, there are large concentration effects. These observations reflect the different chemistries of DNA alkylation and platination. In the former, hydrolysis reactions deactivate the alkylating agent to further reaction with DNA, whereas for cisplatin hydrolysis products are intermediates which further react with donor atoms on DNA. Other investigations of the DNA targeted hypothesis (8,33–38) have not examined the time course of reaction with DNA.

Linker chain length

The linker chain length should affect the reaction as this controls accessibility to the nucleophilic centres. The effect of linker alkyl chain length was examined and the iodo complexes showed more changes than the chloro complexes. As the linker chain length was decreased with the iodo complexes, the concentration needed to significantly damage DNA also decreased. However, no discernible pattern was found for the chloro complexes as the linker chain length was varied.

Chloro versus iodo complexes

Very little work has been carried out on iodo analogues of cisplatin, mainly because they are much less water soluble than their chloro counterparts. Although iodide is generally a poorer leaving group than chloride (39), platinum(II) diodo complexes will still undergo hydrolysis reactions in aqueous solution or participate in substitution reactions with donor atoms such as the DNA base nitrogens. As a result, we have examined the present set of diodo complexes where the presence of the cationic intercalator will aid aqueous solubility. We find that the iodo complexes do indeed damage DNA although the pattern of sequence specificity was more varied than for the chloro
complexes (Table 1). A number of changes in sequence specificity was found for the iodide complexes as the linker chain length increased but variation was more limited for the chloro complexes.

Conclusions
Evidence has been presented in this paper that targeting of platinum to DNA by its attachment to an intercalating chromophore has resulted in a marked increase in rate of platinum binding to DNA. It is possible that this could be due to a novel reaction mechanism in which the N-7 atoms of guanine directly displace the chloride leaving groups of the platinum complexes. This latter point could lead to novel properties of a cisplatin analogue in a clinical environment. The platinum phenanthridinium chloro complexes show activity in a tumour-bearing mouse assay system (Whittaker et al., manuscript in preparation). Thus these compounds are strong lead compounds that could be further improved to give more effective cancer chemotherapeutic agents.

ACKNOWLEDGEMENTS
Support of this work by the NHMRC and Australian Research Council is gratefully acknowledged.

REFERENCES