Metalated DNA

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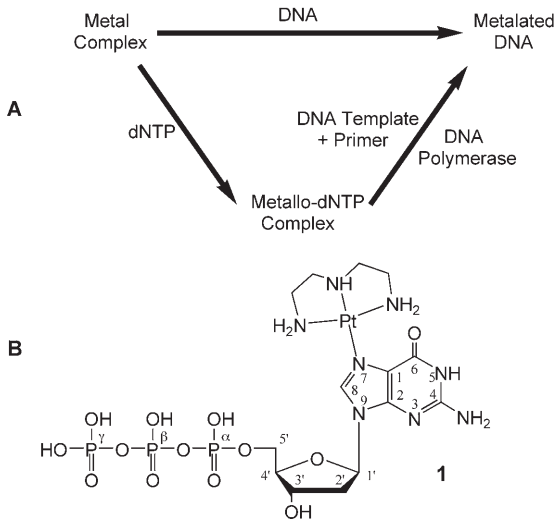
Experimental Evidence That a DNA Polymerase Can Incorporate N7-Platinated Guanines To Give Platinated DNA**

*Michele Benedetti, Cosimo Ducani, Danilo Migoni, Daniela Antonucci, Vita M. Vecchio, Antonella Ciccarese, Alessandro Romano, Tiziano Verri, Giuseppe Ciccarella, and Francesco P. Fanizzi**

Incorporation into DNA of nucleotides linked to functional groups has a variety of potential applications. Several organic reporter groups, covalently linked to nucleotides, are already widely used as experimental tools for nucleic acid modification.[1] Extensive studies on the synthesis, structure, and properties of metalated nucleotides, oligonucleotides, DNA, and RNA, mainly but not solely cisplatin related, have been reported.[2,3] Such metalation is generally achieved by direct reactions of the polynucleotide substrate with a metal complex.

Herein, we report experimental evidence that a DNA polymerase can incorporate N7-platinated guanines to give platinated DNA. A new method to obtain site-specific metalation, by incorporating metal-modified nucleotides into the DNA, is proposed. The possibility of obtaining a metal, covalently linked to a nucleotide, in a specific position of a polynucleotide sequence is expected to present a wide range of applications, both pharmacological (for example, drug design) and technological (for example, DNA-template-based production of metal arrays).[3]

Herein we show, for the first time, that metalated purines can be inserted into DNA by DNA polymerases during an in vitro synthetic process (Scheme 1 A). In our experiments we used: 1) a model DNA polymerase, that is, Taq polymerase, and 2) a simple model molecule, namely, the complex [Pt(dien)(*N*7-5′-dGTP)] (**1**; dien = diethylenetriamine, 5′-dGTP = 5′-(2′-deoxy)guanosine triphosphate; Scheme 1 B). Complex **1** was chosen because, as a result of the lack of labile chloride ligands, it is unable to give chelates, as in the case of similar cisplatin derivatives.[2–4] The insertion of platinated purine bases into the complementary DNA chain during a DNA synthetic process is shown by using two different experimental approaches.



Scheme 1. A) The known mechanism of direct DNA platination compared with the proposed mechanism of DNA platination mediated by DNA polymerases. B) Structure of the model complex [Pt(dien)(*N*7-5′-dGTP)] (**1**).

In the first experiment, we performed a primer extension assay using a synthetic DNA primer/template substrate (Figure 1 A). The presence of only one C residue between the 40 T and 40 G residues in the DNA template allowed Taq DNA polymerase to insert only one G in the newly synthesized complementary strand. We prepared two reaction mixtures, both containing the above DNA primer/template and the Taq DNA polymerase. Moreover, in one reaction the four standard triphosphate nucleotides, 5′-dNTP (dNTP = deoxynucleotide triphosphate), including α-[^{32}P]-dATP (dATP = deoxyadenosine triphosphate) were added, whereas in the other 5′-dGTP was substituted by a mixture of **1** and 5′-dGTP in about 95:5 ratio. Such a mixture was the product of the synthesis of **1** performed in the presence of a small excess of 5′-dGTP with respect to [PtCl(dien)]Cl.[5] Under these conditions, the introduction of Pt into the newly synthesized DNA chain is necessarily controlled by the polymerase. The results of the DNA polymerization process were visualized by autoradiography (Figure 1 B) after separation of the primer extension products by electrophoresis on 13% (v/v) polyacrylamide gel.

For both the mixture containing the standard 5′-dNTPs and that containing **1** and 5′-dGTP in about 95:5 ratio, autoradiography showed a band corresponding to a 100-

[*] Prof. M. Benedetti, Dr. C. Ducani, Dr. D. Migoni, Dr. D. Antonucci, Dr. V. M. Vecchio, Prof. A. Ciccarese, Dr. A. Romano, Prof. T. Verri, Prof. F. P. Fanizzi
Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento
Via Provinciale Lecce-Monteroni, 73100 Lecce (Italy)
Fax: (+39) 0832-298-626
E-mail: fp.fanizzi@unile.it
Prof. G. Ciccarella
Dipartimento di Ingegneria dell'Innovazione
Università del Salento
Via Provinciale Lecce-Monteroni, 73100 Lecce (Italy)

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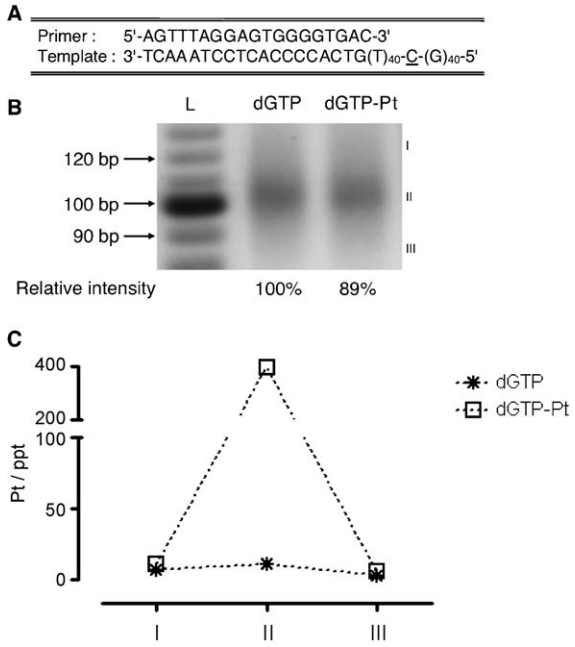

Figure 1. A) Sequence of the DNA primer/template pair used in primer extension. The primer is oriented 5′ to 3′ and the template 3′ to 5′ so that the complementary region can be readily visualized. The single template cytosine residue where 5′-dGTP or **1** will be incorporated is underlined. B) The ability of Taq DNA polymerase to extend the primer/template was evaluated in the presence of all four natural dNTPs (positive control: lane marked dGTP) or of a dNTP mixture containing **1** instead of natural 5′-dGTP (lane marked dGTP-Pt). The DNA substrate (100 pmol), Taq DNA polymerase (1 U), dNTPs (12.5 μM each), and α-[^{32}P]-dATP (20 μCi) were incubated in a primer extension assay. The reaction products were resolved on 13% (v/v) polyacrylamide gel and visualized by autoradiography. The intensity of DNA bands was quantified using a BioRad GS-700 imaging densitometer and the Molecular Analyst software package 1.5 (Bio-Rad Laboratories, Hercules, CA, USA) and reported as percentage of the control value (dGTP lane). A ten-base-pair (bp) oligonucleotide ladder (Invitrogen) was 5′-[^{32}P]-labeled by using polynucleotide kinase (Invitrogen) and γ-[^{32}P]-ATP (lane L). Broadening of the 100-mer band in the dGTP and dGTP-Pt lanes is a result of the higher loaded volumes used for the samples with respect to the standard ladders L. C) Determination of platinum content of gel fragments corresponding to the area above (I), centered onto (II), and below (III) the observed oligomer bands by ICP-AES.

nucleotide oligomer (Figure 1B). To assess the presence of incorporated platinated G nucleotides in the 100-bp synthesized oligomer, quantitative analysis of Pt by inductively coupled plasma atomic emission spectrometry (ICP-AES) was performed (Figure 1C). Fragments of the gel reported in Figure 1B corresponding to the bands observed in the dGTP and dGTP-Pt lanes (II), and the adjacent portions of the gel above (I) and below (III) the visible oligomer bands, were digested in 70% HNO_3 and analyzed for the presence of Pt. The platinum level observed for the band in the dGTP-Pt lane (II) was invariably higher (roughly 40 times) than that in the adjacent portions above (I) and below (III) within the same lane and each portion of the dGTP lane (I–III). In particular, for the band (II) in the lane marked dGTP-Pt the number of Pt atoms per 100-mer reaches the value of 0.57 with an overall yield of platination of about 62%. These results confirm the ability of Taq DNA polymerase to insert previously platinated nucleotides (that is, **1**), although with a reduced efficiency with respect to the standard bases.

In the second experiment, we adopted a PCR-based assay to evaluate competition between 5′-dGTP and **1** for incorporation into plasmidic DNA (pUC19, Figure 2A). We used Taq DNA polymerase and a mixture containing essentially **1** (90–95%) as modified nucleobase in the presence of [PtCl-(dien)]Cl (5–10%; Figure 2B). Such a mixture was the product of the synthesis of **1** performed in the presence of a small excess of [PtCl(dien)]Cl with respect to 5′-dGTP. Excess [PtCl(dien)]Cl ensured both complete consumption of traces of 5′-dGTP and inhibition of the possible dissociation of **1** as a result of the presence of chloride.[5] A separate check demonstrated that excess [PtCl(dien)]Cl did not alter the capacity of Taq DNA polymerase to amplify the specific PCR product and only weakly affected the PCR amplification efficiency (Figure 2C).

Competition between 5′-dGTP and **1** for Taq DNA polymerase catalyzed incorporation into DNA was evaluated in a series of six PCR-based assays by using increasing amounts (20% steps from 0 to 100%) of the platinum-modified base with respect to dGTP (20% steps from 100 to 0%). In all cases, pUC19 DNA was used as a substrate together with Taq DNA polymerase, a pair of pUC19-specific primers, and the natural dNTPs ($N \neq G$). Agarose gel electrophoresis of the reaction products after 30 PCR cycles, followed by ethidium bromide staining and UV visualization, showed a single PCR product in all lanes.

However, the PCR product in lane 100 was visible only at very high UV exposure times (Figure 2B). In particular, on passing from 0 to 100% **1** it is clear that there is 1) a significant electrophoretic shift and 2) a significant reduction in the amount of the synthesized product (Figure 2B). The former observation is explained well by the fact that the newly synthesized oligomer exhibits a progressively lower charge-to-mass ratio because of the presence of the platinated base. The second finding is evidence of the lower efficiency of Taq DNA polymerase in the presence of increasing amounts of **1**, as already observed for other N7-modified nucleobase insertion experiments.[1] These results are also consistent with the well-known concept that platinated DNA templates are able to severely repress DNA polymerase activity.[6] However, it is evident that when only complex **1** is available, the Taq DNA polymerase activity is greatly reduced but not completely quenched.

Herein, we have demonstrated that free platinated purines can be incorporated into DNA synthesized in vitro by using Taq DNA polymerase. The limits for the application of these findings to other polymerases (including eukaryotic polymerases) and metal complexes bearing purine bases (including those more closely related to cisplatin, such as *cis*-[PtCl(NH_3)$_2$(*N7*-5′-dGTP)][4]) need to be defined. However, from the perspective of Scheme 1A, our results also suggest a possible alternative mechanism for DNA platination in living cells treated with platinum-based antitumor drugs, which may parallel the accepted direct DNA platination process. Such a novel approach, if confirmed in vivo, might open the

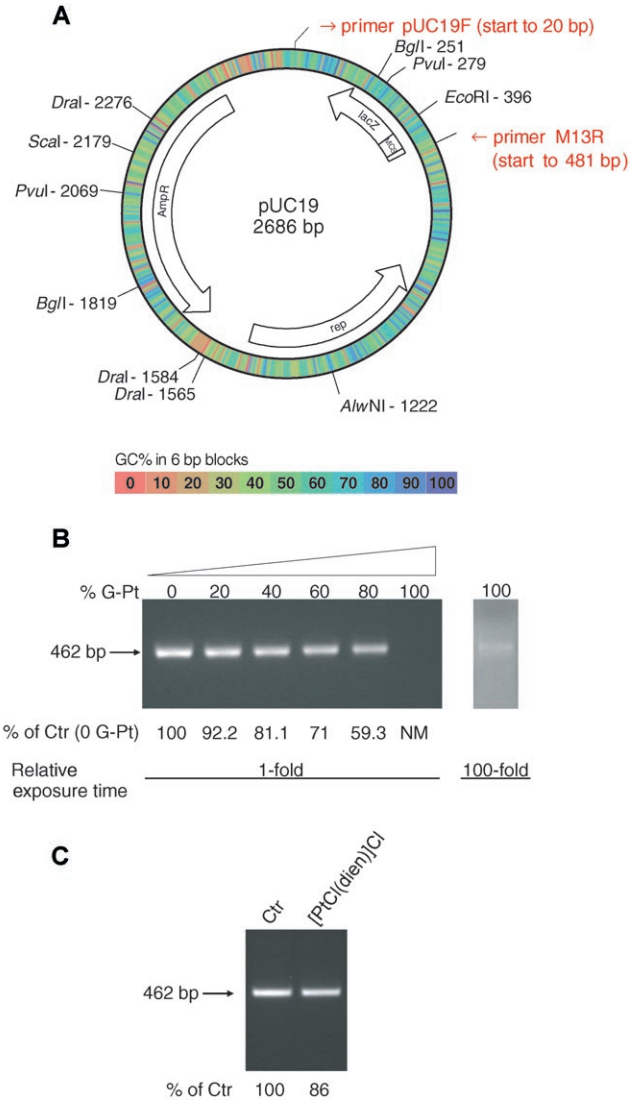

Figure 2. A) Map of the pUC19 plasmid used as a natural DNA template in the PCR-based assay. The primers used to amplify the 462-bp fragment corresponding to the lacZ gene are indicated. B) Competition between 5′-dGTP and **1** for incorporation into DNA catalyzed by Taq DNA polymerase. pUC19 plasmid DNA substrate (2 fmol), Taq DNA polymerase (2 U), and the four natural dNTPs (0.2 mM each) were used in a PCR-based assay. Natural 5′-dGTP (positive control, lane 0) was gradually (20% steps) substituted by modified **1** until it was completely replaced (lane 100). After the reaction, the DNA synthesis products were separated on 1% agarose gel and visualized with ethidium bromide using a Gel-Doc 2000 gel documentation system (Bio-Rad Laboratories). Band intensities were quantified with the Quantity One 4.1.1 software (Bio-Rad Laboratories) and are reported (below the picture) as the percentage of the positive control value. PCR products in the presence of 100% **1** were visualized only with higher exposure times (100 fold). NM = not measurable. C) To control a possible nonspecific effect of excess [PtCl(dien)]Cl, the capacity of Taq DNA polymerase to amplify pUC19 plasmid DNA template (2 fmol) was evaluated in the absence (positive control; Ctr) or presence of [PtCl(dien)]Cl (20 μM; corresponding to [PtCl(dien)]Cl present in the PCR-based assay reported in (B); lane marked [PtCl-(dien)]Cl). [PtCl(dien)]Cl was added to the PCR mixture immediately before starting the reaction. The intensity of DNA bands was reported as percentage of the control value (Ctr lane). Addition of [PtCl(dien)]Cl (20 μM) did not alter the capacity of Taq DNA polymerase to amplify the specific PCR product, and only weakly affected the PCR amplification efficiency.

possibility of developing a new generation of platinum drugs based on a different rationale and design. Moreover, it cannot be excluded that this DNA platination mechanism could be useful for insertion of metal centers into specific positions of oligonucleotides, which may lead to new molecular devices for technological applications.

Experimental Section

All solvents and reagents, unless otherwise stated, were purchased from Aldrich Chemical Company and used as received. [PtCl-(dien)]Cl was prepared from potassium tetrachloroplatinate, as previously described.[7] NMR spectra were recorded on a Bruker Avance DPX 400 spectrometer using deuterated solvents. Spectra were referenced to the residual HOD signal (^{1}H) or 85% H_3PO_4 (^{31}P) as internal and external standard, respectively. Liquid chromatography–mass spectrometry (LC-MS) of **1** dissolved in H_2O (pH 7) was performed on an Agilent Technologies LC-MS ion-trap VL_01002 system. The negative-ion mode (electrospray) was used at a drying-gas temperature of 350°C. The sample was injected by infusion.

1: [PtCl(dien)]Cl (4.58 mg, 0.0124 mmol) was dissolved in D_2O (600 μL) in a 5-mm NMR tube. The pH* value (pH* denotes the uncorrected meter readings of solutions in D_2O) was adjusted to about 3.5 by addition of DCl diluted in D_2O. Increasing amounts of a 5′-dGTP mother solution containing 5′-dGTP (0.04 mmol) in D_2O (2 mL; pH* adjusted to 3.5) were added to the solution in the tube and the formation of **1** was followed by ^{1}H and ^{31}P NMR spectrometry. Additions were stopped when NMR monitoring (integration of the H8 ^{1}H NMR signals of free and N7-bonded 5′-dGTP) showed the presence of a slight excess of unreacted free nucleotide (5–10%) with respect to **1**. The pH of the solution was then adjusted to 7.1 by addition of diluted NaOD, and the final volume of the solution was increased to approximately 2000 μL by addition of distilled water. Mother solutions of **1** containing 5–10% excess of [PtCl(dien)]Cl with respect to **1** were also prepared by a similar procedure. Pure **1** was isolated when the reaction of [PtCl(dien)]Cl and 5′-dGTP was conducted under stoichiometric conditions. ^{1}H NMR (D_2O, pH* 7.1): $\delta = 8.76$ (s, 1H; CH), 6.39 (t, $^3J_{H,H} = 6$ Hz, 1H; CH), 4.92 (m, 1H; CH), 4.78 (m, 2H; CH), 4.26 (m, 3H; CH), 3.32 (m, 1H; CH), 3.12 (m, 4H; CH), 2.89 (m, 1H; CH), 2.74 (m, 1H; CH), 2.62 ppm (m, 1H; CH); ^{31}P NMR (D_2O, pH* = 7.1): $\delta = -11.99$ (d, $^2J_{P,P} = 52$ Hz, 1P; γ-PO_4), −12.18 (d, $^2J_{P,P} = 48$ Hz, 1P; α-PO_4), −24.15 ppm (t, $^2J_{P,P} = 48$ Hz, 1P; β-PO_4); LC-MS of **1**: exact mass calcd for anions $C_{14}H_{25}N_8NaO_{13}P_3Pt$: 824.03 amu; ESI: *m*/*z* found: 824 $[M]^-$, 722.5 $[M-NaPO_3]^-$; exact mass calcd for anions $C_{14}H_{26}N_8O_{13}P_3Pt$: 801.04 amu; ESI: *m*/*z* found: 801 $[M]^-$, 722.5 $[M-HPO_3]^-$.

The primer extension assay was performed with the 100-nucleotide DNA template 5′-$(G)_{40}$-C-$(T)_{40}$GTCACCCCACTC-CTAAACT-3′ and the 19-nucleotide primer 5′-AGTTTAG-GAGTGGGGTGAC-3′. Two reaction mixtures were prepared, both containing standard 1X buffer and $MgCl_2$ (1.5 mM), primer/DNA template (100 pmol), α-[^{32}P]-dATP (20 μCi), and Platinum Taq DNA polymerase (1 U; Platinum is a commercial name not related to platinum metal; Invitrogen Corp., Carlsbad, CA, USA). Moreover, the first mixture contained dNTPs (12.5 μM each), whereas in the second mixture dGTP was substituted by **1**/5′-dGTP with a 95:5 ratio. Both mixtures were used to perform primer extension with a thermocycler PCR Sprint apparatus (Hybaid Ltd., Teddington, UK). After activation of Taq DNA polymerase (at 94°C for 2 min), denaturation was performed at 94°C for 30 s, annealing at 55°C for 30 s, and synthesis at 72°C for 20 s. The reaction mixture (10 μL) and 10-bp DNA ladder (5 μL) labeled with ^{32}P (Invitrogen) were loaded on 13% polyacrylamide gel. After electrophoresis at 40 mA, the primer extension products were visualized by autoradiography. Fragments of similar size of the dried gel, which corresponded to the bands of the newly synthesized DNA strands, were digested in

70% HNO_3. Acid (2 mL) was added to each gel sample, which was then treated at high temperature (200 °C) and pressure (200 psi) in a microwave oven for 10 min. The samples were diluted with Millipore water (18 MΩ) to a final volume of 5.0 mL. Finally, platinum levels (reported in Figure 1C as concentration in the final 5-mL solutions) were measured with a Varian VISTA AX (torch axial configuration) ICP-AES instrument.

PCR-based assay: Six reaction mixtures were prepared, all containing standard 1X buffer and $MgCl_2$ (1.5 mM), pUC19 plasmid DNA substrate (2 fmol), Platinum Taq DNA polymerase (2 U; Invitrogen), the primers (0.4 μM each) pUC19F (5′-CGGTGAAAACCTCTGACACAT-3′) and M13 reverse (5′-CAGGAAACAGCTATGACC-3′; Invitrogen), dCTP (0.2 mM), dTTP (0.2 mM), and dATP (0.2 mM). dGTP (0.2 mM) was gradually substituted in 20% steps by **1** until complete replacement (0.2 mM dGTP/0 mM **1**, lane 0; 0.16 mM dGTP/0.04 mM **1**, lane 20; 0.12 mM dGTP/0.08 mM **1**, lane 40; 0.08 mM dGTP/0.12 mM **1**, lane 60; 0.04 mM dGTP/0.16 mM **1**, lane 80; 0 mM dGTP/0.2 mM **1**, lane 100; see Figure 2B). After Platinum Taq polymerase activation at 94 °C for 2 min, 30 cycles of PCR were performed as follows: denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and synthesis at 72 °C for 5 min. A final polymerization step was performed at 72 °C for 5 min. PCR products were denatured at 65 °C for 3 min and then loaded on 1% agarose gel. After the run, the gel image was acquired by a UV lamp (Gel Doc system; Bio-Rad Laboratories) and the associated software Quantity One (Bio-Rad).

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