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Studies of interaction of trichloro{η²-*cis*-*N*,*N*-dimethyl-1-[6-(*N*′,*N*′-dimethyl-ammoniummethyl)-cyclohex-3-ene-1-yl]-methyl-ammonium}platinum(II) chloride with DNA: Effects on secondary and tertiary structures of DNA. Cytotoxic assays on human ovarian cancer cell lines, resistant and non-resistant to cisplatin

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Abstract—The studies of interaction with DNA and the cytotoxic activity of a new organometallic platinum(II) compound are presented. The ability of this new platinum complex to modify secondary DNA structure was explored by circular dichroism (CD). Electrophoretic mobility showed changes in tertiary DNA structure, and atomic force microscopy (AFM) revealed morphological changes of plasmid DNA (pBR322). This compound breaks the traditional structure–activity rules for *cis*-platinum compounds, but it could be of interest because of its different kinetics. An organometallic bond normally shows a *trans*-effect higher than that of an amine ligand, and that fact, a priori, could contribute to a higher DNA binding rate. Several ovarian cancer cell lines, resistant and non-resistant to cisplatin, were exposed to increasing concentrations of cisplatin and complex **5** for 24 h, after which time the cell number/viability was determined by the colorimetric MTT assay. A lower cytotoxicity but also a lower resistant factor was observed for organometallic compound **5** than for cisplatin, against A2780 and A2780cisR cell lines. This result is consistent with the DNA interaction degree observed by the aforementioned techniques.

1. Introduction

Although cisplatin is a widely used anticancer drug,[1,2] its clinical utility has been limited due to the frequent development of drug resistance,[3,4] severe side effects and the limited spectrum of tumours against which cisplatin is active. Cisplatin is highly effective in the treatment of testicular and ovarian cancer, and is also employed for treating bladder, cervical, head and neck, oesophageal and small cell lung cancer. However, some tumours such as colorectal cancer have intrinsic resistance to cisplatin, while others such as ovarian cancer develop acquired resistance.[5] Thousands of cisplatin analogues have been synthesized by varying the nature of the leaving groups and the carrier ligand, but only four platinum compounds are currently registered for clinical use (cisplatin, carboplatin, oxaloplatin and nedaplatin) even though there are several candidates under clinical trials.[6] Precise mechanism of antitumour action of platinum drugs is not completely understood, but DNA is believed to be the main target of this kind of compounds by forming bifunctional adducts.[7] The major adduct formed by cisplatin and its analogues is an intrastrand d(GpG) adduct with platinum cross-linking N7 atoms of neighbour guanine residues of DNA.[8] Thus, DNA undergoes an unstacking of the bases that leads to a pronounced bend in DNA at the site of

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platination.[9–11] It has been also shown that carrier amine ligands of cisplatin analogues appear to modulate the antitumour properties of this class of drugs. There are many possible roles for the carrier ligand of the platinum antitumour compounds, such as modification of the rate of DNA binding, influence on the recognition of DNA by repair enzymes or on pharmacokinetics. However, most of cisplatin analogues form similar adducts with DNA and surrounding proteins interact with the molecular lesion in a similar way; thus, they often have a parallel spectrum of activity. A possible approach to overcome these problems is to focus on platinum compounds with structures quite different from that of cisplatin with the aim that they could interact in a different way with DNA, and consequently show a different spectrum of activity and toxicity profile.

As part of our research project we are working in the design of new carrier ligands to establish structure–activity relationship rules. These rules may help us to determine the influence of different functional groups on activity and may help to perform a rational design of new analogues of cisplatin with improved activity, lower toxicity and without resistance effects. In the present work, the synthesis of a new organometallic platinum π-complex, tricloro{η^2-*cis*-*N*,*N*-dimethyl-1-[6-(*N*′,*N*′-dimethyl-ammoniummethyl)-cyclohex-3-ene-1-yl]-methylammonium}platinum(II) chloride, is described as well as the studies of interaction with DNA and of its cytotoxic activity against human ovarian cancer cell lines, resistant and non-resistant to cisplatin. This compound breaks the traditional structure–activity rules, but it could be interesting because of its different kinetics and different coordination to DNA. On one hand, an organometallic bond shows a *trans*-effect higher than that of an amine ligand, which could contribute to a higher DNA binding rate.[12] On the other hand, this complex has more coordination sites than cisplatin. Moreover, cationic character of the complex could facilitate the approach to DNA, though pharmacokinetics could be modified in the sense that charged species generally exhibit minor passive diffusion through cell membrane than neutral species.

2. Results and discussion

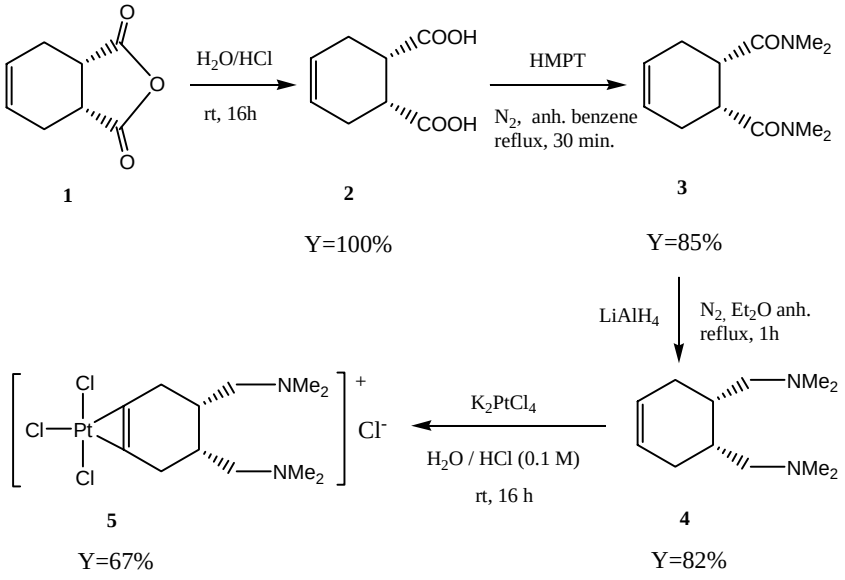
2.1. Synthesis of trichloro {η^2-*cis*-*N*,*N*-dimethyl-1-[6-(*N*′,*N*′-dimethyl-ammoniummethyl)-cyclohex-3-ene-1-yl]-methylammonium}platinum(II) chloride (5)

Synthesis of organoplatinum π-complex **5** was carried out by reacting diamine ligand **4** with K_2PtCl_4 (see Scheme 1).[13a] Ligand **4** was prepared from a commercially available *cis*-3,3,6,6-tetrahydrophthalic anhydride **1**, according to the procedure described in the literature for other substrates different from **4**.[13b,13c] Hydrolysis of **1** led to the *cis*-3,3,6,6-tetrahydrophthalic acid **2**, in quantitative yield. Direct amidation of diacid **2** was easily accomplished by refluxing it with HMPT in benzene. Finally, diamine **4** was obtained by reduction of diamide **3** with $LiAlH_4$ in high yield.

When ligand **4** reacted with K_2PtCl_4 under acidic conditions, coordination to platinum took place by the double bond and a cationic complex was obtained, instead of the usual seven-membered ring chelate, formed by coordination of platinum to both nitrogen atoms of the amino groups. This last type of coordination could be hampered by the steric hindrance exerted by the methyl groups on amino ligands. On the other hand, when the same reaction was carried out under neutral conditions, a mixture of products was obtained. Probably, polymers of different sizes were formed and no single and pure products could be isolated.

2.2. Structure determination of compound 5

Complex **5** was studied, in solid state, by elemental analysis, mass spectrometry and FT-IR spectroscopy, and in solution, by high-field ^{1}H NMR spectroscopy. Its



Scheme 1. Synthetic pathway for the preparation of complex **5**.

structure was confirmed by X-ray diffraction analysis on single crystals.[13a]

Crystallographic data and selected bond distances and angles are quoted in Tables 1 and 2. In Figure 1, an ORTEP view of the X-ray crystal structure of **5** is shown. This molecular representation shows a cationic complex with chloride as a counter-ion. Platinum(II) atom has a square planar coordination geometry. The CH=CH group, and consequently the ligand, is located perpendicular to the plane containing platinum and chlorine atoms. Angles of the CH=CH moiety indicate that the double bond geometry is not exactly coplanar. Moreover, C3′–C4′ length is 1.39 Å, higher than the length expected for a non-coordinated olefin. These two experimental facts are consistent with a back-donation effect. Finally, ammonium groups are located separately to minimize both, the charge repulsion between the N^+ centres and the steric repulsion between methyl groups.

Experimental details, hydrogen coordinates as well as anisotropic thermal parameters are included as supplementary material. Detailed information about the crystal structure determination of compound **5** is given as supporting information, which has been deposited with the Cambridge Crystallographic Data Centre.

2.3. Studies of interaction with DNA

Complex **5** has been evaluated as potential cytostatic agent. Its peculiarly structure could facilitate the

Table 1. Crystal data for compound **5**

Molecular formula	$C_{12}H_{24}Cl_4N_2Pt$
Molecular weight (g/mol)	533.22
T (K)	293(2)
λ (Mo-K$_\alpha$) (Å)	0.71069
Crystallographic system	Orthorhombic
Space group	$P2_12_12_1$
a (Å)	10.8650(10)
b (Å)	12.1680(10)
c (Å)	13.2060(10)
α (°)	90
β (°)	90
γ (°)	90
V (Å^3)	1745.9(3)
Z	4
D_{calcd} (Mg m^{-3})	2.029
μ (mm^{-1})	8.638
F(000)	1024
Rang Θ (°)	2.51–31.60
h, k, l ranges	$0 \leqslant h \leqslant 13$, $0 \leqslant k \leqslant 16$, $0 \leqslant l \leqslant 19$
Number of registered reflections	11,617
Number of independent reflections	2587
[R_{int} (on F)]	0.0490
Method of refinement	Full-matrix least-squares on F^2
Number of data	2587
Number of parameters	173
Goodness-of-fit on F^2	1.089
R final [$I > 2\sigma(I)$]	$R_1 = 0.0377$, $wR_2 = 0.0889$
R index (all data)	$R_1 = 0.0464$, $wR_2 = 0.1008$
Largest difference peak and hole (e Å^3)	0.847 and −0.877

Table 2. Selected bond lengths and angles for compound **5**

Bond lengths (Å)		Angles (°)	
Pt–C(4′)	2.167(10)	C(4′)–Pt–C(3′)	37.3(3)
Pt–C(3′)	2.180(10)	C(4′)–Pt–Cl(3)	93.4(3)
Pt–Cl(3)	2.298(3)	C(3′)–Pt–Cl(3)	98.5(3)
Pt–Cl(2)	2.304(3)	C(4′)–Pt–Cl(2)	89.2(3)
Pt–Cl(4)	2.316(3)	C(3′)–Pt–Cl(2)	85.4(3)
N(1)–C(1‴)	1.484(13)	Cl(3)–Pt–Cl(2)	176.10(12)
N(1)–C(1^{iv})	1.49(2)	C(4′)–Pt–Cl(4)	163.5(3)
N(1)–C(1)	1.494(13)	C(3′)–Pt–Cl(4)	158.4(2)
N(2)–C(1^{v})	1.476(13)	Cl(3)–Pt–Cl(4)	87.94(13)
N(1)–C(1^{vi})	1.48(2)	Cl(2)–Pt–Cl(4)	88.74(12)
N(1)–C(1″)	1.494(13)	C(4′)–C(3′)–Pt	70.8(6)
C(3′)–C(4′)	1.390(13)	C(2′)–C(3′)–Pt	120.0(7)
C(3′)–C(2′)	1.513(13)	C(3′)–C(4′)–Pt	71.9(6)
C(4′)–C(5′)	1.528(13)	C(5′)–C(4′)–Pt	120.9(8)
C(5′)–C(6′)	1.545(12)	C(1‴)–N(1)–C(1^{iv})	110.2(9)
C(1′)–C(6′)	1.519(13)	C(1‴)–N(1)–C(1)	115.8(8)
C(6′)–C(1″)	1.531(14)	C(1)–N(1)–C(1^{iv})	111.1(8)
C(1′)–C(1)	1.55(2)	C(1‴)–N(1)–H(N1)	106.4(5)
C(1′)–C(2′)	1.552(12)	C(1^{iv})–N(1)–H(N1)	106.4(6)
		C(1)–N(1)–H(N1)	106.4(5)
		C(1^{v})–N(2)–C(1^{vi})	112.3(9)
		C(1^{v})–N(2)–C(1″)	109.3(9)
		C(1^{vi})–N(2)–C(1″)	112.3(8)
		C(4′)–C(3′)–C(2′)	121.4(8)
		C(4′)–C(5′)–C(6′)	113.8(8)
		C(1′)–C(6′)–C(1‴)	112.1(8)
		C(3′)–C(4′)–C(5′)	121.5(8)
		C(1)–C(1′)–C(2′)	111.9(8)
		C(1″)–C(6′)–C(5′)	112.1(7)
		C(6′)–C(1′)–C(1)	112.8(7)
		C(6′)–C(1′)–C(2′)	106.4(8)
		C(1)–C(1′)–C(2′)	111.9(8)
		C(3′)–C(2′)–C(1′)	109.5(7)
		N(2)–C(1″)–C(6′)	112.8(9)
		N(1)–C(1)–C(1′)	113.5(7)

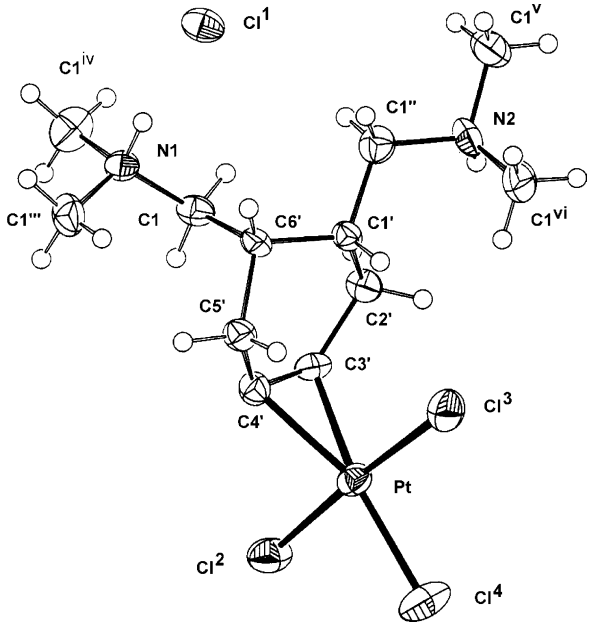


Figure 1. ORTEP view of molecule **5**.

formation of the DNA adduct, which is the main target of platinum compounds. On one hand, carbon–platinum bond has an enhanced *trans*-effect and, on the other hand, this compound has three labile ligands, and consequently three possible coordination sites to DNA.

It is widely believed that the main target of this kind of platinum compound is DNA. Different techniques have been used to study changes in secondary and tertiary DNA structures, and to obtain images of DNA morphological changes. The ability of complex **5** to modify secondary DNA structure was explored by circular dichroism (CD).[14–17] Electrophoretic mobility showed changes in tertiary DNA structure.[18,19] Finally, atomic force microscopy (AFM) revealed morphological changes of plasmid DNA (pBR322).[20] In all cases, cisplatin was used as positive control reference because its effects on DNA structure are well known.

2.4. Circular dichroism

Cisplatin and complex **5** were added separately at different r_i (molar ratio of the complex to nucleotide. See Section 4 for mathematical definition of r_i) to a solution of (calf thymus DNA) (CT DNA). The reactions were run at 37 °C for 24 h in the dark. CD spectra showed changes in DNA secondary structure (Fig. 2). To compare the results with each other, variations of ellipticity of DNA ($\Delta\Theta$) and of wavelength ($\Delta\lambda$) in the maximum of absorption in the CD spectra were calculated and are plotted versus molar ratio r_i (Fig. 3, Table 3), after incubation of DNA with platinum compounds and using DNA alone as a blank or reference. Although complex **5** seemed to have a cisplatin-like behaviour, as deduced from the plot of $\Delta\Theta$ versus r_i, modifications in secondary DNA structure for complex **5** were not as significant as changes observed for cisplatin. On the other hand, a different behaviour was observed in the variation of wavelength $\Delta\lambda$ versus r_i, when DNA was incubated with complex **5**. A bathochromic effect was appreciated at the maximum of CD spectra, instead of the hypsochromic effect observed for cisplatin.

2.5. Agarose gel electrophoresis

Changes in tertiary DNA structure were studied by electrophoresis on agarose gel. The samples were incubated with DNA pBR322 for 24 h at 37 °C in the dark. DNA pBR322 is a plasmid DNA, which has two different predominant forms, open circular (OC) and covalently closed circular (CCC).

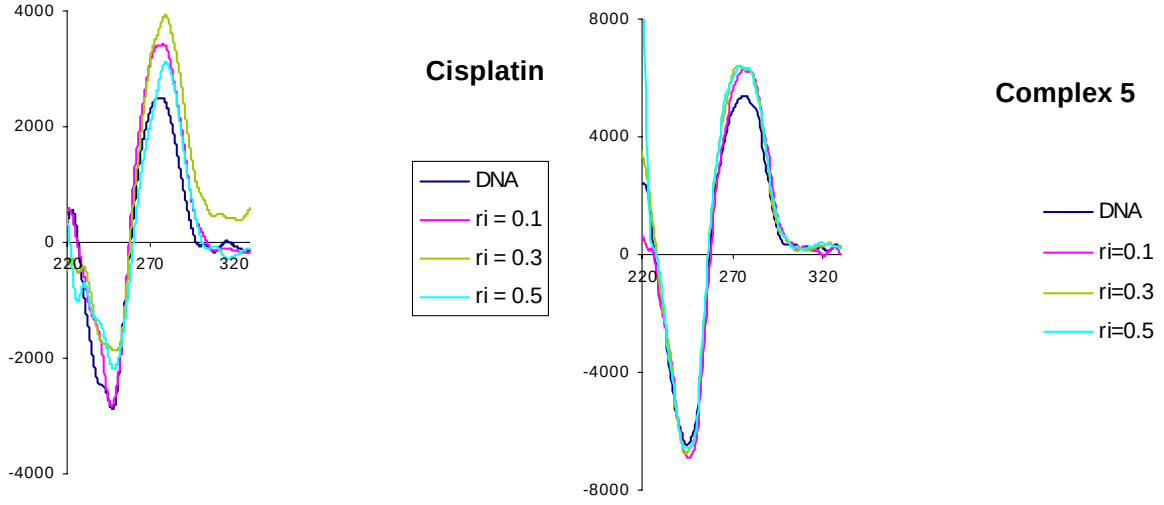


Figure 2. Circular dichroism spectra of DNA and DNA incubated with cisplatin and complex **5** at different r_i.

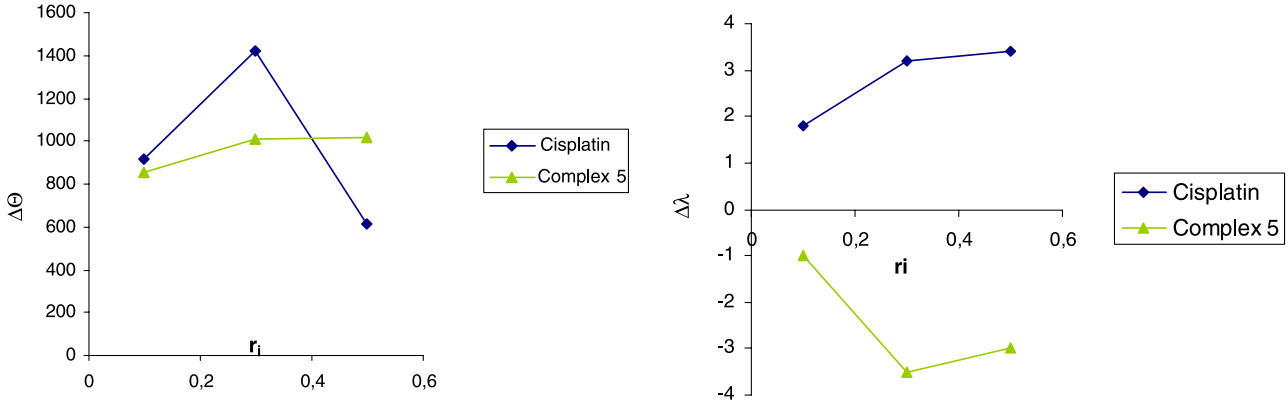


Figure 3. $\Delta\Theta$ versus r_i and $\Delta\lambda$ versus r_i at the maximum of CD spectra.

Table 3. Data from the CD studies for the plasmid DNA as a standard and for DNA incubated with cisplatin, as a reference, and/or with complex **5**

Sample	r_i	Θ_{max} [a]	$\Delta\Theta_{max}/10^3$	λ_{max} [b]	$\Delta\lambda_{max}$	Θ_{min} [a]	λ_{min} [b]
DNA		2.50		277.0		−2.88	247.4
	0.1	3.42	0.92	277.8	1.8	−2.83	246.6
Cisplatin	0.3	3.92	1.42	279.2	3.2	−1.88	249.2
	0.5	3.12	0.61	279.4	3.4	−2.18	248.0
DNA		5.41		276.8		−6.46	244.9
	0.1	6.26	0.85	275.8	−1.0	−6.89	245.4
Complex **5**	0.3	6.42	1.01	273.3	−3.5	−6.75	243.9
	0.5	6.43	1.02	273.8	−3.0	−6.58	244.4

[a] deg cm^2 dmol^{-1} 10^3.
[b] nm.

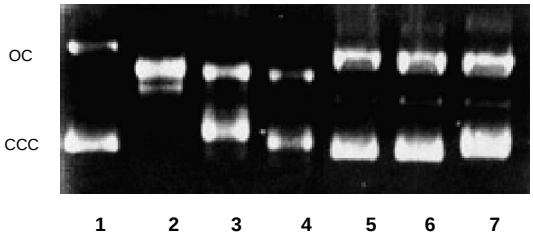


Figure 4. Lane 1, pBR322; lane 2, cisplantin $r_i = 0.5$; lane 3, cisplantin $r_i = 0.3$; lane 4, cisplantin $r_i = 0.1$; lane 5, complex **5** $r_i = 0.1$; lane 6, complex **5** $r_i = 0.3$; lane 7, complex **5** $r_i = 0.5$. Agarose gel electrophoresis of pBR322 alone and pBR322 incubated with cisplatin and complex **5** at different r_i.

In the first lane of the electrophoretic gel, the image shows two DNA forms, CCC and OC, for pBR322 DNA (Fig. 4). The former runs faster because it is more compact. Lanes 2–4 correspond to the same DNA incubated with cisplatin at different r_i. When r_i increased, OC form ran faster and CCC form slower because of cisplatin effect on tertiary DNA structure.[21–23] Lanes 5–7 correspond to the pBR322 incubated with complex **5** at different r_i. Complex **5** was not able to modify electrophoretic mobility of OC and CCC forms, which means that it was not able to modify tertiary DNA structure at this r_i. Only when $r_i = 0.5$ (lane 7) slight changes were observed.

2.6. Atomic force microscopy

The tapping mode atomic force microscopy (TMAFM) is a powerful technique to study biological structures because it does not destroy the sample, it allows performing the studies in both interfaces air-solid and liquid-solid samples, and it makes it possible to know the topographic real relief of the biological structure. On the other hand, by this technique it is possible to directly observe the sample in real time.[24] In the tapping mode AFM, the contact between the tip of the cantilever and the sample is intermittent and weak, in such a way that the lateral forces are avoided as well as the dragging of the sample.[25–27] Many AFM studies have been carried out to observe the effect of cisplatin on several types of DNA.[28] In all these studies, it was possible to appreciate how the DNA molecules compacted and shortened as a consequence of the ability of cisplatin to bind DNA generating foldings.[29]

In our case, samples for AFM were incubated at 37 °C for 24 h in the dark working with a $r_i = 0.5$. Several different samples were studied by this technique: plasmid pBR322 DNA alone, pBR322 DNA incubated in the presence of cisplatin as a standard or activity reference and pBR322 DNA incubated with complex **5**.

Images of the AFM observations for these samples are illustrated in Figure 5 and the effects caused in DNA by complex **5** have been carefully studied. The first image corresponds to pBR322 in an OC form. The second one corresponds to the same DNA incubated with cisplatin and reveals the formation of knots and bendings in DNA structure.[29] Complex **5** seemed to modify DNA in the same way as cisplatin did. Moreover, aggregations of different DNA molecules were observed. This phenomenon could be easy to explain, taking into

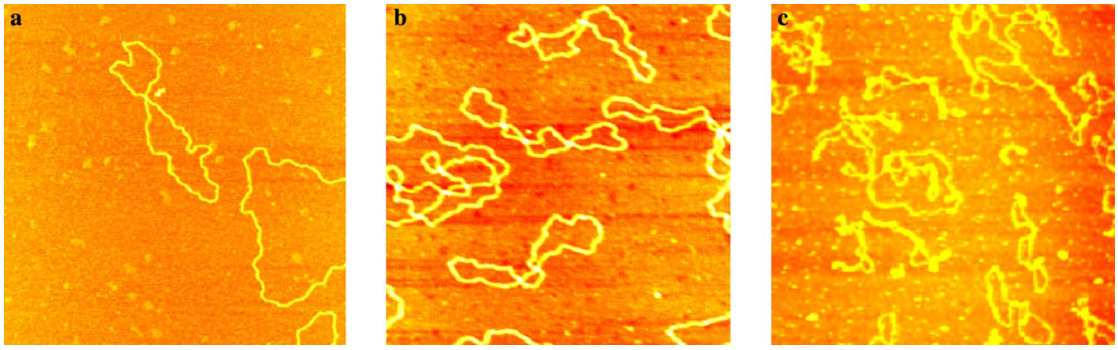


Figure 5. AFM images of: (a) DNA pBR322, (b) DNA pBR322 incubated with cisplatin and (c) DNA pBR322 incubated with complex **5**.

Table 4. IC_{50} (μM) values of different pair cell lines sensitive and resistant to cisplatin

	Cell lines	A2780	A2780cisR	CH1	CH1cisR	41M	41McisR
Complex **5**	IC_{50}	10 ± 2	126 ± 9	24 ± 3	150 ± 12	32 ± 4	187 ± 15
	RF[a]		12.6		6.2		5.8
Cisplatin	IC_{50}	2.2 ± 0.6	38 ± 3	6 ± 1	23 ± 3	26 ± 2	107 ± 8
	RF[a]		17.3		3.8		4.1

[a] Resistance factor (RF) = IC_{50} resistant cell line/IC_{50} sensitive cell line.

account that complex **5** could coordinate DNA by three different sites, the three labile chloro ligands.

2.7. In vitro tumour cell inhibitory activity

Different ovarian cancer cell lines, sensitive (A2780, CH1 and 41M) and resistant (A2780cisR, CH1cisR and 41McisR) to cisplatin, were exposed to increasing concentrations of cisplatin and complex **5** for 24 h. After this time, the cell number/viability was determined by the colorimetric MTT assay. These pairs of cell lines (A2780/A2780cisR, CH1/CH1cisR and 41M/41McisR) were selected to consider the main resistant mechanisms of cisplatin.[30] 41McisR is essentially resistant to the transport of the drug, CH1cisR is resistant to the tolerance and activation of the reparation mechanism of damaged DNA and, finally, A2780cisR is resistant to the combination of the following factors: (a) the decrease of transport through the cell membrane, (b) the activation or reparation mechanism and (c) the increase of GSH (glutathione). The number of cells in the presence of the tested compound was compared to that observed in control cultures and the inhibition of growth (%) was calculated. The IC_{50} (i.e., the concentration producing 50% inhibition of growth) was determined and expressed in μM concentrations (Table 4, Fig. 6). Table 4 also shows the resistance factor (RF), calculated as the ratio IC_{50} resistant cell line/IC_{50} sensitive cell line. The lower activity observed for complex **5** with respect to cisplatin was consistent with the lower interaction with DNA observed in previous comparative studies. However, although complex **5** was not as active as cisplatin, against all the cell lines evaluated, it is worth noting that resistance factor (RF) in the pair of cell lines A2780/A2780cisR was lower for complex **5**. Free ligand **4** was also evaluated for cytotoxic activity against these cell lines and also in front of a panel of cell lines from the National Cancer Institute (Bethesda, Maryland EEUU) and in all cases compound **4** showed to be inactive.

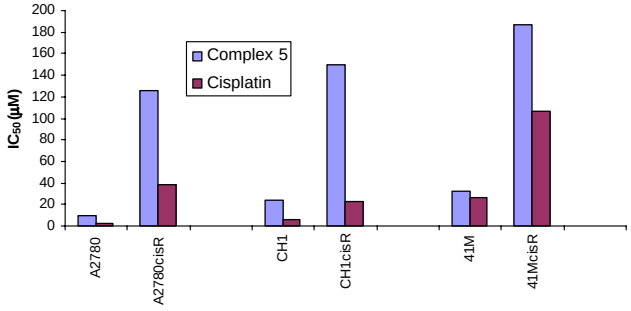


Figure 6. IC_{50} (μM) values for compound **5** and cisplatin for the different cancer cell lines.

3. Conclusions

A new and unexpected platinum(II) π-complex, synthesized by the reaction of 1,2-bis(dimethylaminomethyl)cyclohexene with K_2PtCl_4, has been submitted to studies of interaction with plasmid pBR322 DNA by using three techniques: (a) circular dichroism which showed a certain ability of compound **5** to modify secondary DNA structure compared to cisplatin. (b) Electrophoretic mobility that showed changes in tertiary DNA structure. Thus, in the case of cisplatin incubated with pBR322 DNA, when r_i increases, OC form ran faster and CCC form slower because of cisplatin effect on tertiary DNA structure. In the case of pBR322 DNA incubated with complex **5** at different r_i, it was possible to observe a slight modification of electrophoretic mobility of OC and CCC forms; however, it is worth noting the increase of lineal DNA forms which could be assumed as due to the behaviour of **5**. (c) Atomic Force Microscopy (AFM) revealed morphological changes of plasmid DNA (pBR322) such as formation of knots, folded and supercoiling forms. The images of the AFM observations for these samples illustrated that plasmid DNA, without the presence of Pt(II) compounds, appears in a relaxed OC form. In the presence of cisplatin, some kicks and bending of DNA structure are observed. Complex **5** seems to modify DNA in the same way as cisplatin does.

The lower activity observed for complex **5** with respect to cisplatin is consistent with the lower interaction with DNA observed in previous comparative studies. However, although complex **5** is not as active as cisplatin against all the evaluated cell lines, it is worth noting that resistance factor (RF) in the pair of cell lines A2780/A2780cisR is lower for complex **5**. Efforts to modify this structure to increase its ability to anchor on DNA and concomitant structure–activity relationship studies are under way in our laboratories.

4. Experimental

4.1. Materials and methods

Reactions that required an inert atmosphere were conducted under dry nitrogen and the glassware was oven dried (120 °C). THF, Et_2O and benzene were distilled from sodium/benzophenone prior to use. CH_2Cl_2 was dried by refluxing it over CaH_2 under nitrogen. *Elemental analyses* (C, H, N and S) were carried out on a Carlo Erba EA1108 apparatus. *Infrared spectra* were recorded on a FT-IR NICOLET 510 spectrophotometer in a 4000–400 cm^{-1} range. *NMR spectra* were obtained on a Varian

Gemini-200, a Varian Unity-300 plus or a Varian VXR-500, using $CDCl_3$, DMF-d_7 or DMSO-d_6 as solvents. ^1H NMR spectra were obtained at 200, 300 or 500 MHz frequencies and chemical shifts are given in ppm relative to tetramethylsilane (TMS). ^{13}C NMR and DEPT experiments were recorded at 50 or 75 MHz and were referenced to the 77.0 ppm resonance of $CDCl_3$. *Mass spectra* were run on a Fisons VG Quattro triple quadrupole analyser in the 1800–200 *m/z* range, using MeCN–H_2O as solvent under electrospray (ESP-MS) conditions or by the FAB(+) method using NBA as a matrix, or on a Hewlett–Packard 5890 mass spectrometer using chemical ionisation technique (conditions are specified for each case). *Melting points* were measured with a Galenkamp and a Stuart Scientific SMP3 apparatus.

Circular dichroism (CD) spectra were obtained at room temperature on a JASCO J720 spectropolarimeter with a 450 W xenon lamp using a computer for spectral subtraction and noise reduction. Each sample was scanned three times in a range of wavelength between 220 and 360 nm at a 50 nm min^{-1} rate. The CD spectra obtained are the mean of the three independent scans. The data are expressed as mean residue molecular ellipticity Θ in deg cm^2 $dmol^{-1}$. *Atomic force microscopy* (AFM). The samples were imaged in a Nanoscope III Multimode atomic force microscope (Digital Instrumentals Inc., Santa Barbara, CA) operating in tapping mode in air, at a scan rate of 1–3 Hz. The microscope proves were 125-μm long monocrystalline silicon cantilevers with integrated conical-shaped Si tips (Nanosensors GmbH, Germany) with an average resonance frequency of $f_0 \approx 330$ kHz and spring constant $K \approx 50$ N m^{-1}. The cantilever is rectangular and the tip radius given by supplier is ≈5 nm, a cone angle of 35° and a high aspect ratio. In general, the images were obtained at room temperature (23 ± 2 °C) and the relative humidity (RH) was typically 55%.

4.2. Auxiliary solutions and buffers for in vitro antiproliferative assays

PBS. Commercial solution of phosphate (Dulbecco's phosphate-buffered saline), which was diluted 1/10. *DMEM*. To 500 mL of a solution of DME/HIGH commercial (Dulbecco's modified Eagle's medium), several components were added: 50 mL of FBS (foetal bovine serum) (which is inactivated at 56 °C for 30 min), 5 mL glutamine oil (200 mM), 0.5 mL insulin (10 mg/mL), 2.5 μL hydrocortisone (1 g/mL), 5 mL amphotericin (250 μg/mL) and finally 2.5 mL gentamicin (10 mg/mL). *Trypsin*. Commercial solution trypsin 0.05% in EDTA and 0.02% in PBS, which was preserved at −20 °C. *SRB*. Solution 0.4% (w/v) of sulforodamine B in acetic acid (1% in water). *TrisBase*. A 1 M solution of commercial TrisBase was diluted to 0.01 M, and the pH was adjusted to 10.5 with a NaOH 1 M aqueous solution.

Incubator. Cells were incubated in a Thermo Electron Co., Direct Heat CO_2 incubator. The working conditions were: 5% CO_2, 37 °C and 87% of relative humidity. *Microscopy*. To observe and to count cells, the following microscopes were used: Leitz Wetzlar Diavert Lent (170/-, 4/0.12) Periplan GF 12.5× M and Leitz Wetzlar HM-LUX Lent (170/-, 10/0.25) periplan NF 10×. *Espectrophotometry*. Packard Spectra Count® apparatus was used to measure the absorbance in the SRB tests.

4.3. Synthesis of complex 5

The platinum π-complex **5** was synthesized following the synthetic pathway shown in the discussion section, from tetrahydrophthalic anhydride **1** and according to the procedures described in the literature.[13]

4.4. Crystallographic data

Crystallographic data and experimental details for the structural analyses by X-ray diffraction on single crystals have been deposited with the Cambridge Crystallographic Data Centre as supplementary material, CCDC No. 268228, for compound **5**. Copies of this information may be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ UK [Fax: (int code) +44(1223)336-033 or E-mail: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk].

4.5. Sample preparation for DNA interaction studies by circular dichroism

Stock solution of the platinum complex (1 mg/mL) in a TE (50 nM NaCl, 10 nM Tris–HCl and 0.1 nM EDTA, pH 7.4)/DMSO (98:2) mixture was prepared. A solution of CT DNA in TE was prepared (20 μg mL^{-1}) and kept at 4 °C before use. Drug–DNA complex formation was accomplished by addition of the appropriate volume of the CT DNA solution to aliquots of the compound at different concentrations in TE buffer. The samples were prepared with an input molar ratio of the complex to nucleotide, $r_i = 0.1$, 0.3 and 0.5. The r_i values were calculated from the following equation: $r_i = \mu g_c \cdot M_{nuc} \cdot at_M / C_{DNA} \cdot M \cdot V$, where μg_c is the weight of the compound (μg), M_{nuc} is the average weight per nucleotide (330), at_M is the number of metal atoms in the complex, C_{DNA} is the final concentration of CT DNA, M is the molecular weight of the ligand or the complex and V is the final volume of the sample. The reactions were run at 37 °C for 24 h in the dark.

4.6. Sample preparation for agarose gel electrophoresis

Stock solution of the platinum complex (1 mg/mL) in a TE (50 nM NaCl, 10 nM Tris–HCl and 0.1 nM EDTA, pH 7.4)/DMSO (98:2) mixture was prepared. Drug–DNA complex formation was accomplished by addition of an appropriate volume of the DNA pBR322 (0.25 μg μL^{-1}) solution to aliquots of the compound at different concentrations in TE buffer. The samples were prepared with an input molar ratio of the complex to nucleotide, $r_i = 0.1$, 0.3 and 0.5. The reactions were run at 37 °C for 24 h in the dark. Twenty microlitre aliquots of complex-DNA compounds were subjected to 0.5% agarose gel electrophoresis for 5 h at 1.5 V/cm in 0.5× TBE (45 mM Tris–borate, 1 mM EDTA and pH 8.0) buffer. The gel was subsequently stained in the same buffer containing ethidium bromide (1 mg/mL) and photographed with a Fujifilm FTI-500 system.

4.7. Sample preparation for atomic force microscopy

HEPES solution was prepared (40 mM HEPES, 10 mM $MgCl_2$, pH 7.4). *DNA standard*: 1 μL of pBR322 DNA (Boehringer Mannheim GmbH) was dissolved in 39 μL HEPES, the solution was heated at 60 °C for 30 min, to obtain a high number of OC forms, and incubated at 37 °C for 24 h in the dark. Stock solutions (1 mg/mL) of the complex in HEPES/DMSO (40:60) mixture were prepared. One microlitre of DNA pBR322 (0.25 μg μL^{-1}) and an appropriate aliquot of the stock solution ($r_i = 0.5$) were diluted to 40 μL in HEPES. Finally, the sample was incubated at 37 °C for 24 h in the dark. Samples were prepared by placing a drop (6 μL) of DNA solution or DNA-complex solution onto a freshly cleaved mica (Ashville-Schoonmaker Mica Co., Newport News, V.A). After absorption for 5 min at room temperature, the samples were rinsed for 10 s in a jet of deionised water of 18 $M\Omega\,cm^{-1}$ from a Milli-Q water purification system (Millipore, Molshem, France) directed onto the surface with a squeeze bottle. The samples were blow dried with compressed argon over silica gel for 10 min before imaging by AFM.

4.7.1. Cell culture and cytotoxicity tests. Ovarian cell lines, once defrosted, were incubated at 37 °C for three days in DMEM containing a 10% PBS, under an atmosphere having 10% CO_2 and 80% of relative humidity. The cytotoxicity tests were carried out in 96-well plates using 10,000 cells per well in all cases. To count the number of cells in suspension, a Bürker–Tiefe plate $0.100\ mm \times 0.0025\ mm^2$ was used. The cells were incubated again in the plates for 24 h and the SRB[31] tests were performed on six wells to obtain the T_0 parameter. Stock solutions (1 mg/mL) of compounds to be evaluated (in 2% DMSO and 98% DMEM) were prepared prior to use. From these initial solutions, several diluted solutions (from 1 to 200 μM) were also prepared. For each compound to be evaluated and for each diluted solution, 100 μL aliquots were added on the wells (previous incubation for 24 h) and the cells were incubated in the resultant medium, containing the cytotoxic agents, for three days. Each assay was repeated three times. Afterwards, the MTT or the SRB tests were performed to quantify the number of alive cells.

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