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Synthesis, Characterization and Biological Activity of *trans*-Platinum(II) and *trans*-Platinum(IV) Complexes with 4-Hydroxymethylpyridine

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The synthesis and chemical characterization of two new trans *platinum complexes,* trans-*[$PtCl_2NH_3$(4-hydroxymethylpyridine)] (**1**) and* trans-*[$PtCl_4NH_3$(4-hydroxymethylpyridine)] (**2**) are described. Their ability to interact with 5′-GMP by themselves and in the presence of reducing agents in the case of* trans-*[$PtCl_4NH_3$(4-hydroxymethylpyridine)] were tested. Circular dichroism, electrophoretic mobility in agarose gel, and atomic force microscopy studies showed that the interaction of complex **1** with DNA is stronger than that of complex **2**. Cytotoxicity tests against HL-60 tumor cells also showed higher activity for* trans-*[$PtCl_2NH_3$(4-hydroxymethylpyridine)] than for* trans-*[$PtCl_4NH_3$(4-hydroxymethylpyridine)]. Complex **1** presents similar behavior to cisplatin, but with a lower IC_{50} at 24 h. Complex **1** also showed high apoptosis induction.*

Introduction

In his well-known experiments, Rosenberg demonstrated the antitumor activity of the *cis* isomer of $[PtCl_2(NH_3)_2]$.[1,2] In contrast, the *trans* isomer does not seem to cause such an effect on the same kind of tumor cell lines. For several decades, researchers connected the antitumor activity of platinum compounds with the *cis* geometry and a chemical structure analogous to that of cisplatin.[3–7] However, in the last few years, several platinum compounds that break established rules, among them that of *cis* geometry, have been successfully studied, and their antitumor behavior against tumor cell lines has been confirmed.[8–38] In particular, series of *trans*-Pt^{II} complexes with different types of ligands, planar amines,[11–22] iminoethers,[23–27] asymmetric aliphatic amines[28–30] and *trans*-$Pt^{II}Cl_2$ polynuclear complexes,[29,31,32,35,36] have been designed, synthesized, structurally characterized, and tested against tumor cell lines. Most of them show antitumor properties and apoptotic behavior,[22,37,38] The modes of binding to DNA and other biochemical effects are also being investigated.[39–41]

In addition, Rosenberg's experiments also detected antitumor activity for some octahedral platinum(IV) complexes, such as *cis*-$[PtCl_4(NH_3)_2]$.[1,2] Pt^{IV} complexes present some advantages; mainly a higher water solubility and, as a consequence, the possibility of being orally administered, as well as greater cellular uptake and milder side effects. Nevertheless, none of the three Pt^{IV} compounds that have reached clinical trials, *tetraplatin*, *iproplatin* and JM216, presents better activity than cisplatin in humans, in spite of the fact that they have much more active "in vitro" assays.[42–46]

Recently, the synthesis of *trans*-$[PtCl_2NH_3$(3-hydroxymethylpyridine)] and *trans*-$[PtCl_2NH_3$(4-hydroxymethylpyridine)] (**1**) has been published.[47] Although tests against cisplatin-resistant cell lines were performed without producing noticeable results, crystal structures and other aspects of biochemical properties were obtained, which had not been described before.

In this paper, we report the synthesis, spectroscopic and crystallographic characterization, interaction with 5′-GMP and DNA, and cytotoxicity of the water-soluble *trans*-platinum complexes, **1** and *trans*-$[PtCl_4NH_3$(4-hydroxymethylpyridine)] (**2**). The results confirm the activation of *trans* structures by using planar ligands and the activation of Pt^{IV} compounds by reduction with ascorbate and glutathione.

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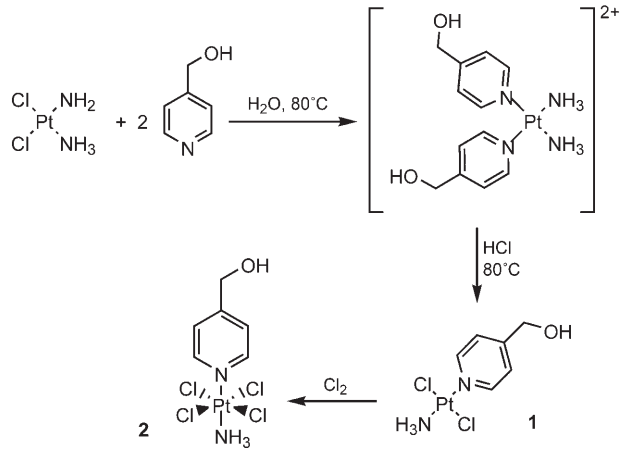
Results and Discussion

Chemistry

To synthesize compounds **1** and **2**, 4-hydroxymethylpyridine was added to an aqueous solution of cisplatin, and the mixture was stirred for 5 h. Addition of concentrated HCl precipitated the *trans*-Pt^{II} compound. Complex **1** was dissolved in a 0.1 M hydrochloric solution, and compound **2** was obtained with a stream of gaseous Cl_2 (Scheme 1).



Scheme 1. Synthesis of complexes **1** and **2.**

The structure was confirmed by ^{1}H and ^{195}Pt NMR spectroscopy and X-ray diffraction. The NMR data for the ligand and compounds **1** and **2** are shown in Table 1. All the signals appear deshielded in the complexes; especially in Pt^{IV} compound, which is more electronegative. The value of ^{195}Pt is typical for these kind of compounds, as has been previously described.[12,28,47–51]

Table 1. ^{1}H and ^{195}Pt parameters δ (ppm) of the ligand and its complexes.

Compounds	CH_2	^{1}H Aromatics	^{195}Pt
4-hydroxymethyl-pyridine	4.64(s)	$H_{A,A'}$:8.26(dd); $H_{B,B'}$:7.18(dd)	
compound **1**	4.71(s)	$H_{A,A'}$:8.45(dd); $H_{B,B'}$:7.23(dd)	−2034 ppm
compound **2**	4.75(s)	$H_{A,A'}$:8.78(dd); $H_{B,B'}$:7.43(dd)	−153 ppm

Crystal and molecular structure determination

The molecular structure of the complexes *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] and *trans*-[$PtCl_4NH_3$(4-hydroxymethylpyridine)] are shown in Figure 1. Crystallographic data and structure refinement are presented in Table 2, while bond lengths and angles are summarized in Table 3.

The structure of **1** consists of discrete molecules of *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)], in which platinum is bound to the pyridinic N of the ring and to a molecule of NH_3 in a *trans* arrangement. The other two positions are occupied by the chloride atoms. Platinum is in a slightly distorted square-planar environment, due to the repulsion of the bulky ring; this causes opening of the N(2)–Pt–Cl angle, which becomes higher than 90°, and closing of the N(1)–Pt–Cl angle, which is lower than 90°. Average values for Pt–Cl and Pt–N bond lengths are 2.298 and 1.985 ?, respectively, in agreement with typical values for this type of bond. The distance between consecutive pyridinic rings is 3.74 Å; this suggests a slight

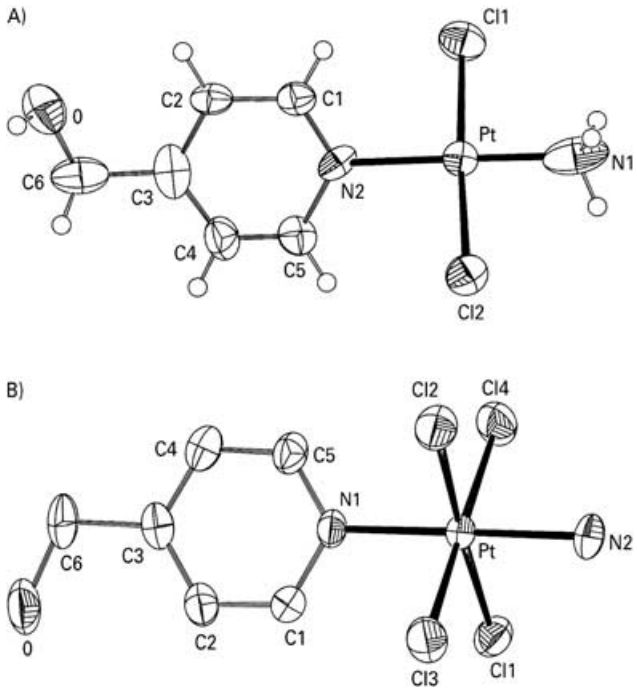


Figure 1. Molecular structure of the platinum compounds **1** (A) and **2** (B).

Table 2. Crystallographic data for compounds **1** and **2**.

	Compound (**1**)	Compound (**2**)
formulae	$C_6H_{10}Cl_2N_2OP$	$C_6H_{10}Cl_4N_2OP$
M_W	392.15	463.05
T [K]	293(2)	293(2)
λ [Å]	0.71069	0.71069
crystal system	monoclinic	monoclinic
spatial group	$P2_1/c$	$P2_1/c$
a [Å]	7.211(2)	9.2340(10)
b [Å]	18.711(2)	10.3310(10)
c [Å]	8.183(2)	13.2010(10)
α [°]	90	90
β [°]	114.43	105.74
γ [°]	90	90
V [Å^3]	1008.5(4)	1212.1(2)
ρ [mg m^{-3}]	2.583	2.537
absorption coefficient [nm^{-1}]	14.399	12.428
F(000)	720	856
size [mm]	0.1×0.1×0.2	0.1×0.1× 0.2
2θ limit	3.29 to 29.98	2.29 to 31.64
measured reflections	2883	9505
independent reflections	2883	3144
R_1	0.0465	0.0468
wR_2	0.0402	0.1200

Table 3. Selected bond distances and angles for compounds **1** and **2**.

Compound **1**				Compound **2**			
Length [Å]		Angles [°]		Length [Å]		Angles [°]	
Pt–N(1)	1.921(13)	N(1)–Pt–N(2)	177.7(5)	Pt–N(1)	2.057(5)	N(1)–Pt–N(2)	178.9(2)
Pt–N(2)	2.048(13)	N(1)–Pt–Cl(2)	86.7(4)	Pt–N(2)	2.038(5)	N(2)–Pt–Cl(2)	89.1(2)
Pt–Cl(1)	2.311(5)	N(2)–Pt–Cl(2)	91.9(4)	Pt–Cl(1)	2.3120(18)	N(1)–Pt–Cl(2)	91.98(17)
Pt–Cl(2)	2.286(5)	N(1)–Pt–Cl(1)	90.1(4)	Pt–Cl(2)	2.306(2)	N(2)–Pt–Cl(1)	87.9(2)
N(2)-C(1)	1.280(18)	N(2)–Pt–Cl(1)	91.4(4)	Pt–Cl(3)	2.3186(19)	N(1)–Pt–Cl(1)	91.06(17)
N(2)-C(5)	1.40(2)	Cl(2)–Pt–Cl(1)	176.71(17)	Pt–Cl(4)	2.3128(19)	N(2)–Pt–Cl(4)	88.9(2)
		C(1)-N(2)–Pt	119.9(12)	N(1)-C(1)	1.354(9)	N(1)–Pt–Cl(4)	90.76(17)
		C(5)-N(2)–Pt	120.6(11)	N(1)-C(5)	1.325(10)	Cl(2)–Pt–Cl(4)	90.28(9)
						Cl(1)–Pt–Cl(4)	89.43(8)
						Cl(2)–Pt–Cl(3)	89.12(9)
						Cl(1)–Pt–Cl(3)	91.13(8)
						N(2)–Pt–Cl(3)	90.1(2)
						N(1)–Pt–Cl(3)	90.23(17)

"stacking" interaction.[52,53] The crystal packing is also determined by the hydrogen bonding established between the OH and NH_3 groups of different molecules.

The structure of **2** can be defined as a 3D arrangement of discrete molecules of *trans*-[$PtCl_4NH_3$(4-hydroxymethylpyridine)]. The metal ion has an octahedral environment in which the hydroxymethylpyridine and amine ligands occupy the axial positions, and the four chlorine atoms the equatorial sites. In this case, the repulsion of the bulky 4-hydroxymethylpyridine also produces distortion of the octahedral geometry. In fact, the N(2)–Pt–Cl angle is less than 90° and N(1)–Pt–Cl is greater than 90°. The pyridine plane is oriented over the equatorial plane between two chlorine atoms and, as a consequence, the angles Cl(1)–Pt–Cl(4) and Cl(2)–Pt–Cl(3) are also greater than 90°. The average values for Pt–Cl and Pt–N bonds are 2.312 and 2.048 Å, respectively; these are in good agreement with the typical values for this kind of bond. The spatial distribution of molecules gives a value of 4.369 Å for the distance between the rings and 5.753 Å for the Pt–Pt separation. So, stacking or intermetallic interactions must be discarded. Some hydrogen-bonding interactions were also detected between the NH_3 groups and the Cl^- ligands of different molecules.

Interaction with 5′-GMP

Studies of the mechanism of Pt drugs have shown a preference of the metal ion to bind DNA through purine bases, mainly through the N(7) of guanines, due to the higher basicity of this particular N atom,[54,55] and the formation of hydrogen bonds between the carrier ligands and the nucleobase.[56,57]

There is much evidence that the interaction between the Pt atom and DNA nucleotides of tumor cells is responsible for the interruption of cell replication. Thus, this interaction can be studied in order to gain a better understanding of the mechanisms of action of Pt compounds. Reactions between Pt drugs and 5′-GMP can be followed by the shifting of the H(8) resonance in the ^{1}H NMR spectra. In the free nucleotide 5′-GMP, the H(8) signal appears at 8.0–8.1 ppm while, in the adduct Pt-5′-GMP, it shifts to 8.5–8.6 ppm. The results of this type of study of the interaction of compounds **1** and **2** with 5′-GMP are shown in Figure 2.

The reaction of compound **1** with 5′-GMP gives rise to two new H(8) signals. The one at 8.75 ppm corresponds to the monofunctional adduct, where a labile Cl^- ligand is replaced by a molecule of 5′-GMP. This signal is the main one after a short reaction time. After three hours of reaction, a new signal appears at 8.84 ppm, which is assigned to the bifunctional adduct. This one is the only signal present after a long reaction time. Changes in the signal assigned to the aromatic protons of 4-hydroxymethylpyridine are also observed simultaneously with the formation of adducts with 5′-GMP. In spite of the reaction time being increased to 72 h, no changes were observed in the reaction of the complex **2** with 5′-GMP. The kinetic inertness of the Pt^{IV} compounds is well-known. It has been postulated that platinum(IV) compounds act as prodrugs and that their reduction to Pt^{II} inside the organisms is the trigger to activate them as antitumor agents.[58–62] So, experiments to verify this were planned. The addition of several reducing agents, present in the cell as ascorbic acid, methionine, or glutathione (Scheme 2), to the Pt^{IV} complex was performed. The spectral changes observed over the course of the Pt^{IV} complex–5′-GMP reaction are shown in Figure 3.

In the presence of ascorbic acid (ascorbate at pH 7) and glutathione, the Pt^{IV} compound becomes reactive against 5′-GMP. These species reduce the *trans*-[$PtCl_4NH_3$(4-hydroxymethylpyridine)] compound to the corresponding analogue *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)], which reacts with 5′-GMP, as was tested before. The presence of both the monofunctional and bifunctional adducts was verified. Nevertheless, the rate of formation of the adducts was slower than that of the Pt^{II} complex.

It is known that concentrations of glutathione higher than those stoichiometrically required to reduce platinum(IV) can deactivate the corresponding platinum(II) compound by coordination of the thiol groups to the metal ions.

The first step in the reaction is the reduction of Pt^{IV} to Pt^{II} by the glutathione, followed by the coordination of the excess of glutathione to Pt^{II}; this causes partial inactivation and only monofunctional adducts with 5′-GMP are formed. (Further in-

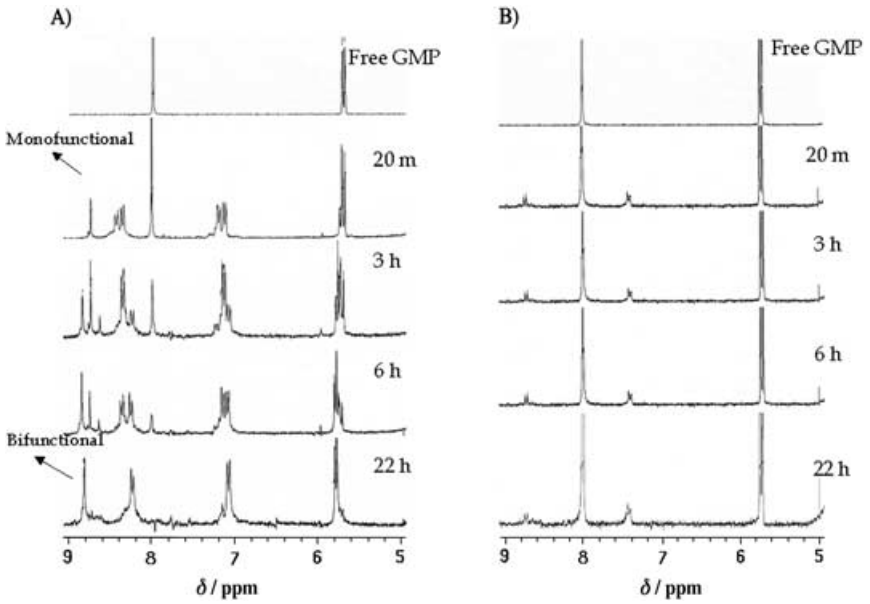
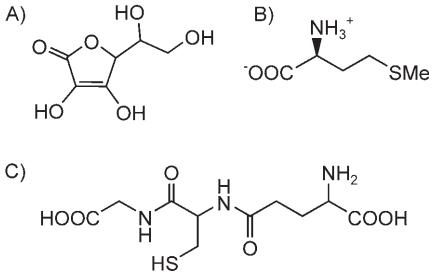


Figure 2. ^{1}H NMR spectra, registered in D_2O, of the interaction of compounds **1** (A) and **2** (B) with 5′-GMP. New signals appear, in the case of the complex **1,** at about 8.7 ppm; this suggests an interaction at the N7 position. The lack of new signals for H8 in complex **2** suggests the inability of **2** to interact by itself with 5′-GMP.



Scheme 2. Reducing agents used in the experiment. A) ascorbic acid, B) methionine and C) glutathione.

formation from this study is given in Figures S2–S4 in the Supporting Information.)

Biological studies

The key role of DNA in cell replication is to convert this biomolecule in the target of the majority of anticancer drugs. Three different techniques have been used to measure the changes in DNA structure induced by compounds **1** and **2**: circular dichroism (CD), atomic force microscopy (AFM), and electrophoretic mobility in agarose gel.

CD studies

The modifications caused in the molar ellipticity of calf thymus DNA by 4-hydroxymethylpyridine, compounds **1** and **2**, and the products of reduction by the above-mentioned reducing agents of **2**, are summarized in Table 4. The ligand, compound **2**, and its reduction product by methionine do not produce significant changes. Therefore, they do not modify the secondary structure of DNA. However, complex **1** and the products of reduction of complex **2** by ascorbic acid and glutathione behave completely differently. The positive values of ellipticity decrease and, likewise, a shift in the ellipticity maximum towards higher wavelengths (bathochromic effect) is observed. These modifications clearly indicate a transformation from the B form of DNA to the C form.[63–66]

It is known that *trans*-platinum compounds forming monofunctional adducts with DNA slightly modify DNA ellipticity. Thus, the formation of pseudo-bifunctional adducts, already described for *trans*-platinum compounds with planar amines,[11–13,22] could be also effective in this case.

In conclusion, CD results show that complex **2** does not change the secondary structure of the DNA. The methionine does not seem to reduce **2**, and, consequently, the behavior of **2** in the presence of methionine is the same as in its absence. Finally, in the presence of ascorbic acid and glutathione, the product of reduction of **2** modifies the secondary structure of the DNA.

Electrophoretic mobility

In Figure 4, the electrophoretic mobility patterns of native DNA pBR322, DNA+**1**, DNA+**2**, and DNA+**2** in the presence of reducing agents are shown.

Complex **2** slightly modifies the electrophoretic mobility of both open circular (OC) and covalently closed circular (CCC) forms of the plasmid DNA pBR322. However, complex **1** significantly modifies electrophoretic mobility: a coalescence of the two forms OC and CCC can be observed. Compound **2** produces a similar effect in the presence of ascorbic acid and glutathione in equimolar ratios. However, an excess of glutathione (lane 7) does not produce a similar delay, and a new pattern appears, probably due to the formation of a new complex of Pt^{II} in which the thiol groups are bound to the metal ions. The addition of methionine does not significantly alter the activity of complex **2**, since electrophoretic mobility is not substantially modified. In conclusion, the changes observed in electrophoretic mobility indicate modifications in the tertiary structure of the DNA molecule.

Tapping-mode atomic force microscopy (TMAFM)

AFM images of pBR322 DNA, DNA incubated with cisplatin, DNA incubated with **1** and with **2** are shown in Figure 5.

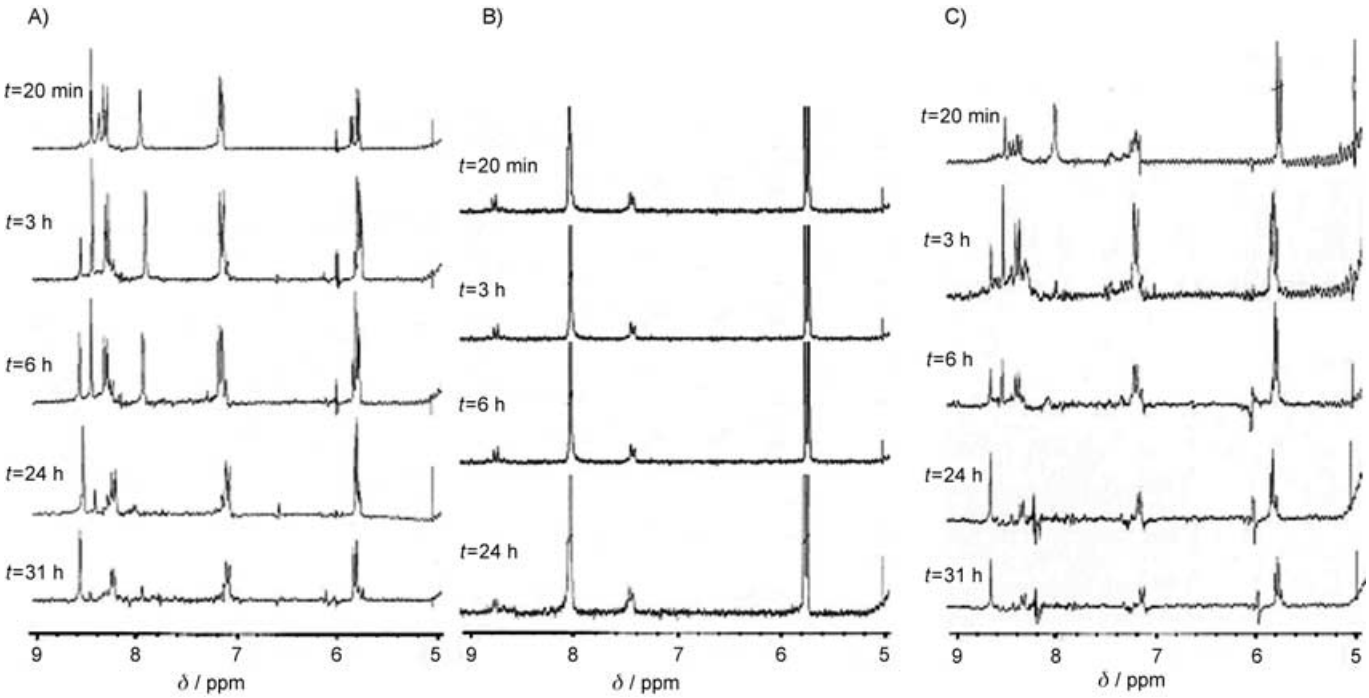


Figure 3. ^{1}H NMR spectra, registered in D_2O, for the reaction of the compound **2** with 5′-GMP in the presence of A) ascorbic acid, B) methionine, and C) glutathione. Complex **2** is activated by reduction to the Pt^{II} analogue by ascorbic acid and glutathione, and this Pt^{II} analogue is able to interact with 5′-GMP.

Table 4. Values of ellipticity for DNA–4-hydroxymethylpyridine, DNA–**1** and DNA–**2** adducts. The effect of reducing agents on DNA–**2** is also shown.

Compound	r_i	Θ_{max}[a]	λ_{max}[b]	Θ_{min}[a]	λ_{min}[b]
DNA		3696	274.2	−4921	246.4
DNA + 4-hydroxymethylpyridine	0.1	3698	274	−4915	246.2
	0.3	3685	274.2	−4928	246.2
	0.5	3690	274	−4920	246.6
DNA + *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)]	0.1	3611	276.4	−5285	243.8
	0.3	2851	278.2	−4592	246.4
	0.5	2825	278	−4078	247.2
DNA + *trans*-[$PtCl_4NH_3$(4-hydroxymethylpyridine)]	0.1	3917	276.6	−4953	246.2
	0.3	3872	276	−5598	243.6
	0.5	3648	277.2	−4979	244.8
DNA + *trans*-[$PtCl_4NH_3$(4-hydroxymethylpyridine)] + ascorbic acid	0.1	3182	275	−4856	244.8
	0.3	2779	280.2	−5438	246.2
	0.5	2591	278.6	−4932	245.8
DNA + *trans*-[$PtCl_4NH_3$(4-hydroxymethylpyridine)] + methionine	0.1	3754	275.8	−5059	245.8
	0.3	3141	271.2	−5257	246
	0.5	3681	279.4	−5330	244.6
DNA + trans-[$PtCl_4NH_3$(4-hydroxymethylpyridine)] + glutathione 1:1	0.1	3560	274.6	−5023	244.6
	0.3	2989	278	−4963	245.6
	0.5	2950	278.2	−4920	244.2
DNA + *trans*-[$PtCl_4NH_3$(4-hydroxymethylpyridine)] + glutathione in excess	0.1	3762	275.6	−5012	244.8
	0.3	3658	276.4	−4986	245.6
	0.5	3720	275.2	−5023	244

The AFM images show morphological changes in the forms of the plasmid DNA. Cisplatin produces supercoiling and microfolds in DNA.[67] The effect caused by complex **1** is very strong and not only supercoiling but also kinks can be observed. In addition, due to its *trans* geometry, the metal ion binds to different DNA strands and association between chains can also be detected.[67–69] The effect caused by compound **2** is not as dramatic as that produced by **1**. The CD and electrophoretic mobility results have shown that this complex does not modify the secondary and tertiary structures of pBR322 DNA. The AFM images agree with these results, because morphological changes are not detected.

The addition of methionine to complex **2** (Figure 6B) did not significantly modify the AFM image of pBR322 treated with complex **2** (Figure 5D). However, the addition of ascorbic acid or glutathione to compound **2** was able to activate this

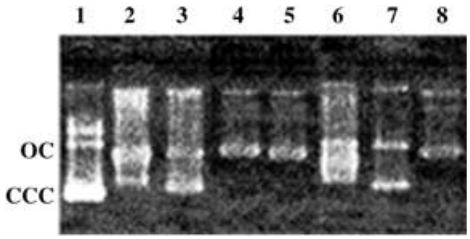


Figure 4. Agarose gel electrophoretic mobility of DNA pBR322 (lane 1) and DNA treated with cisplatin (lane 2), compound **2** (lane 3), compound **1** (lane 4), compound **2** plus ascorbic acid (lane 5), compound **2** plus methionine (lane 6), compound **2** plus excess glutathione (lane 7), and with compound **2** plus glutathione (lane 8).

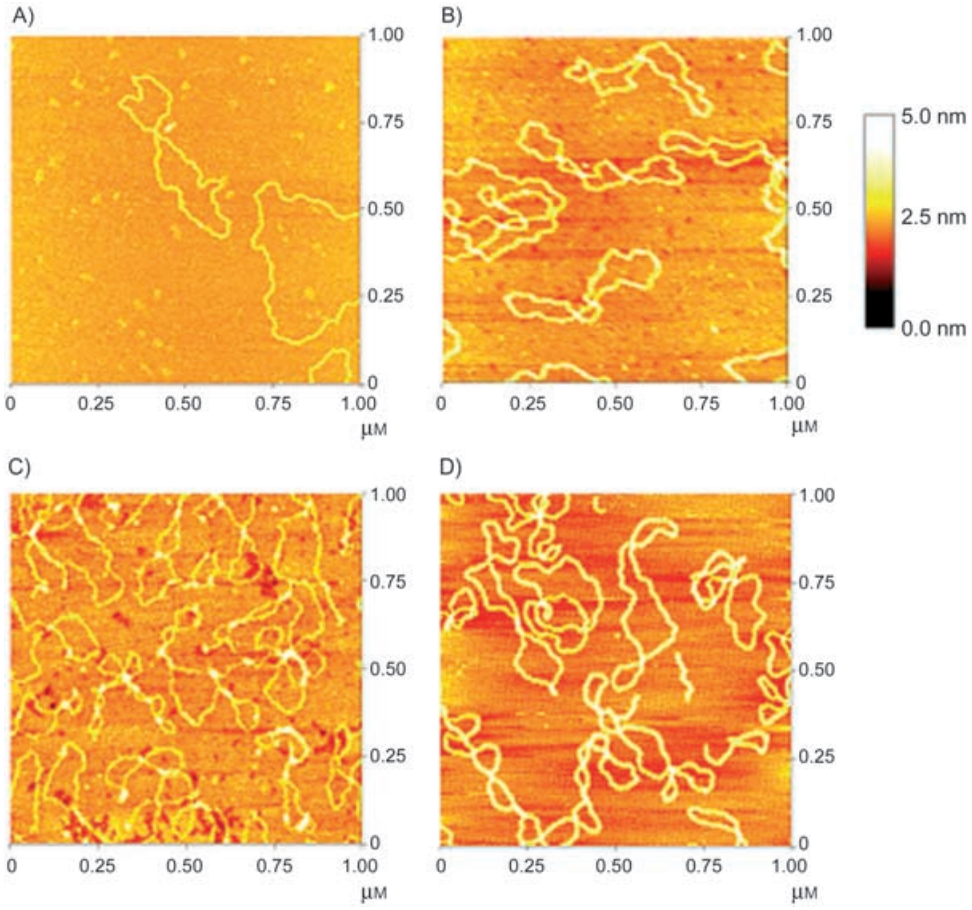


Figure 5. TMAFM images of A) pBR322 DNA, B) DNA with cisplatin, C) DNA with compound **1**, and D) DNA with compound **2**. Complex **1** significantly alters the morphology of DNA pBR322, in contrast to complex **2**.

metal complex by reducing it to the Pt^{II} analogue. Several types of coiling in the forms can be observed; this is in agreement with the result of electrophoretic mobility. The images obtained for these cases are shown in Figure 6A and C. These images are comparable with that corresponding to complex **1** (Figure 5C).

Cytotoxicity of the platinum complexes against HL-60 cells

The effect of the platinum complexes on human leukemia cancer cells (HL-60) was examined by using the MTT assay, a colorimetric determination of cell viability during in vitro treatment with a drug. The assay, developed as an initial stage in drug screening, measures the amount of MTT reduction by mitochondrial dehydrogenase and assumes that cell viability (corresponding to the reductive activity) is proportional to the production of purple formazan that is measured spectrophotometrically. A low IC_{50} value implies cytotoxicity or antiproliferation at low drug concentrations.

The compounds tested in this experiment were cisplatin and the two *trans* platinum complexes **1** and **2**. Cells were exposed to each compound continuously for a 24 h or a 72 h period and then assayed for growth by using the MTT endpoint. Figure 7 shows the dose–response curves of these drugs in terms of the drug effect on the growth of HL-60 cells. The IC_{50} values of **1**, **2**, and cisplatin for the inhibition of growth of HL-60 cells are shown in Table 5.

The IC_{50} value of cisplatin for growth inhibition of HL-60 cells for 24 h exposition was 15.61 ± 2.47 μM, which is approximately 4.5-fold greater than that of **1** and very similar to that of **2**. The cytotoxicities of the Pt complexes were also determined after 72 h. As shown in Table 5, **1** exhibits cytotoxicity comparable to that of cisplatin, and it is approximately 3.5-fold more toxic than compound **2**.

After a shorter time, complex **2** has the same cytotoxic activity as cisplatin, but complex **1** still showed strong activity against HL-60. After 72 h, complex **1** and cisplatin have similar activities that are stronger than that of **2**. In fact, complexes **1** and **2** do not modify their activities significantly, even when the the time of treatment is increased: only cisplatin changes its activity (Figure 7).

Quantification of apoptosis by annexin V binding and flow cytometry

We also analyzed with annexin V–propidium iodide flow cytometry whether compounds **1** and **2** are capable of inducing apoptosis in HL-60 cells after 24 h of incubation at equitoxic

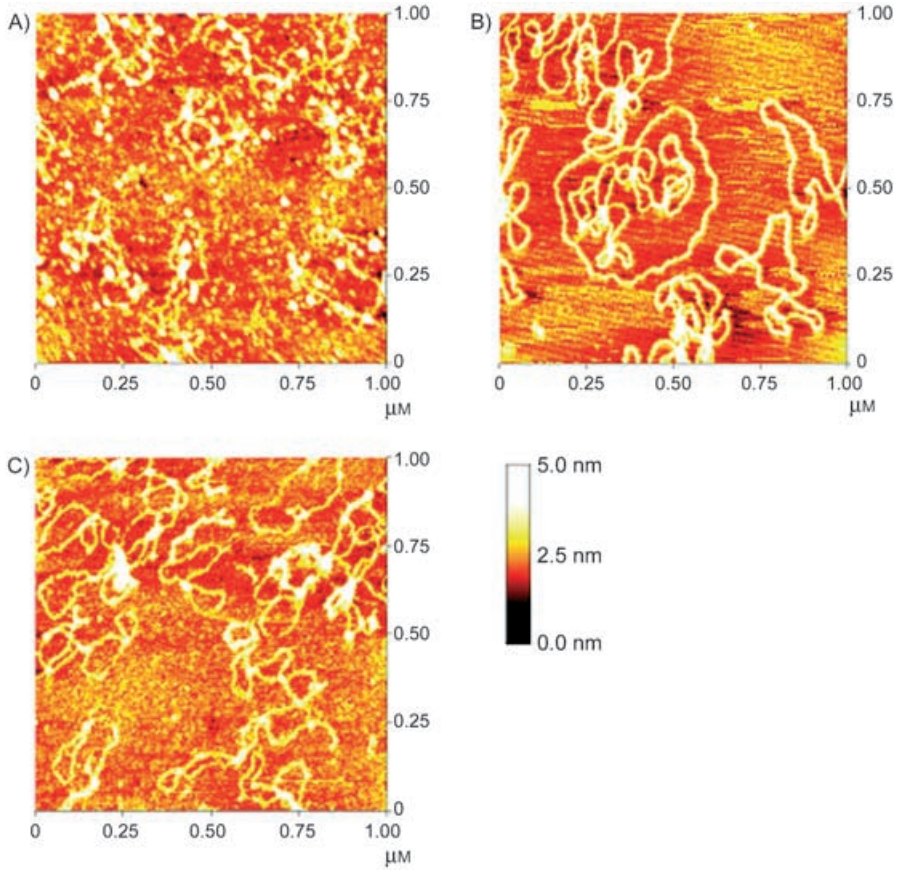


Figure 6. TMAFM images of DNA with compound **2** and A) ascorbic acid, B) methionine, C) glutathione. The addition of ascorbic acid or glutathione to complex **2** activates the Pt^{IV} complex by reducing it, and visual morphological changes are clearly observed. Methionine does not alter the activity of complex **2**.

Table 5. IC_{50} values of **1**, **2**, and cisplatin against various HL-60 cells. The IC_{50} value of complex **1** is about fourfold less than those of complex **2** and cisplatin after 24 h of incubation; this suggests that *trans*-Pt^{II} is the most active. After 72 h of interaction, cisplatin and complex **1** have about the same activity.

Complex	IC_{50} [μM] 24 h	IC_{50} [μM] 72 h
1	3.48 ± 0.89	3.35 ± 1.05
2	16.68 ± 1.76	11.69 ± 1.55
cisplatin	15.61 ± 2.47	2.15 ± 0.51

concentrations (IC_{50} values). Annexin V binds phosphatidyl serine residues, which are asymmetrically distributed toward the inner plasma membrane, but migrate to the outer plasma membrane during apoptosis.[70] The data show that, at concentrations equal to the IC_{50} values, cisplatin induced early apoptotic cell death in 47.25% and late apoptotic cell death in 4.26% of HL-60 cells. Therefore, at concentrations equal to their IC_{50} values, compounds **1** and **2** also induced early apoptotic cell death in 40% and late apoptotic cell death in 37.5 and 27.5% of the HL-60 cells, respectively, as shown in Table 6 and Figure 8.

Conclusion

We have synthesized two similar platinum compounds with different oxidation states. The Pt^{II} compound was shown to be highly soluble in water and other common organic and inorganic solvents, while the Pt^{IV} compound is poorly soluble. We demonstrated by CD, AFM, EF, and essays against tumor cell lines that *trans*-Pt^{II} compounds can be more active than cisplatin. The Pt^{IV} compound studied is less active on its own, but can be easily activated by reduction with some common reducing agents in cells, such as ascorbate or glutathione.

Experimental Section

***trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] (1)**. Cisplatin (0.15 g, 0.5 mmol) was suspended in distilled H_2O (20 mL), and this suspension was treated with a slight excess of 4-hydroxymethylpyridine (0.112 g, 1.03 mmol). The mixture was stirred for 5 h at 90 °C. When the solution became colorless, concentrated HCl (5 mL) was added, and the mixture was stirred for 6 h at 80 °C. The solution turned yellow. Then, it was concentrated to 5 mL and cooled in an ice bath until a yellow precipitate formed. The precipitate was filtered off, washed with cool water, and dried in air. Yield: 65–70%. Suitable crystals for X-ray study were obtained by crystallization from HCl 0.01 N. Elemental analysis calcd (%) for $PtC_6H_{10}N_2OCl_2$: C 18.36, N 7.14, H 2.57; found: C 18.38, N 6.93, H 2.82.

***trans*-[$PtCl_4NH_3$(4-hydroxymethylpyridine)] (2)**. Complex **1** (0.078 g, 0.2 mmol) was dissolved in HCl 0.01 N (10 mL). The oxidation to the corresponding Pt^{IV} compound was carried out under a stream of chlorine gas (generated with concentrated HCl and MnO_2) at RT for 20 min. The yellow precipitate formed was filtered off, washed with cool water and acetone, and dried in air. Yield: 58 mg (60–65%). Suitable crystals for X-ray study were obtained from slow evaporation of the solution. Elemental analysis calcd (%) for $PtC_6H_{10}N_2OCl_4$: C 15.56, N 6.05, H 2.18; found: C 15.13, N 6.08, H 2.72.

NMR solution studies with 5′-GMP. NMR spectra were recorded on a GEMINI 200 MHz spectrometer. All samples were studied in D_2O at pH 7. Complexes **1** (4 mg, 0.01 mmol) and **2** (4 mg, 8.63 μmol) were mixed with a slight excess of 5′-GMP (9 mg and 8 mg, respectively) in D_2O (1 mL) at 37 °C.

X-ray diffraction. A prismatic crystal (0.1 × 0.1 × 0.2 mm) of complex **1** was selected and mounted on an Enraf–Nonius CAD4 four-circle diffractometer. Unit-cell parameters were determined from auto-

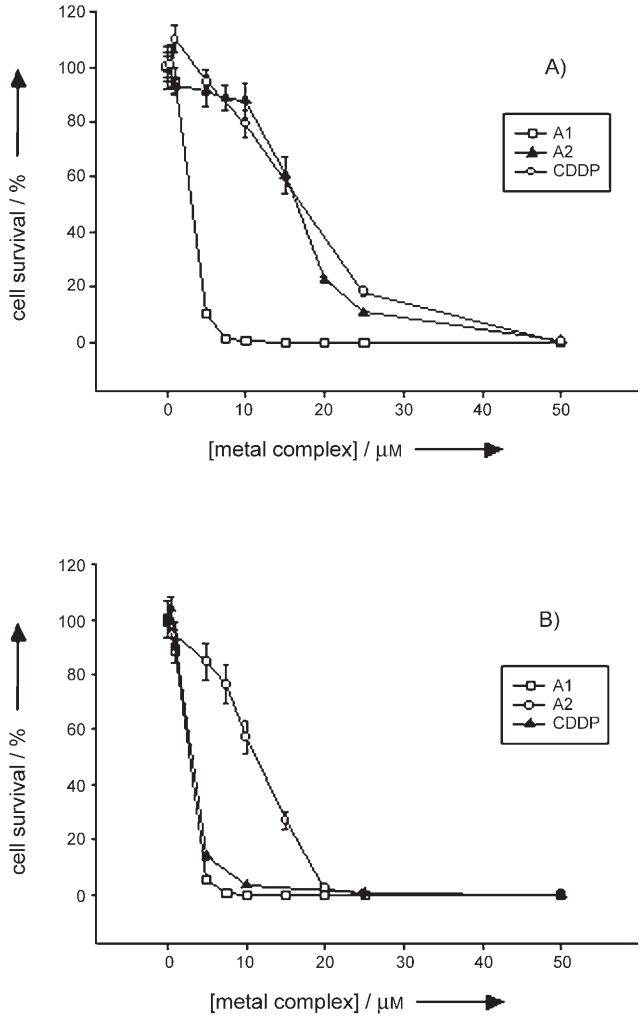


Figure 7. The growth inhibitory effect was determined by MTT assay after A) 24 or B) 72 h of exposure to platinum complexes at various concentrations. The error bars indicate one standard deviation of the averaged cell percent viability. Complex **1** is rather more active than complex **2** and cisplatin after 24 h of incubation. After 72 h of incubation, complex **1** and cisplatin have about the same activity.

matic centering of 25 reflections ($12<\theta<21°$) and refined by the least-squares method. Intensities were collected with graphite monochromatized $Mo_{K\alpha}$ radiation by using the $\omega/2\theta$ scan technique. 2883 total reflections were measured in the range $3.29\leq\theta\leq29.98$, of which 858 were assumed to be observed by applying the condition $I>2\sigma(I)$. Three reflections were measured every two hours, as an orientation and intensity control, and no significant intensity decay was observed. Lorentz-polarization but no absorption corrections were made. A prismatic crystal ($0.1\times0.1\times0.2$ mm) of complex **2** was selected and mounted on a MAA345 diffractometer with an image plate detector. Unit-cell parameters were determined from 10200 reflections ($3<\theta<31°$) and refined by the least-squares method. Intensities were collected with graphite monochromatized $Mo_{K\alpha}$ radiation by using the $\omega/2\theta$ scan technique. 9505 total reflections were measured in the range $2.29\leq\theta\leq31.64$, of which 3144 were nonequivalent by symmetry (R_{int}(on I) = 0.042). 2832 reflections were assumed to be observed by applying the condition $I>2\sigma(I)$. Lorentz-polarization but no absorption corrections were made.

The structures were solved by direct methods by using the SHELXS computer program and refined by the full-matrix, least-squares method with the SHELX97 computer program by using 2883 and 3144 reflections for **1** and **2**, respectively, (very negative intensities were not assumed).[71] The functions minimized were $\Sigma w||F_o|^2-|F_c|^2|^2$, here $w=[\sigma^2(I)]^{-1}$, for complex **1** and $w=[\sigma^2(I)+(0.0400P)^2+0.5360P]^{-1}$ for complex **2**, and $P=(|F_o|^2+2|F_c|^2)/3$; f, f' and f'' were taken from the International Tables of X-ray Crystallography.[72] All H atoms were computed and refined by using a riding model, with an isotropic temperature factor equal to 1.2 times the equivalent temperature factor of the atoms that are linked. For complex **1**, the final R (on F) factor was 0.046, wR(on $|F|^2$) = 0.040, and goodness of fit = 0.742 for all observed reflections. The number of refined parameters was 118. The maximum shift/esd = 0.00, mean shift/esd = 0.00. Maximum and minimum peaks in the final difference synthesis were 0.763 and -0.612 e Å^{-3}, respectively. In the case of complex **2**, the final R (on F) factor was 0.023, wR(on $|F|^2$) = 0.060, and goodness of fit = 1.070 for all observed reflections. The number of refined parameters was 127. Maximum shift/esd = 0.00, mean shift/esd = 0.00. Maximum and minimum peaks in final difference synthesis were 0.766 and -0.856 e Å^{-3}, respectively.

CCDC 273554 and 273555 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data@request/cif

Formation of drug–DNA complexes. Stock solutions of each compound (1 mg mL^{-1}) in Tris–EDTA were freshly prepared before use. Drug–DNA-complex formation was accomplished by addition of calf thymus DNA (CT DNA) to aliquots of each of the compounds at different concentrations in TE buffer (50 mM NaCl, 10 mM Tris.HCl, 0.1 mM EDTA, pH 7.4). The amount of drug added to the DNA solution was designated as r_i (the input molar ratio of Pt or 4-hydroxymethylpyridine to nucleotide). The mixture was incubated at 37 °C for 24 h.

CD spectroscopy. The CD spectra of the DNA complexes (DNA concentration 20 mg mL^{-1}, r_i = 0.1, 0.3, 0.5) were recorded at room temperature on a JASCO J720 spectropolarimeter with a 450 W xenon lamp by using a computer for spectral subtraction and noise reduction. Each sample was scanned twice over a range of

Table 6. Quantification of apoptosis after 24 h of exposure to concentrations equal to the IC_{50} values of **1**, **2**, and cisplatin against various HL-60 cells. Complex **1** is able to induce approximately 80% apoptotic death at a concentration equal to the IC_{50} parameter, in contrast to 65% for complex **2** and 50% for cisplatin.

Treatment (IC_{50} 24 h [μM])	Vital cells (R1) [%]	Early apoptotic cells (R2) [%]	Late apoptotic/dead cells (R3) [%]	Damaged cells (R4) [%]
control	87.23	5.73	6.07	0.94
cisplatin (15.6)	46.81	47.25	4.26	1.67
1 (3.5)	21.64	40.71	33.47	4.19
2 (16.7)	32.25	40.36	25.88	1.52

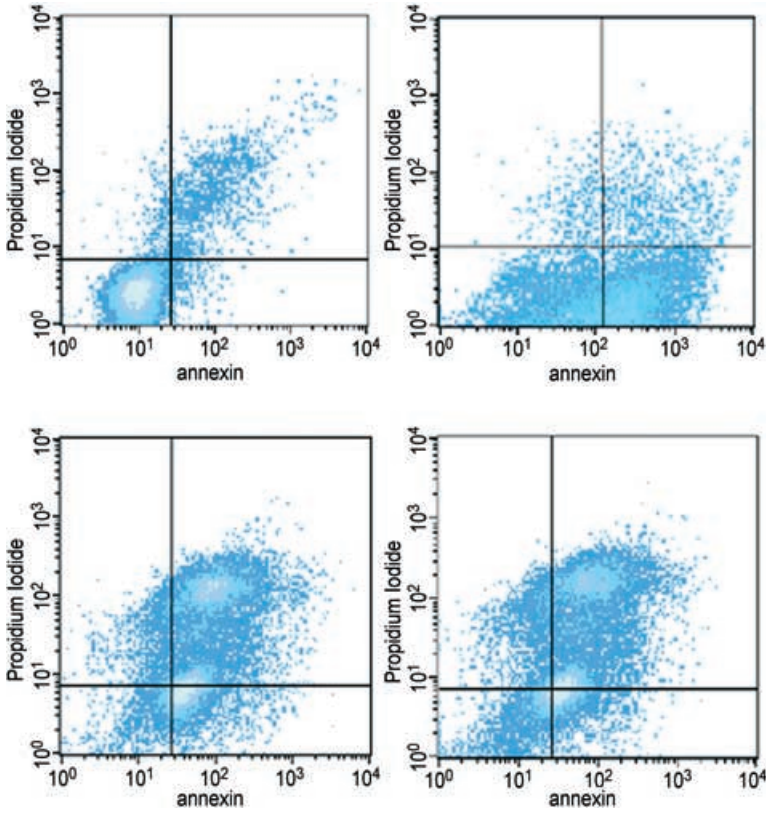


Figure 8. Quantification of apoptosis after 24 h of exposure to concentrations equal to the IC_{50} values of the platinum drugs. Representative annexin V/PI fluorescence scattergrams showing HL-60 cells. A) Control and treatment with B) cisplatin, C) compound **1**, and D) compound **2**.

wavelengths between 220 and 330 nm. The CD spectra drawn are the mean of three independent scans. The data are expressed as mean residue molecular ellipticity (θ) in $°\,cm^2\,dmol^{-1}$.

Electrophoretic mobility in agarose gel. pBR322 plasmid DNA (0.25 $\mu g\,\mu L^{-1}$) was used for the experiments. Charge maker (4 μL) was added to aliquots (20 μL) of the DNA complex that had previously been incubated at 37 °C for 24 h. The mixtures were separated by electrophoresis in agarose gel (1% in Tris–borate–EDTA buffer) for 5 h at 1.5 $V\,cm^{-1}$. Afterwards, the DNA was dyed with a solution of ethidium bromide in TBE (0.5 $\mu g\,mL^{-1}$) for 20 min. Samples of DNA and cisplatin–DNA adduct were used as a control. The experiment was carried out in an ECOGEN horizontal tank connected to a PHARMACIA GPS 200/400 variable potential power supply.

Atomic force microscopy (TMAFM). pBR322 DNA was heated at 60° for 10 min to obtain the OC form. A stock solution in HEPES buffer (1 $mg\,mL^{-1}$) was used. Each sample contained DNA pBR322 (1 μL, 0.25 $\mu g\,\mu L^{-1}$) for a final volume of 40 μL. The amount of drug added was expressed as r_i, the ratio between the molar concentration of the drug to the number of base pairs.

Images were obtained with a NANOSCOPE III MULTIMODE AFM, (Digital Instruments Inc., Buffalo, NY) operating in the tapping mode.

Tumor cell lines and culture conditions. The cell line used in this experiment was the human acute promyelocytic leukemia cell line HL-60 (American Type Culture Collection (ATCC)). Cells were routinely maintained in RPMI-1640 medium supplemented with 10% (*v*/*v*) heat-inactivated, fetal bovine serum, glutamine (2 $mmol\,L^{-1}$), penicillin (100 $U\,mL^{-1}$), and streptomycin (100 $\mu g\,mL^{-1}$; Gibco BRL, Invitrogen, Netherlands) in a highly humidified atmosphere of 95% air with 5% CO_2 at 37 °C.

Cytotoxicity assay. The growth inhibitory effects of platinum complexes on the leukemia HL-60 cell line were measured by using the microculture tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT] assay.[73] Briefly, cells growing in the logarithmic phase were seeded in 96-well plates (10^4 cells per well) and then were treated with varying doses of platinum complexes and the reference drug, cisplatin, at 37 °C for 24 or 72 h. Four wells were used for each of the variants tested. Aliquots of MTT solution (20 μL) were then added to each well. After 3 h, the color formed was quantified by a spectrophotometric plate reader (Labsystems iEMS Reader MF, Somerset, NJ) at 490 nm wavelength. The percentage cell viability was calculated by dividing the average absorbance of the cells treated with a platinum complex by that of the control; IC_{50} values were obtained by using GraphPad Prism software, version 4.0.

In vitro apoptosis assay. Induction of apoptosis in vitro by **1** and **2** was determined by a flow cytometric assay with annexin V–fluorescein isothiocyanate (FITC) by using an annexin V–FITC apoptosis detection kit (Roche).[74] Exponentially growing HL-60 cells in 6-well plates (5×10^5 cells per well) were exposed to concentrations equal to the IC_{50} of the platinum drugs for 24 h. After the cells had been stained with the annexin V–FITC and propidium iodide, the amount of apoptotic cells was analyzed by flow cytometry (FACSCalibur, BD Biosciences, San José, CA).

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