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Phosphodiester-Mediated Reaction of Cisplatin with Guanine in Oligodeoxyribonucleotides

Meghan A. Campbell and **Paul S. Miller***
Department of Biochemistry and Molecular Biology Johns Hopkins Bloomberg School of Public Health 615 North Wolfe Street Baltimore MD, 21205

Abstract

The cancer chemotherapeutic agent cis-diamminedichloroplatinum(II) or cisplatin reacts primarily with guanines in DNA to form 1,2-Pt-GG and 1,3-Pt-GNG intrastrand cross-links, and to a lesser extent, G-G interstrand cross-links. Recent NMR evidence has suggested that cisplatin can also form a coordination complex with the phosphodiester internucleotide linkage of DNA. We have examined the effects of the phosphodiester backbone on the reactions of cisplatin with oligodeoxyribonucleotides that lack or contain a -GTG- sequence. Cisplatin forms a stable adduct with TpT that can be isolated by reversed phase HPLC. The *cis*-Pt-TpT adduct contains a single Pt, as determined by atomic absorption spectroscopy (AAS) and by electrospray ionization mass spectrometry (ESI-MS), and is resistant to digestion by snake venom phosphodiesterase. Treatment of the adduct with sodium cyanide regenerates TpT. Similar adduct formation was observed when $T(pT)_8$ was treated with cisplatin, but not when the phosphodiester linkages of $T(pT)_8$ were replaced with methylphosphonate groups. These results suggest that the platinum may be coordinated with the oxygens of the thymine and possibly with those of the phosphodiester group. As expected reaction of a 9-mer containing a -GTG- sequence with cisplatin yielded an adduct that contained a 1,3-Pt-GTG intrastrand cross-link. However, we found that the number and placement of phosphodiesters surrounding a -GTG- sequence significantly affected intrastrand cross-link formation. Increasing the number of negatively charged phosphodiesters in the oligonucleotide, increased the amount of -GTG- platination. Surrounding the -GTG- sequence with non-ionic methylphosphonate linkages reduced or eliminated cross-link formation. These observations suggest that interactions between cisplatin and the negatively charged phosphodiester backbone may play an important role in facilitating platination of guanine nucleotides in DNA.

Reactions of the chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (cisplatin or *cis*-DDP) with DNA have been studied for many years. Although it is widely accepted that the therapeutically relevant lesion is a 1,2-Pt-GG intrastrand cross-link, which links the N7s of the two guanines (1-3), the precise pathway leading to formation of this cross-link, as well as to 1,3-Pt-GNG intrastrand and G-G interstrand cross-links, is still under investigation. It is thought that *cis*-DDP is converted to *cis*-diammine diaquaplatinum(II) upon leaving the high concentration of chloride in the blood and entering the low concentration of chloride in the cell (3-5). This dicationic, aquated species then reacts with the DNA bases and other nucleophiles within the cell.

*Address correspondence to: Paul S. Miller Department of Biochemistry and Molecular Biology Johns Hopkins Bloomberg School of Public Health 615 North Wolfe Street Baltimore, MD 21205 phone: 410-955-3489 fax: 410-955-2926 e-mail: pmiller@jhsph.edu.

Supporting Information Available: Figures S1-S7 (as referred to in the text) are available free of charge via the Internet at http://pubs.acs.org.

While the reactions of cisplatin with nucleobases have been studied extensively, interactions with the DNA backbone have been largely overlooked. Platinum, a “soft” metal, preferentially binds to other “soft” ligands, such as amines and thiols (6,7). This binding preference does not, however, exclude the possibility that platinum can form coordination complexes with “harder” oxygen ligands, such as those found in the DNA sugar-phosphate backbone, although the products of such interaction would presumably be less stable than those formed when platinum reacts with one of its typical targets such as the N7 of guanine. A number of studies have shown that cisplatin reacts with phosphate buffer (8-10) and there has been extensive characterization of the products formed between platinum and nucleoside phosphomonoesters (8,9,11-15). For example, a crystal structure shows that platinum interacts with the N3 of cytosine and the phosphate of 5'-CMP in a head-to-tail fashion (16).

When the therapeutic mechanism of cisplatin was first investigated, there was speculation that reaction with the DNA phosphate backbone could be important (14,16-20). Although it eventually became clear that therapeutically relevant lesions resulted from reaction with purine bases, primarily guanine, it was still noted that addition of phosphate groups increased the rate of platination of purine nucleosides (21,22). A possible explanation for this rate acceleration is the formation of an N7-Pt-O(P) macrochelate that would facilitate platination of the purine base. Indeed, extensive NMR investigations have revealed the formation of such macrochelates between platinum and 5'-IMP (23), 5'-GMP (23,24), 5'-AMP (24,25), 5'-ITP (26), and 5'-GTP (26). More recently, using NMR, Kozelka and Barre observed similar macrochelate formation when cisplatin was mixed with d-TpG in dimethylformamide (DMF) (27). This observation was the first experimental evidence for macrochelate formation in the context of an oligonucleotide, rather than a nucleoside mono- or triphosphate.

There is also evidence that cisplatin can bind to phosphodiester internucleotide linkages in the absence of guanine residues. Kozelka and Barre observed changes in the ^{1}H and ^{31}P NMR spectra of d-TpT in DMF in the presence of an equimolar amount of cisplatin (27). These changes were consistent with coordination of platinum to the phosphodiester group. Furthermore, Kozelka and co-workers observed that in a low ionic strength, aqueous solution, cisplatin altered the [^{1}H,^{15}N] HMQC NMR signals of d-T(pT)$_{19}$, consistent with formation of a coordination complex between *cis*-[Pt(H$_2$O)$_2$(NH$_3$)$_2$]$^{2+}$, the hydrolysis product of *cis*-DDP, and the non-bridging phosphodiester oxygen atoms (28). These workers also showed that the polyanionic phosphate backbone of DNA affects the rate of aquation of cisplatin. It seems then that the phosphate backbone can participate in reactions of *cis*-DDP and could affect the rate of guanine platination by recruiting the positively charged platinum and/or by forming a platinum-phosphodiester intermediate.

In this report, we examine the reaction of cisplatin with oligodeoxyribonucleotides composed of thymidine, some of which contain a -GTG- sequence. We show that cisplatin forms stable adducts with oligothymidylates and demonstrate that substitution of the phosphodiester linkages with nonionic methylphosphonate linkages eliminates reaction of these oligonucleotides with cisplatin. We also show that the number and position of the phosphodiester linkages surrounding the -GTG- sequence affects platination of the guanine bases in the oligonucleotide. The results of this study are consistent with a model in which interactions between cisplatin and the DNA backbone contribute to formation of -GNG- cisplatin intrastrand cross-links.

EXPERIMENTAL PROCEDURES

5'-O-Dimethyoxytritylthymidine- and 5'-O-dimethoxytrityl-N^2-isobutryl-3'-*O*-bis (diisopropylamino)-β-cyanoethylphosphoramidites, 5' nucleoside-derivatized controlled-pore glass (CPG) supports, 4,5-dicyanoimidazole, 1-*H*-tetrazole, 5-ethylthiotetrazole, Cap Mix A,

Cap Mix B, and oxidizing solution were purchased from Glen Research Inc., Sterling, VA. Protected 2'-deoxyribonucleoside-3'-*O*-(N, N-diisopropylamino)-methylphosphonamidites were obtained from JBL Scientific Inc., San Luis Obispo, CA. Phosphoramidite and methylphosphonamidite solutions were prepared using synthesis-grade acetonitrile (Fisher Scientific) that was dried and stored over calcium hydride. C-18 buffer A consisted of 2% acetonitrile in 50 mM sodium phosphate, pH 5.8. A preaquated solution of *cis*-diamminedichloroplatinum(II) dichloride (*cis*-DDP) was prepared as follows: a 5 mM solution of *cis*-DDP (Sigma-Aldrich, Inc.) was incubated in the dark with 10 mM silver nitrate overnight at 37 °C. The resulting precipitate was centrifuged, and the supernatant, which contains 5 mM *cis*-diammine diaquaplatinum(II) dinitrate, was stored in the dark at room temperature. MALDITOF analysis was performed in the AB Mass Spectrometry/Proteomics Facility at The Johns Hopkins School of Medicine, with support from the National Center for Research Resources Shared Instrumentation, Grant 1S10-RR14702.

Synthesis of oligonucleotides

The oligonucleotides, whose sequences are shown in Table 1, were synthesized on an ABI DNA synthesizer (model 392 or 3400). Phosphoramidite solutions were prepared in dry acetonitrile at a concentration of 0.15 M; 5-ethylthiotetrazole was used as the activating agent, and coupling times were 120 s. The oligonucleotides were deprotected and removed from their supports by incubating the CPG in a solution containing 300 μL of concentrated ammonium hydroxide and 100 μL of 95% ethanol for 3.5 h at 55 °C.

Oligonucleotides containing methylphosphonate linkages were synthesized using a 0.25 M solution of 1*H*-tetrazole as the activating agent and a coupling time of 120 s. The oxidizing solution contained 1.25 g of iodine, 37.5 mL of tetrahydrofuran, 12.5 mL of 2,6-lutidine, and 100 μL of water; Cap A contained 10% acetic anhydride, 10% pyridine in tetrahydrofuran (v/v); and Cap B contained 0.5 g of 4-dimethylaminopyridine in 20 mL of anhydrous pyridine. The methylphosphonamidites were dissolved in dry acetonitrile or in the case of G, in a 50% acetonitrile/tetrahydrofuran solution. The oligonucleotides were first removed from the CPG by incubation with 0.4 mL of concentrated ammonium hydroxide for 2.5 h at room temperature. The supernatant was recovered and after evaporation, the residue was treated with a solution containing 5 μL of water, 22.5 μL of acetonitrile, 22.5 μL of 95% ethanol, and 50 μL of ethylenediamine for 6.5 h at room temperature. The solution was cooled on ice; neutralized by addition of 600 μL of ice cold 2 N hydrochloric acid; and desalted on a C-18 reversed phase Clarity desalting tube (Phenomenex, Inc., Torrance, CA).

Oligonucleotides containing four or more phosphodiester linkages were purified by strong anion exchange (SAX) high performance liquid chromatography (HPLC). Oligomers **1p**, **2p**, **4p-*in***, and **4p-*ex*** were purified with a linear gradient of 0-0.5 M NaCl, **3p-*in*** with a gradient of 0-0.15 M NaCl, and **3p-*ex*** with a gradient of 0-0.1 M NaCl. Methylphosphonate oligonucleotides (**2mp**, **5p-*in***, **5p-*ex***) were purified by C-18 reversed phase HPLC using a linear gradient of 2-30% acetonitrile. The concentrations of the purified oligomers were determined from their UV absorbance at 260 nm, using calculated extinction coefficients based on nearest neighbor approximations (29). The compositions of oligonucleotides were confirmed by MALDI-TOF mass spectrometry as shown in Table 1.

Reaction of platinated TpT with cyanide

A solution of TpT (0.6 OD, 39 nmol) was evaporated to dryness and 60 μL of 5 mM *cis*-DDP was added. A 20 μL aliquot was removed and quenched in 20 mM Tris. The remaining solution was incubated at 37 °C for 24 h, when another 20 μL aliquot was removed and quenched in 20 mM Tris. A 100 μL aliquot of 200 mM sodium cyanide was added to the remaining 20 μL of solution and the solution was incubated at 37 °C for 24 h. The first two aliquots and this

remaining solution were each analyzed on a Gemini C-18 reversed phase HPLC column (Phenomenex, Inc., Torrance, CA) using a linear gradient of 0-52.5% methanol in 20 mM Tris pH 8.5.

Isolation of cis-Pt-TpT

A solution of 3 OD of TpT (193 nmol) was evaporated to dryness and the residue was treated with 300 μL of 5 mM *cis*-DDP at 37 °C for 24 h. The reaction mixture was injected onto a Gemini C-18 reversed phase HPLC column and the column was eluted with a linear gradient of 0-52.5% methanol in 20 mM Tris, pH 8.5. The cluster of peaks that eluted with approximately 30% methanol was collected and the solvent was evaporated. The residue was redissolved in 200 μL of MilliQ water and the solution was desalted on the Gemini C-18 column using a linear gradient of 0-52.5% methanol in MilliQ water.

Enzymatic digestion of TpT and cis-Pt-TpT

Samples containing 0.1 OD (6.4 nmol) of TpT or *cis*-Pt-TpT, isolated as described above, were dissolved in 18 μL of enzyme buffer that consisted of 10 mM Tris pH 8, 2 mM $MgCl_2$. One set of samples was diluted with 200 μL of C-18 buffer A. The other set was treated with 1 μL (0.14 units) of snake venom phosphodiesterase and 1 μL (10 units) of calf intestinal phosphatase for 1 h at 37 °C after which the digests were diluted with 200 μL of C-18 buffer A. Each sample was analyzed by C-18 reversed phase HPLC using a linear gradient of 2-20% acetonitrile in C-18 buffer.

Atomic absorption spectroscopy

Samples of TpT and *cis*-Pt-TpT were prepared in water at a concentration of 554 μg/L and 776 μg/L, respectively. The platinum contents of these samples were analyzed using a graphite furnace AAnalyst 600 spectrometer (Perkin Elmer Instruments, Waltham, MA) and the amounts of platinum determined using a standard curve generated from platinum standards containing 0, 50, 100, 500 and 1000 μg/L Pt.

Electrospray mass spectrometry

Purified *cis*-Pt-TpT was diluted with 35% methanol and 0.5% formic acid in MilliQ water to a concentration of 19.7 μg/mL. ESI mass spectra were collected on a Finnegan-MAT LCQ Classic spectrometer. Samples were infused at a flow rate of 10 μL/min under positive ionization conditions and a capillary temperature of 200 °C.

Platination of oligonucleotides

The oligonucleotide was incubated with a solution of *cis*-DDP, in either water or 10 mM Tris pH 7.5, at 37 °C. Aliquots of the reaction mixture were removed and analyzed by C-18 reversed phase or SAX HPLC. The columns were monitored at 260 nm. The percentage of platinated oligonucleotide was determined by comparing the areas under the peaks of the platinated and non-platinated oligonucleotides.

RESULTS

Platination of TpT and oligothymidylates

As shown in Figure 1, when TpT was incubated with 5 mM *cis*-DDP, new products formed that appeared on a C-18 reversed phase column as two close moving peaks with retention times of approximately 12 min. Further treatment of the reaction mixture with 167 mM sodium cyanide regenerated TpT, as shown in Figure 1c. Because cyanide readily displaces other ligands from platinum coordination complexes (30), this result suggests that the products at 12 min are platinum adducts of TpT. The phosphodiester group of TpT appears to be involved

in formation of these adducts, because essentially no reaction was observed when thymidine was incubated with *cis*-DDP under the same conditions (see Figure S1). A small peak observed at 2.5 min in Figure S1 may represent the Pt-N3 adduct of thymidine. Although formation of this adduct requires deprotonation of the thymine ring (22,31), Pt-OH could act as base to carry out this deprotonation, even under the acidic conditions of the experiment.

The *cis*-Pt-TpT adducts could be isolated by reversed phase HPLC and proved to be quite stable. Thus examination of a solution of *cis*-Pt-TpT stored at 4°C for 6 months by HPLC showed no degradation of the adduct (see Figure S2). Analysis of this material by atomic absorption spectroscopy (AAS) showed the presence of 1.147 ± 0.004 Pt/dimer. This ratio suggests that there is one cisplatin group coordinated with each TpT dimer. Unmodified TpT gave a background signal of 0.02 ± 0.004 Pt/dimer.

As shown in Table 2, the results of electrospray ionization (ESI) mass spectrometry analysis of *cis*-Pt-TpT were consistent with those obtained by AAS. The calculated mass of *cis*-Pt-TpT, in which the Pt is coordinated to a single ligand and contains a single positive charge, is 792 m/z. A parent ion (M^+) with this mass was observed in low abundance when the adduct was analyzed in positive ionization mode. A more prominent peak was observed at 774 m/z. This peak corresponds to loss of the water ligand from the cisplatin group ($M^+ - H_2O$). Such loss could be a consequence of the ionization technique. Alternatively the M^+-H_2O peak could represent a species in which Pt is coordinated to a second ligand on the TpT. Such coordination could occur through formation of a macrochelate between a non-bridging phosphodiester oxygen and an exocyclic oxygen of T or the N3 of T, through coordination of the Pt with an exocyclic O of one thymine and the N3 of the neighboring thymine of the dimer, or through coordination of platinum with both non-bridging phosphate oxygens. NMR studies of the products formed in solution between *cis*-DDP and TpT or d-T$(pT)_{19}$ provide evidence for adducts in which Pt is coordinated to a single non-bridging oxygen of the phosphodiester group and for adducts in which Pt is coordinated to O^4 and N3 atoms of adjacent thymine residues in d-T$(pT)_{19}$ (27,28). Adduct formation involving coordination of Pt with one of the phosphodiester oxygens would produce two diastereoisomers, which could account for the appearance of the two peaks of approximately equal intensity in the C-18 chromatogram (see Figure 1).

The M^+ - H2O peak at 774 m/z was the more abundant of a set of three peaks whose masses each differed by 1 mass unit (see Table 2). These mass differences correspond to the differences between the three natural abundance isotopes of platinum and are diagnostic for the presence of platinum. Two other prominent groups of peaks were observed in the spectrum. These peaks, which also occurred as triplets, arose from loss of one ammonia ($M^+ - H_2O - NH_3$) or two ammonia ($M^+ - H_2O - 2NH_3$) groups from the Pt coordinate. The lability of ammonia groups on platinum dinucleotide complexes when subjected to ESI mass spectrometry has been shown previously (32). Additionally, the mass spectrum contained peaks corresponding to the sodium derivatives of each of the species described above.

Treatment of the isolated *cis*-Pt-TpT with sodium cyanide resulted in 85%-90% conversion to TpT. Based on the observations of Raudaschl-Sieber and Lippert (30), who showed that Pt-N3 adducts of thymidine resist reaction with cyanide, our results would suggest that it is unlikely that the platinum is coordinated with the N3 of T. However, the unconverted compound that remained, even after 48 hrs incubation with cyanide, may represent material in which the platinum is coordinated to one or both of the thymine N3s of the dimer.

As shown by the chromatograms in Figure S3, *cis*-Pt-TpT appears to resist hydrolysis by snake venom phosphodiesterase (SVP). When incubated with a combination of SVP and calf intestinal phosphatase (CIP), TpT was completely digested to T within 1 hr as analyzed by

C-18 reversed phase HPLC. In contrast, only 32% of the *cis*-Pt-TpT was digested and a new peak that did not correspond to either the original *cis*-Pt-TpT or to T appeared in the chromatogram (see Figure S2). This peak may correspond to a platinated thymidine 5′-monosphosphate, i.e. *cis*-Pt-pT. Interestingly, one of the *cis*-Pt-TpT peaks was digested to a greater extent than the other peak. If these peaks correspond to diastereoisomers of *cis*-Pt-TpT as a result of Pt-phosphodiester coordination, then perhaps one the diastereoisomers is more susceptible to hydrolysis by SVP than the other. Similar preferential hydrolysis is observed when the diastereoisomers of oligonucleotide phosphorothioates are digested by SVP (33).

The extent of platination of TpT is very dependent upon the concentration of *cis*-DDP. As shown in Figure 2, after 96 hr approximately 70% of the TpT is converted to *cis*-Pt-TpT when the dimer is incubated with 2.5 mM *cis*-DDP at 37°C. However only 14% conversion to *cis*-Pt-TpT is observed in the presence of 0.5 mM *cis*-DDP and essentially no platination occurs at lower *cis*-DDP concentrations, even after 96 hrs incubation. In these reactions, the ratio of *cis*-DDP to dimer ranged from 1:1 up to 50:1.

Although relatively high concentrations of *cis*-DDP are required to platinate TpT, thymidine oligonucleotides, which contain multiple phosphodiester groups, are effectively platinated at much lower concentrations. For example, nonamer, **1p** (see Table 1), which contains eight phosphodiester linkages, was incubated with 0.15 mM *cis*-DDP for 72 hrs and the reaction was analyzed by strong anion exchange (SAX) HPLC. As shown in Figure 3a and 3b, new product peaks were observed that eluted before the peak corresponding to **1p**. The two major product peaks, which represent approximately 30% of the total, were isolated and analyzed by MALDI-TOF mass spectrometry (see Figure S4). The masses of these peaks were consistent with a derivative of **1p** that contained a single platinum. The additional earlier eluting peaks most likely represent oligonucleotide derivatized with two or more platinums. In contrast to this behavior, no platination was observed when the methylphosphonate analog of $T(pT)_8$, **1mp**, was incubated with 0.15 mM *cis*-DDP at 37°C for 72 hrs (see Figure 3c and 3d). These results further demonstrate that the phosphodiester groups facilitate platinum adduct formation with the thymidine oligonucleotides.

Platination of guanine-containing oligodeoxyribonucleotides

Guanine bases are targets for platination by *cis*-DDP, which forms intrastrand cross-links with -GG- and -GNG- sequences in DNA. As shown in Figure 4, we examined the platination of thymidine oligonucleotide **1p** and oligonucleotide **2p**, which contains a central -GTG- sequence. These 9-mers were incubated with 36 μM *cis*-DDP at 37°C. Aliquots were removed over a 24 hr time period and analyzed by SAX HPLC. Under these conditions, approximately 8% of **1p** was converted to platinated products after 24 hrs, whereas 90% of **2p** reacted. Analysis of the product from **2p** by MALDI-TOF mass spectrometry confirmed that it was a singly platinated species (see Figure S4) and most likely contains a 1,3-Pt-GTG intrastrand cross-link (34). In support of this conjecture, we have found that both guanines of a -GTG- sequence in a platinated oligonucleotide no longer react with dimethylsulfate, a result that is consistent with coordination of platinum with the N7s of these guanines (data not shown). Unlike **2p**, its methylphosphonate derivative, **2mp**, showed no reaction with 36 μM *cis*-DDP after incubation for 24 hrs at 37°C. To confirm that the unchanged peak observed in the C-18 reversed phase chromatogram of the reaction was not a mixture of platinated and unplatinated **2mp**, the entire peak was isolated and subjected to analysis by MALDI-TOF mass spectrometry. As shown in Figure S5, the major peak in the spectrum corresponded to **2mp**. Platinated **2mp** comprised only about 1-2% of the material seen in the mass spectrum, indicating that the amount of platinated **2mp** in this reaction is negligible. These results suggest that the phosphodiester backbone facilitates platination of the -GTG- sequence in the oligonucleotide.

To further investigate the effects of phosphodiesters on guanine platination, -GTG- oligonucleotides were prepared that contained mixtures of phosphodiester and methylphosphonate linkages (see Table 1). Oligomer **3p-*in*** contained four internal phosphodiester linkages that surround the -GTG- sequence, whereas the four phosphodiester linkages of oligomer **3p-*ex*** are external to the -GTG- sequence, which is surrounded by methylphosphonate groups. Oligomer **4p-*in***, a 13-mer, contained four internal phosphodiester linkages surrounding the -GTG- sequence and two additional phosphodiester linkages at each end of the oligomer. Oligomer **4p-ex**, on the other hand, had four external phosphodiester linkages at each end of the oligonucleotide and four methylphosphonate groups surrounding the -GTG- sequence. Finally, oligomer **5** contained a single phosphodiester linkage that was positioned either internally between the guanines of the -GTG- sequence, **5p-*in***, or the 3′-end of the oligonucleotide, **5p-*ex***.

As shown in Figure 5, both **3p-*in*** and **3*p*-*ex***, each of which contained four phosphodiester linkages, were platinated when incubated with 36 μM *cis*-DDP at 37°C. However the extent of platination was significantly less than that observed for **2p**, which contained a total of eight phosphodiester linkages. This result is consistent with the previous observation that phosphodiester linkages facilitate guanine platination.

Placement of the phosphodiester linkages near the target guanines enhances platination. This was shown by comparing the extent of platination of oligomers **3p-*in*** and **3p-*ex***. Oligomer **3p-*in***, which contained four internal phosphodiester linkages, was platinated to the extent of 33%, whereas only 17% platination was observed for oligomer **3p-*ex***, which contained four externally placed phosphodiester groups.

Like oligomer **2p**, oligomer **4p-*in*** contained the same -GTG- sequence surrounded by four phosphodiester groups and the same total number of phosphodiester groups. However the extent of platination of **4p-*in*** was less than that observed for **2p**. It appears that the intervening methylphosphonate groups of **4p-*in*** interfered with the platination reaction.

The extent of platination of oligomer **4p-*ex*** was approximately 45% less than that of **2p** and 25% less than that of **4p-*in***. Although all three oligonucleotides had the same net negative charge, the presence of the methylphosphonate groups surrounding the -GTG- sequence in **4p-*ex*** significantly reduced the rate of guanine platination. This reduction paralleled that previously seen between oligomers **3p-*in*** and **3p-*ex***. However unlike nonionic **2mp** whose -GTG- sequence was surrounded by methylphosphonates, and which was not platinated under these conditions, both **3p-*ex*** and **4p-*ex*** were platinated to some extent. These results are consistent with the idea that electrostatic interactions between *cis*-DDP and phosphodiester groups, even those remote from the -GTG- platination site, can serve to increase the effective local concentration of the platinating agent, which results in an increased rate of platination.

To see if a single phosphodiester could facilitate guanine platination, we incubated oligomers **5p-*in*** and **5p-*ex***, which contained respectively a single phosphodiester immediately 5′- of the second guanine of the -GTG- sequence and a phosphodiester at the 3′-end of the oligonucleotide, with 36 μM *cis*-DDP. The reaction mixtures were then analyzed by C-18 reversed phase HPLC after incubation for 24 hrs at 37°C. As shown in Figure 5, no platination of either oligomer was detected under these conditions. This result suggests that a single phosphodiester is not sufficient to trigger guanine platination, at least at the low concentration of *cis*-DDP used here. Lack of platination is perhaps not surprising given that essentially no platination of TpT was seen in the presence of 50 μM *cis*-DDP (see Figure 2). Raising the *cis*-DDP concentration to 1 mM did result in significant platination of both **5p-*in*** and **5p-*ex*** (see Figure S6). However, the platinated products of these oligonucleotides were not sufficiently resolved from the starting material on the C-18 column to enable quantification

and thus it was not possible to determine if platination of **5p-in** proceeded to a greater extent than that of **5p-*ex***. Some of the observed platination most likely results from direct reaction of guanines with *cis*-DDP because oligomer **2mp** was also platinated under these conditions (see Figure S7).

DISCUSSION

Platinum compounds are important chemotherapeutic agents whose primary DNA target appears to be guanines. In addition to reacting with guanine in DNA, NMR experiments have suggested that platinum can also interact with the phosphodiester group of oligonucleotides (27). A model of these interactions, based on that proposed by Kozelka and Barre (27), leading to phosphodiester-facilitated guanine platination is shown in Figure 6. Initially, the anionic phosphodiesters attract dicationic *cis*-diamminediaquaplatinum through formation of an outer-sphere complex. The resulting increased local concentration of platinum increases the likelihood of guanine monoadduct formation. Alternatively, the platinum could coordinate with a non-bridging oxygen of the phosphodiester internucleotide bond. In the absence of an adjacent guanine, the coordinated platinum could then exchange ligands with a neighboring phosphodiester thus causing the platinum to in effect “walk” along the phosphodiester backbone. If this platinum subsequently encounters a guanine residue, it could form a phosphodiester-N7 macrochelate (23,27), which in the presence of a neighboring guanine could go on to form an intraor interstrand cross-link. Our experiments with oligothymidylates show that at suitably high concentrations, *cis*-DDP reacts with TpT and with $T(pT)_8$ to form stable adducts that can be isolated by HPLC. The observation that under the same conditions thymidine displays little reactivity with *cis*-DDP and that adducts are not formed when the phosphodiester groups in these oligonucleotides are replaced with non-ionic methylphosphonate groups, shows that the phosphodiester group plays a crucial role in adduct formation. Although the structure of the *cis*-Pt-TpT adduct requires further investigation, the observation that treatment with sodium cyanide readily converts this adduct back to TpT suggests that the platinum may not be coordinated with the N3 of T in the dimer.

Positively charged *cis*-DDP can interact electrostatically with the negatively charged phosphodiester groups in the DNA sugar-phosphate backbone. The extensive platination of -GTG- in oligonucleotide **2p** by low concentrations of *cis*-DDP, but the lack of platination of its methylphosphonate analog, **2mp**, suggests that electrostatic interactions between *cis*-DDP and the phosphodiester groups of the oligonucleotide play an important role in the guanine platination reaction. This idea receives support by comparison of the relative levels of platination of oligomers **2p**, **3p-*in***, and **4p-*in***. Oligomer **3p-*in***, which contains four phosphodiester groups, is platinated much less extensively than either oligomer **2p** or **4p-*in***, each of which contains eight phosphodiester groups. It appears that the more highly charged oligonucleotides are better able to attract the positively charged *cis*-DDP which increases the local concentration of the *cis*-DDP and results in a faster rate of guanine platination.

The total net charge is not the only factor that enhances the rate of platination, however. Placement of the charges near the guanine target site is also important. This effect is shown by comparing the relative levels of platination of **4p-*in*** and **4p-*ex***. Both oligonucleotides have eight phosphodiester groups. However, the level of platination of **4p-*ex***, whose -GTG- platination site is surrounded by nonionic methylphosphonate groups, is approximately 36% less than that of **4p-*in***, whose -GTG- platination site is surrounded by negatively charged phosphodiester groups.

Even though the -GTG- platination site of oligomer **4p-*in*** is surrounded by phosphodiester groups and its net negative charge is the same as that of oligomer **2p**, the level of platination of **4p-*in*** is approximately 22% less than that of **2p**. In these experiments *cis*-DDP is in 2-fold

molar excess of the oligonucleotide. However, because the oligonucleotide contains eight phosphodiester bonds, there is a 4-fold molar excess of phosphodiesters to *cis*-DDP, and the *cis*-DDP is therefore more likely to initially interact with a phosphodiester in the oligonucleotide. If *cis*-DDP first encounters a phosphodiester group at the end of the oligomer, it could then migrate along the oligomer backbone as originally suggested by Kozelka and Barre (27), and shown in Figure 6. When it eventually encounters a guanine, platination of the N7 could occur. In the case of **2p** such migration can occur unimpeded. In the case of oligomer **4p-*in***, such migration would be blocked by the intervening methylphosphonate groups located on either side of the -GTG- sequence. Such blockage would contribute to the overall decrease in the rate of guanine platination that was seen with these oligomers.

In addition to enhancing platination rates as a consequence of electrostatic interaction, our experiments suggest that a phosphodiester group located immediately adjacent to a guanine nucleoside may further facilitate platination. It is not clear from our experiments if this facilitated platination takes place as a consequence of formation of a phosphodiester-Pt-guanine macrochelate, of the type shown schematically in Figure 6. We tried to examine this possibility by comparing the reaction of oligomers **5p-*in*** and **5p-*ex*** with *cis*-DDP. Both oligomers contain a single phosphodiester group located either next to a guanine (**5p-*in***) or at the 3′-end of the oligomer (**5p-*ex***). Although both oligomers were platinated, it was not possible to determine if there were differences in the amount of platination.

Our results clearly demonstrate that interactions between *cis*-DDP and the negatively-charged phosphodiester backbone play an important role in the platination of single-stranded oligonucleotides. Although our data are consistent with the model proposed in Figure 6 for phosphodiester-mediated guanine platination, further experiments need to be carried out to fully test this model. It seems reasonable that similar phosphodiester interactions could contribute to platination of guanine bases in cellular DNA. In the cell, *cis*-DDP is more likely to initially encounter negatively charged phosphodiesters than a guanine base. These phosphodiesters could facilitate migration of the *cis*-DDP to its ultimate guanine target thus enhancing the effectiveness of this platinating agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cis-DDP, *cis*-diamminedichloroplatinum(II); AAS, atomic absorption spectroscopy; ESI-MS, electrospray ionization mass spectrometry; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SAX, strong anion exchange; SVP, snake venom phosphodiesterase; CIP, calf intestinal phosphatase.

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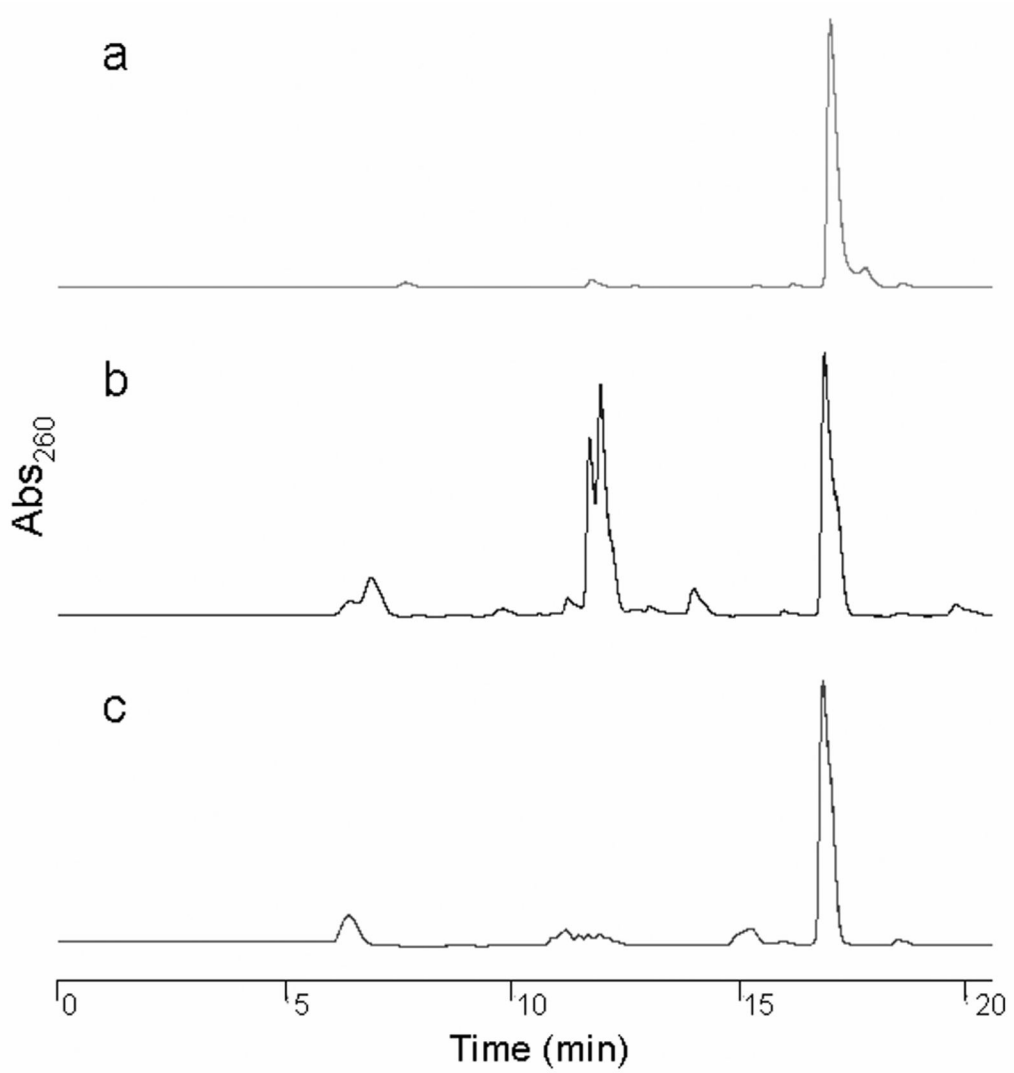

Figure 1. Reaction of TpT with cisplatin and reversal with cyanide

(a) TpT incubated 0 hr with *cis*-DDP, (b) TpT incubated 24 hr with 5mM *cis*-DDP, (c) TpT incubated 24 hr with 5 mM *cis*-DDP followed by incubation with 167 mM sodium cyanide for 24 hr. The reaction mixtures were analyzed by C-18 reversed phase HPLC using a 0-52.5% MeOH gradient in 20mM Tris pH 8.5.

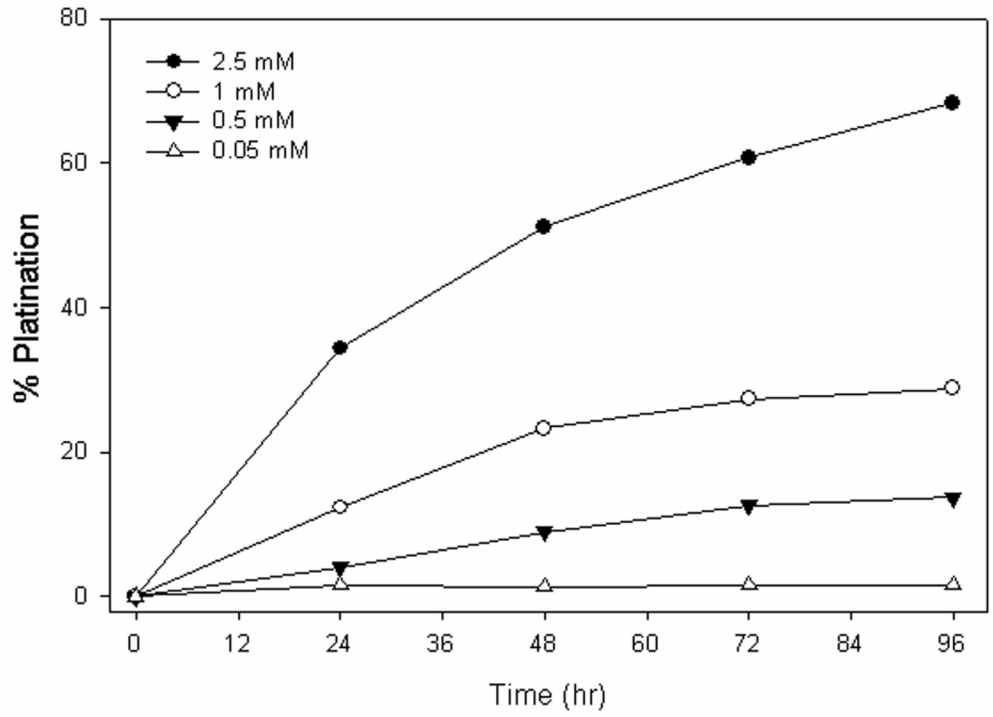

Figure 2. Kinetics of TpT platination as a function of cis-DDP concentration

TpT, 0.05 mM, was incubated with 2.5 mM (closed circles), 1 mM (open circles), 0.5 mM (closed triangles), or 0.05 mM (open triangles) *cis*-DDP.

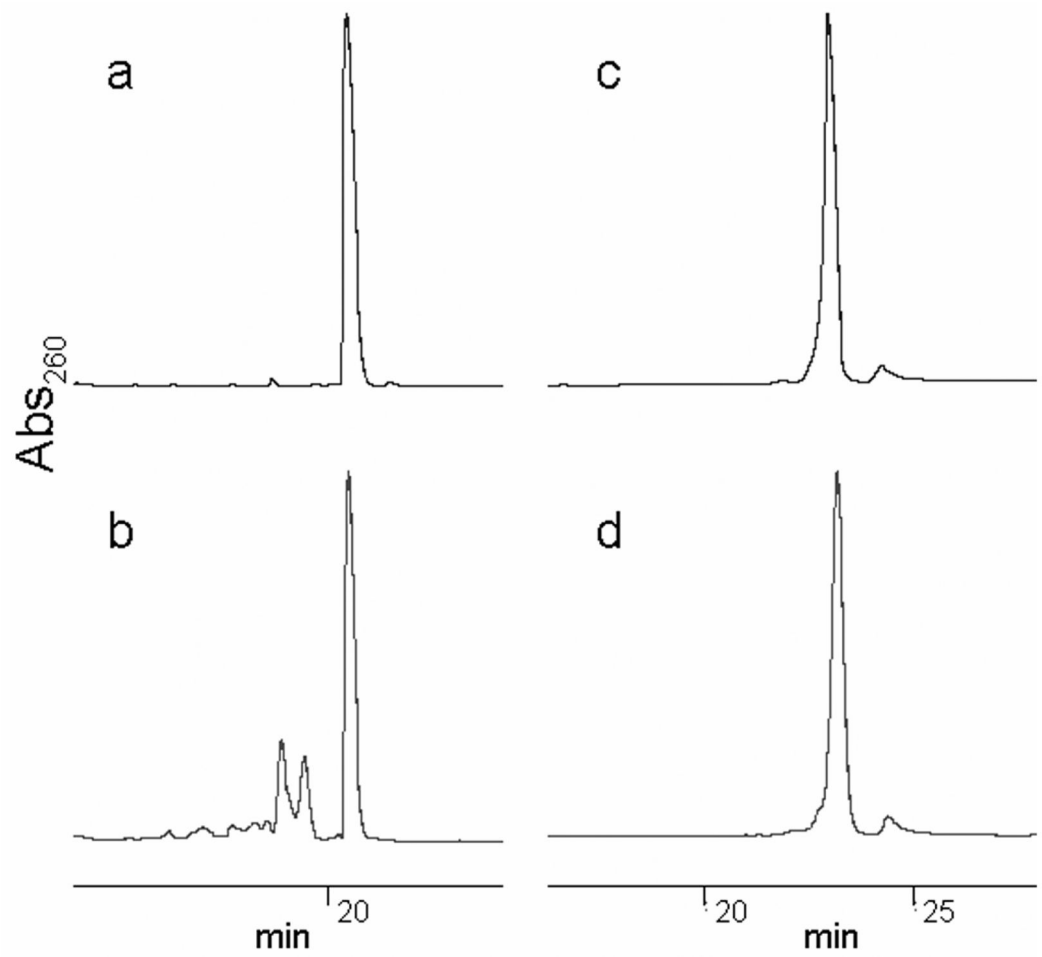

Figure 3. Platination of oligonucleotides with phosphodiester or methylphosphonate linkages
Oligomer **1p** (a & b) or **1mp** (c & d) was incubated with 150 μM cisplatin and the reactions were analyzed by SAX or C-18 HPLC, respectively, after 0 hr (a & c) and 72 hr (b & d).

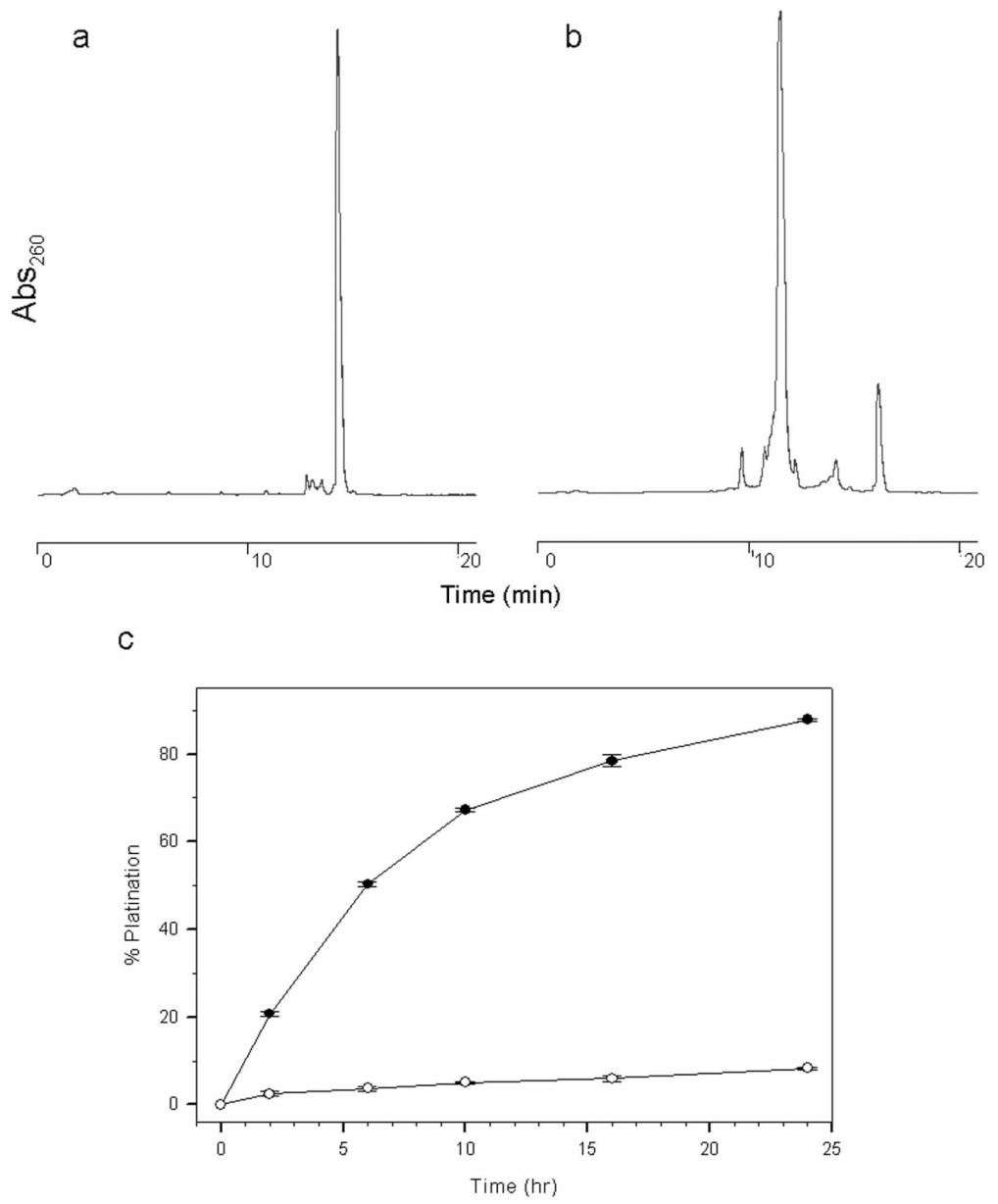

Figure 4. Platination of oligodeoxyribonucleotides

(a) Oligonucleotide **1p** was incubated with 36 μM *cis*-DDP at 37 °C for 24 hr. (b) Oligonucleotide **2p** was incubated with 36 μM *cis*-DDP at 37 °C for 24 hr (c) Time course of reaction of 36 μM *cis*-DDP with 18 μM **1p** (closed circles) and 18 μM **2p** (open circles).

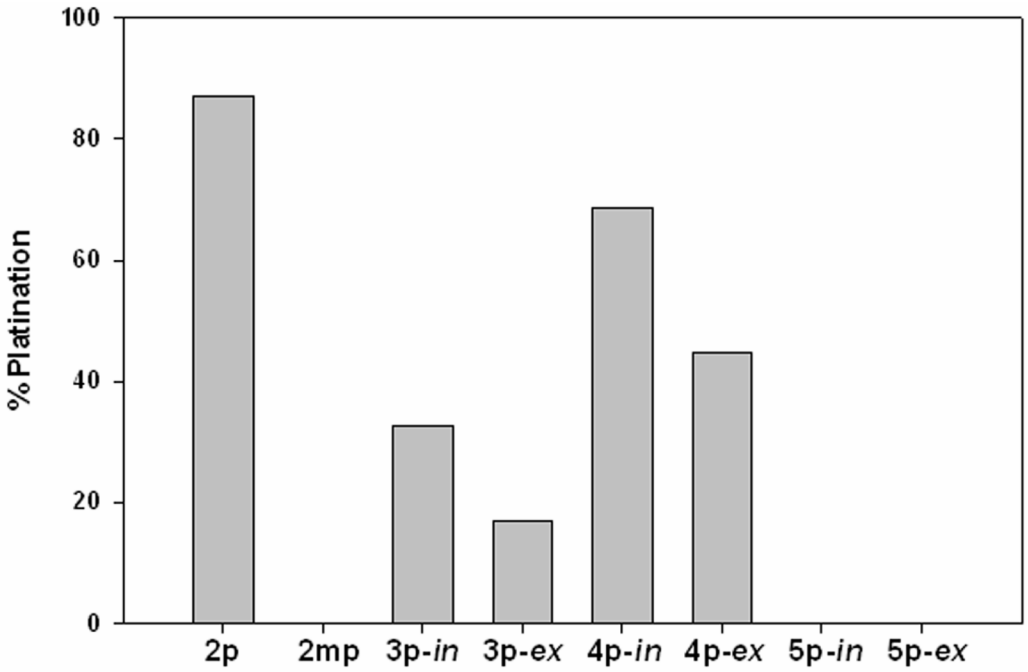

Figure 5. Effect of methylphosphonates on -GTG- platination

Percent platination of oligonucleotides incubated with 36 μM *cis*-DDP for 24 hr.

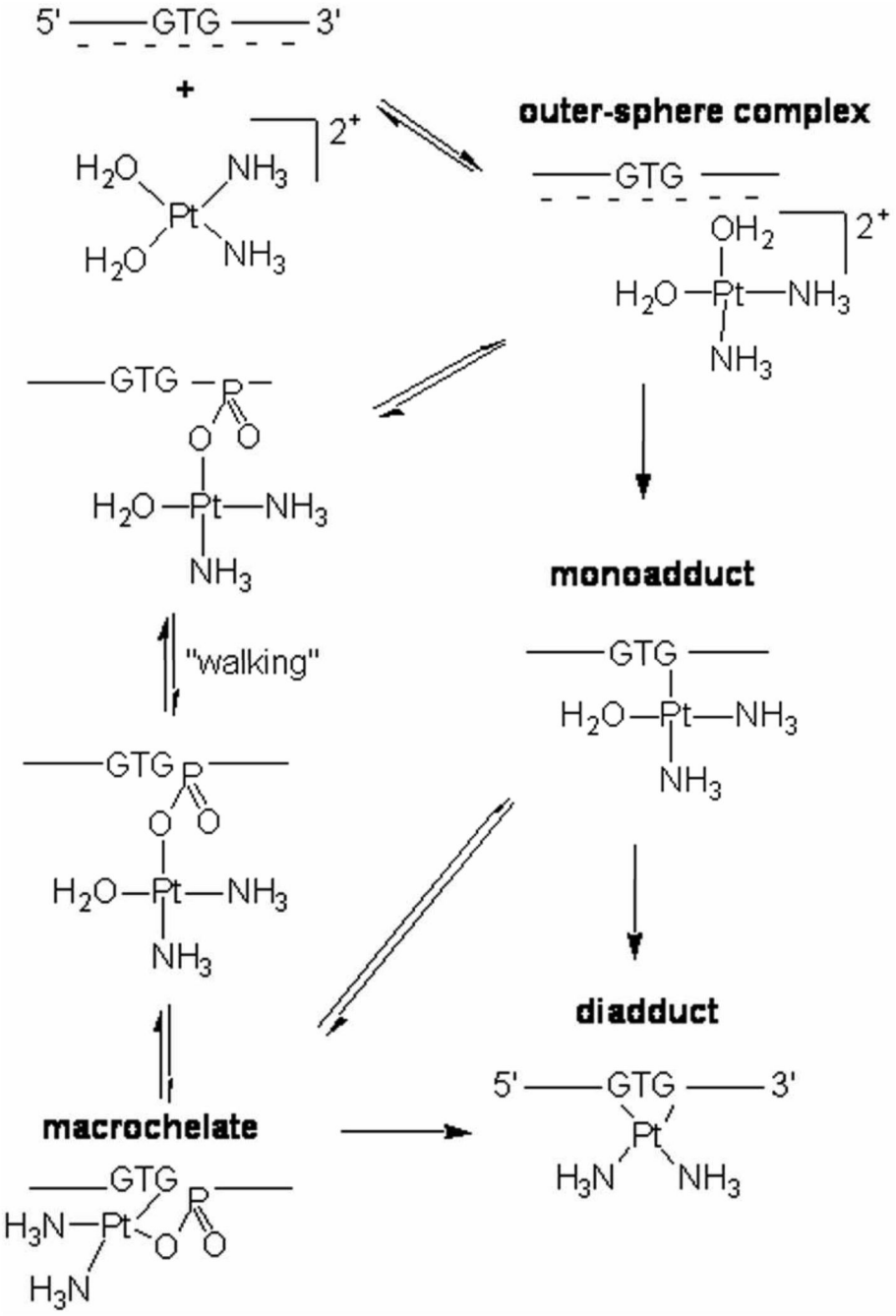

Figure 6.
Phosphodiester-facilitated guanine platination (Adapted from Kozelka and Barre, (27))

Table 1

Sequences of Oligonucleotides

Oligodeoxyribonucleotide [(a)]	Abbreviation	Net Charge	m/z	
			calcd	observed
5'-TpTpTpTpTpTpTpTpT	**1p**	-8	2674.40	2673.50
5'-TpTpTpTpTpTpTpTpT	**1mp**	0	2658.66	2659.97
5'-TpTpTpGpTpGpTpTpT	**2p**	-8	2724.42	2725.96
5'-TpTpTpGpTpGpTpTpT	**2mp**	0	2708.42	2709.66
5'-TpTpTpGpTpGpTpTpT	**3p-*in***	-4	2720.42	2717.96
5'-TpTpTpGpTpGpTpTpT	**3p-*ex***	-4	2720.42	2719.25
5'-TpTpTpTpTpGpTpGpTpTpTpTpT	**4p-*in***	-8	3936.58	3937.37
5'-TpTpTpTpTpGpTpGpTpTpTpTpT	**4p-*ex***	-8	3936.58	3938.08
5'-TpTpTpGpTpGpTpTpT	**5p-*in***	-1	2709.42	2711.31
5'-TpTpTpGpTpGpTpTpT	**5p-*ex***	-1	2709.42	2711.80

[(a)] p = phosphodiester linkage; p = methylphosphonate linkage

Table 2

ESI-MS analysis of cis-Pt-TpT

m/z	Species
792	M^+
774 (773, 775)	M^+-H_2O
757 (756, 758)	M^+-H_2O-NH_3
740(739,741)	M^+-H_2O-2NH_3