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Influence of the position of substituents in the cytotoxic activity of *trans* platinum complexes with hydroxymethyl pyridines

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Abstract—The synthesis and chemical characterization of two *trans* platinum complexes, (**1**) *trans*-[$PtCl_2NH_3$(2-hydroxymethylpyridine)] and (**2**) *trans*-[$PtCl_2NH_3$(3-hydroxymethylpyridine)], are described. The structures and chemical behaviour of these compounds have been compared to those of their isomer (**3**) *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] previously studied. X-ray structures of all of them were solved and some interesting differences were found. The values of the dihedral angle (85°, 57° and 42° for **1**, **2** and **3**, respectively) demonstrate how important is the position of substituent from a structural point of view. Studies of circular dichroism (CD), electrophoretic mobility (EM) in agarose gel and atomic force microscopy (AFM) showed differences in the modifications caused by the three complexes on DNA. Studies of antiproliferative activity of complexes **1** and **2** against cell tumour lines (HL-60) and apoptosis assays have also been carried out, showing that **1** as well as **2** are far less active than the previously described complex **3** (IC_{50} = 19; 19 and 3 μM, respectively). This fact probes that slight modifications on the drug's design may generate significant differences in the final antitumour activity by modifying the DNA–drug adducts, performance of resistance mechanisms and all the factors that play a fundamental role in Pt complexes' cytotoxicity.

1. Introduction

Modifications in DNA structure when platinum drugs bind covalently seem to be the reason for the anticancer activity of these compounds. Until now *cis*-diamminedicholoroplatinum(II) (cisplatin)[1] and its analogue *cis*-diammineycyclobutanedicarboxylatoplatinum(II) (carboplatin) have been the most used drugs against tumours, but they have some limitations, mainly the narrow range of tumours where they are effective and natural or developed resistance against these platinum compounds. The administration of platinum complexes can also originate several side effects including nausea, vomiting and nephrotoxicity. In order to improve the use of platinum drugs in chemotherapy many different platinum complexes have been investigated and tried against different tumour cell lines. Several new unconventional platinum compounds which violate the original structural rules have been synthesised.

The ineffectiveness of transplatin did not allow the development of *trans* compounds until 1989 when the first active *trans* analogue was described.[2] Up to now, several new complexes with this geometry have exhibited an enhanced cytotoxicity in tumour cell lines, equivalent to or even better than *cis* isomers and cisplatin itself.[3–6] Examples of this kind of compounds are those with general formula *trans*-[$PtCl_2(NH_3)$(L)] where L could be a N-heterocycle, an iminoether or an aliphatic amine.

Previous studies have shown that *trans* compounds with planar N-heterocycles can mainly form monofunctional adducts 1,3-intrastrand bifunctional adducts, and interstrand bifunctional adducts.[7,8] They can bind monofunctionally to DNA with a rate similar to that of transplatin, then the rearrangement to bifunctional

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adducts is also similar to that observed in the case of transplatin and it is a process relatively slow.[9] Differently to transplatin these kind of compounds with planar ligands have the ability to form more interstrand cross-links (30–40% after 48 h). However, a significant fraction of adducts remain monofunctional after long reaction times (30–40% after 48 h of reaction).[10,11]

Similar to cisplatin, transplatin analogues with planar N-heterocycles terminate in vitro RNA synthesis transcription at guanine residues at similar sites to the adducts of cisplatin.[10] It is very interesting to emphasize two points of DNA modified by the *trans*-platinum compounds. First of all, these lesions are recognised by cisplatin specific antibodies and not by transplatin ones, which suggest a similar behaviour to cisplatin; and second, in the majority of DNA adducts of transplatin analogues the planar ligand is very well positioned to interact via stacking with the duplex,[10,12] so it is accepted that monofunctional covalent interaction with Pt combined with stacking interaction of the ligand with DNA bases can produce a similar effect to that of cisplatin.

For some *trans* Pt compounds with planar heterocycles such as thiazole, it has been shown that their adducts are recognised by cellular proteins and repair systems differently from the monofunctional adduct formed by [PtCl(dien)]Cl.[13] The damage produced by these kind of compounds is very similar to that created by 1,2-intrastrand adducts of cisplatin, and it makes them be recognised by HMG proteins and removed by nuclear scission repair (NER) in a similar way as well.

It is also well known that steric hindrance can also increase the antitumour activity of some platinum complexes. This is the case of the complex *cis*-[$PtCl_2(NH_3)$(2-picoline)] (AMD473) in clinical trials and whose activity differs significantly from that of cisplatin.[14,15] The 2 methyl group makes difficult axial approach to Pt(II) and, as a result, hydrolysis occurs approximately four times more slowly than for cisplatin.[16]

Recently, some interesting complexes with general structure $[(\eta^6\text{-arene})Ru(X)(Y)(Z)]^+$ have displayed very interesting activity against tumour cell lines depending on the structure of the arene ligand and the X, Y, Z ligands. In general, the best activity was found for bulky arenes able to intercalate DNA and when X, Y were a chelating ligand.[17–19] These kind of complexes, similar to our *trans* platinum compounds, are also able to interact with DNA through their arene ligands upon covalent coordination of the metal complex.

In this work, we describe two *trans* platinum compounds with planar N-heterocycles as 2-hydroxymethylpyridine and 3-hydroxymethylpyridine,[20] and we compare their activity to the activity of the previously described complex with 4-hydroxymethylpyridine.[20,21] This platinum complex forms on double-stranded DNA stable intrastrand and interstrand crosslinks which distort DNA conformation in a unique way.[22] Downstream effects modulated by recognition and binding of p53 protein to DNA distorted by 4-hydroxymethylpyridine *trans* platinum complex and transplatin are not likely the same. It has been suggested that these different effects might contribute to different antitumour effects of these two *trans* platinum complexes.[22]

The main objective of the work is to observe how differences in the stereochemistry of the compound make the three complexes, *trans*-[$PtCl_2NH_3$(2-hydroxymethylpyridine)] (**1**), *trans*-[$PtCl_2NH_3$(3-hydroxymethylpyridine)] (**2**) and *trans*-[$PtCl_2NH_3$(4-hydroxymethyl pyridine)] (**3**), exhibit different effects on primary, secondary and tertiary structures of DNA and also different cytotoxic activity. It means the way in which they interact with DNA is probably different. Time and again, this is the most important factor which determines the activity of platinum compounds.

2. Results and discussion

2.1. Results

2.1.1. Chemistry. Synthesis of **1** and **2** is analogous to the synthesis described for **3**.[21] Compounds **2** and **3** have been prepared before using a different procedure,[20] while compound **1** was not obtained. Crystal structures of **1** and **2** have not been yet described.

Molecular and crystal structures were confirmed by X-ray diffraction. Structural data of **3** have been previously published.[21]

2.1.2. Crystal and molecular structure determination. The molecular structures of the complexes *trans*-[$PtCl_2NH_3$(2-hydroxymethylpyridine)] (**1**) and *trans*-[$PtCl_2NH_3$(3-hydroxymethylpyridine)] (**2**) are shown in Figure 1.

Crystallographic data and structure refinement are presented in Table 1. Bond lengths and angles are collected in Table 2.

Complex **2** has a very similar structure to that described for complex **3** with a trans arrangement around platinum centre.[21] Differently, the crystal structure is formed by the packing of three different discrete molecules of *trans*-[$PtCl_2NH_3$(3-hydroxymethylpyridine)] which slightly differ just in some angles. In all of them the average values for Pt–Cl distances and Pt–N distances are 2.295(2) and 2.017(5) Å, respectively. Repulsions with aromatic ring distort the square planar structure making N(ring)–Pt–Cl angles higher than 90°, and N(ammonia)–Pt–Cl angles lower than 90°, likewise in complex **3**. Distances between consecutive pyridinic aromatic rings and between nearest Pt atoms are 4.33 and 3.76 Å, which discard any kind of 'stacking' or intermetallic interactions.[23] The average dihedral angle is 57°, which is logical due to the higher repulsion of the hydroxymethyl group in meta position, in comparison to complex **3** with the same group in para position.

Complex **1** consists of a package of two kind of discrete molecules of *trans*-[$PtCl_2NH_3$(2-hydroxymethylpyri-

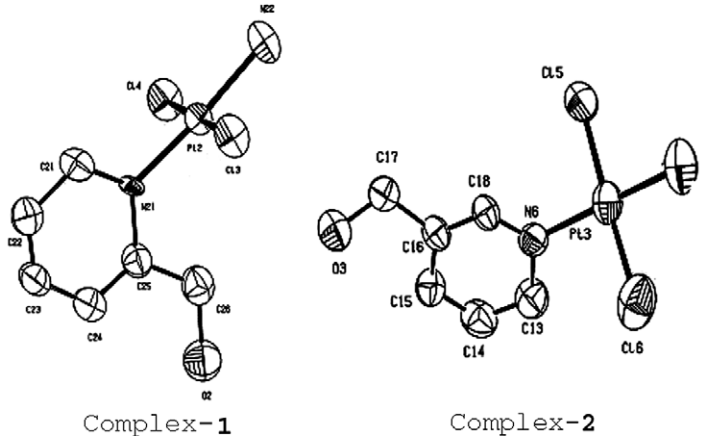

Figure 1. Molecular structures of the complex **1** and complex **2**.

Table 1. Crystal parameters and structure refinement data of complexes **1** and **2**

Compound	Complex-**1**	Complex-**2**
Formula	$C_6H_{10}Cl_2N_2OPt$	$C_6H_{10}Cl_2N_2OPt$
Molecular weight	392.15	392.15
Crystal size (mm)	$0.1 \times 0.1 \times 0.2$	$0.1 \times 0.1 \times 0.2$
Crystal system	Monoclinic	Monoclinic
Space group	$P2_1/c$	$P2_1/c$
a, b, c (Å)	$a = 20.2640(10)$	$a = 12.0441(10)$
	$b = 12.4440(10)$	$b = 28.295(2)$
	$c = 7.9310(10)$	$c = 8.8814(11)$
α, β, γ (°)	$\beta = 91.920(10)$, $\alpha = \gamma = 90$	$\beta = 90.389(6)$, $\alpha = \gamma = 90$
Volume (Å^3)	1998.8(3)	3026.6(5)
Z	8	12
Density (calcd) (Mg m^{-3})	2.606	2.582
Absorption coefficient (mm^{-1})	14.530	14.394
F(000)	1440	2160
Temperature (K)	293(2)	293(2)
Collected reflexions	8021	57331
Independent reflexions	4208 ($R_{int} = 0.0385$)	8696 ($R_{int} = 0.0371$)
Refined parameters	217	325
Final R_1 (wR_2) [$I > 2\sigma$ (I)]	0.0357	0.0377

dine)] which slightly differ between them in some angles. Average values for Pt–Cl distances and Pt–N distances are 2.272(2) and 2.012(5) Å, respectively. The planar square is distorted as in the isomers **2** and **3**. The distance between the nearest aromatic rings is 3.97 Å, which is too long to suggest 'stacking' interactions. Pt–Pt distance is 4.59 Å, which allows to discard intermetallic interactions. As it was expected, dihedral angle is, on average, 85°. Small differences in the structure could mean big differences in cytotoxic activity. The unit cells of complexes **1** and **2** are shown in Figure 2.

2.1.3. Biochemical studies. Three different techniques to probe the changes on DNA structure induced by the complexes **1** and **2** have been used: circular dichroism (CD), atomic force microscopy (AFM) and gel electrophoresis (EF). Despite the fact that the results for the complex with 4-hydroxymethylpyridine and its ligand have been previously published,[21] the electrophoretic mobility, AFM images and cytotoxicity studies have been included here in order to compare the activity of the complexes obtained with the two other isomers.

2.1.3.1. Circular dichroism. Modifications caused by the ligands 3-hydroxymethylpyridine and 2-hydroxymethylpyridine and their compounds of Pt(II) are collected in Table 3. The comparison of the ellipticity values obtained demonstrates that ligands do not produce significant changes in ellipticity which means that they are not inducing modifications in the secondary structure of DNA. However, platinum(II) complexes behave completely differently. Complex **2** is able to decrease ellipticity values of the positive side of the spectra. In addition, a shift of the ellipticity maximum towards upper wavelengths (bathochromic effect) is observed. These modifications are described as a transformation from B form of DNA to C form.[24–27] Complex **1** increases ellipticity in the positive side and it means that this complex displays a different effect on DNA, probably due to a different interaction from a covalent bond. In sum, the three compounds are able to modify the secondary structure of the biomolecule, differently from ligands.

2.1.3.2. Electrophoretic mobility. As it can be seen in Fig. 3, cisplatin has the known effect at $r_i = 0,5$, that is

Table 2. Bond distances and angles for metal complexes **1** and **2**

Complex-**1**		Complex-**2**	
Lengths (Å)		Lengths (Å)	
Pt(1)–N(11)	2.023(6)	Pt(1)–N(1)	2.016(5)
Pt(1)–N(12)	2.075(5)	Pt(1)–N(2)	2.013(5)
Pt(1)–Cl(2)	2.257(2)	Pt(1)–Cl(1)	2.292(2)
Pt(1)–Cl(1)	2.291(2)	Pt(1)–Cl(2)	2.306(2)
Pt(2)–N(21)	1.928(4)	Pt(2)–N(3)	2.046(6)
Pt(2)–N(22)	2.020(7)	Pt(2)–N(4)	2.009(5)
Pt(2)–Cl(3)	2.274(2)	Pt(2)–Cl(3)	2.288(2)
Pt(2)–Cl(4)	2.265(2)	Pt(2)–Cl(4)	2.287(2)
		Pt(3)–N(5)	2.001(1)
Angles (°)		Pt(3)–N(6)	2.014(7)
N(11)–Pt(1)–N(12)	177.5(2)	Pt(3)–Cl(5)	2.297(3)
N(11)–Pt(1)–Cl(2)	90.1(2)	Pt(3)–Cl(6)	2.297(4)
N(12)–Pt(1)–Cl(2)	88.4(2)		
N(11)–Pt(1)–Cl(1)	91.1(2)	Angles (°)	
N(12)–Pt(1)–Cl(1)	90.5(2)	N(1)–Pt(1)–N(2)	178.7(2)
Cl(2)–Pt(1)–Cl(1)	178.4(1)	N(2)–Pt(1)–Cl(1)	92.0(2)
N(21)–Pt(2)–N(22)	174.7(3)	N(1)–Pt(1)–Cl(1)	89.2(2)
N(21)–Pt(2)–Cl(4)	89.2(2)	N(2)–Pt(1)–Cl(2)	89.3(2)
N(22)–Pt(2)–Cl(4)	91.6(2)	N(1)–Pt(1)–Cl(2)	89.5(2)
N(21)–Pt(2)–Cl(3)	91.1(2)	Cl(1)–Pt(1)–Cl(2)	178.6(1)
N(22)–Pt(2)–Cl(3)	88.0(2)	N(3)–Pt(2)–N(4)	177.9(2)
Cl(4)–Pt(2)–Cl(3)	178.9(1)	N(3)–Pt(2)–Cl(4)	88.4(2)
		N(3)–Pt(2)–Cl(3)	90.2(2)
		N(4)–Pt(2)–Cl(4)	90.8(2)
		N(4)–Pt(2)–Cl(3)	90.6(2)
		Cl(3)–Pt(2)–Cl(4)	178.3(1)
		N(5)–Pt(3)–N(6)	179.6(4)
		N(5)–Pt(3)–Cl(5)	88.9(4)
		N(6)–Pt(3)–Cl(5)	91.3(2)
		N(5)–Pt(3)–Cl(6)	88.6(4)
		N(6)–Pt(3)–Cl(6)	91.2(2)
		Cl(5)–Pt(3)–Cl(6)	177.5(1)

CCC form and OC form migrate at about the same average speed (coalescence point), while *trans* platinum complexes **2** and **3** exhibit a noticeable effect since they are able to make CCC and OC migrate at exactly the same average speed. It means that *trans* complexes are able to originate microfolders on OC forms of DNA and they achieve a more compacted structure, whereas the CCC form becomes more relaxed. The effect on tertiary structure of complex **1** is apparently different from the effect of complex **2** and complex **3** as it happened with secondary structure. It seems that the different behaviour of complex **1** makes it to be less effective against DNA, since it does not reach the coalescence point.

2.1.3.3. Atomic force microscopy (AFM). AFM pictures of DNA pBR322, DNA with cisplatin, DNA with *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)], DNA with *trans*-[$PtCl_2NH_3$(3-hydroxymethylpyridine)] and DNA with *trans*-[$PtCl_2NH_3$(2-hydroxymethylpyridine)] are shown in Figure 4.

AFM images show morphologic changes on tertiary structure of DNA. Cisplatin makes appear stress and microfolds in DNA. *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] has a more dramatic effect. In addition, its geometry allows it to form cross points between chains. With reference to metal complexes **1** and **2** it is seen that they do not produce the same lesions as the complex with 4-hydroxymethylpyridine. Their ability to interact with DNA seems to be less effective. Changes in the structure of metal complexes make them interact in a different way with DNA and it means a different effect.

2.1.4. Pt cell uptake. The results of Pt uptake for complexes **1**, **2** and **3** are given in Table 4.

These results correspond to the average of three independent measurements. A direct relationship between Pt uptake and activity has not been described yet and it is not clearly observed in our measurements,[28,29] since cisplatin is the complex with greater uptake at 24 h, but is not the metal complex with the highest cytotoxicity at this time. With reference to the *trans* complexes, the compound, *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)], exhibits the best capacity to reach the cell nucleus.

2.1.5. Cytotoxicity of the platinum complexes against HL-60 cells. The effect of the platinum complexes was examined on human leukaemia cancer cells (HL-60) using the MTT assay, a colorimetric determination of cell viability during in vitro treatment with a drug. The assay, developed as an initial stage of 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} is desired and implies cytotoxicity or antiproliferation at low drug concentrations.

The drugs tested in this experiment were cisplatin, complex *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] (**3**), [previously studied][21] and the other two *trans* platinum complexes **1** and **2**. Navarro et al.[20] tested compounds **2** and **3** against A2780/A2780cisR and CH1/CH1cisR cell lines but they did not show cytotoxic activity.

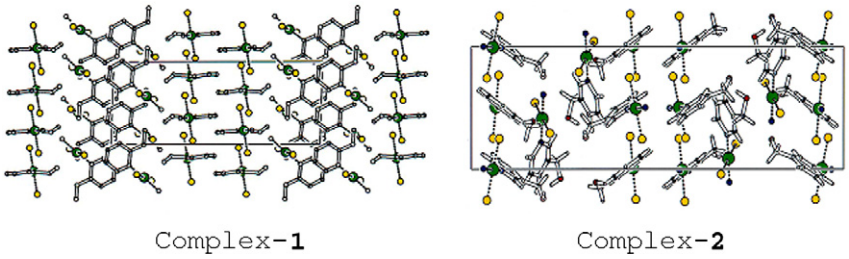

Figure 2. Unit cell of complexes **1** and **2** observed from (0,1,0) and (1,0,0) planes, respectively.

Table 3. Values of ellipticity for the ligands 3-hydroxymethylpyridine, 2-hydroxymethylpyridine and their Pt(II) complexes

Ligands or metal complexes	r_i	Θ_{max}[a]	λ_{max} (nm)	Θ_{min}[a]	λ_{min} (nm)
DNA	—	5105	276.4	−5471	246
3-Hydroxymethylpyridine	0.1	5091	274.4	−5418	245.8
	0.3	5010	274	−5760	245.6
	0.5	4496	276.6	−4825	245.8
2-Hydroxymethylpyridine	0.1	5100	274.2	−5601	245.8
	0.3	5085	274	−5653	246
	0.5	5090	274	−5680	246
DNA	—	4512	276.6	−5052	245.6
Complex-**1**	0.1	4882	278	−4897	246.8
	0.3	5492	278.2	−4344	247.2
	0.5	5269	278.2	−4990	247.8
DNA	—	3003	275	−4893	245.4
Complex-**2**	0.1	2545	279.2	−4836	245.8
	0.3	2426	281	−4987	247.2
	0.5	2192	281	−4235	248.2

[a] degree cm^2 $dmol^{-1}$.

Figure 3. Agarose gel electrophoresis of DNA treated with Cisplatin (lane 1), DNA pBR322 (lane 2), complex **3** (lane 3), complex **2** (lane 4) and complex **1** (lane 5); all of them at $r_i = 0,5$.

Cells were exposed to each compound continuously for a 24 h or a 72 h period and then assayed for growth using the MTT endpoint. Figure 5 shows the dose–response curves of these drugs in terms of the drug effect on the growth of the HL-60 cells. The IC_{50} values of complex **1**, complex **2** and cisplatin for the growth inhibition 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 $16 \pm 3\,\mu M$, which is 5-fold greater than *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] (**3**) but a bit better than IC_{50} for complexes **1** and **2** at this time. The cytotoxicities of the Pt complexes were also determined for 72 h. As listed in Table 5, *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] exhibits cytotoxicity comparable to that of cisplatin, and they are approximately 6- and 9-fold more toxic than **1** and **2**, respectively.

Complexes **1**, **2** and **3** have the same activity along the time, only cisplatin increases its activity.

2.1.6. Quantification of apoptosis by annexin V binding and flow cytometry. We have also analysed by Annexin V-PI flow cytometry whether complexes **1**, **2** and **3** are able to induce apoptosis in HL-60 cells after 24 h of incubation at equitoxic concentrations (IC_{50} values). Annexin V binds phosphatidylserine residues, which are asymmetrically distributed towards the inner plasma membrane but migrate to the outer plasma membrane during apoptosis.[30]

As it can be seen in Figure 6 and in Table 6, all metal complexes induce cell death mainly by apoptosis. The most active metal complex is complex **3**, which is able to induce more than 80% of apoptotic death, in comparison to the 50% of cisplatin or the 20% of the other two complexes. Complex **3** is also the complex which can reach the highest Pt uptake and which interacts more effectively with DNA.

2.2. Discussion

Monofunctional binding mode was expected not to be effective against tumour cells as it was observed with some clinically inert compounds [PtCl(dien)]Cl or [$PtCl(NH_3)_3$]Cl. However, the presence of planar ligands changes the structures of Pt–DNA adduct and it gives more possibilities for new biological activity. Therefore, some Pt compounds, which form monofunctional stable adducts, have been described, for example *cis*-[Pt-(Am)$Cl(NH_3)_2$]$^+$, where Am is a derivative of pyridine, pyrimidine, purine or aniline,[31] or a family of compounds with iminoethers of general formula *trans*-[$PtCl_2$(*E*-iminoether)$_2$], which form stable monofunctional adducts preferentially.[32–37]

In the present work, we have shown that monofunctional adducts of our *trans* platinum compounds are able to generate anticancer activity against HL-60 tumour cells, probably because they finish replication and/or transcription. We have also shown how important the stereochemical differences are in order to affect the cytotoxicity of the Pt complexes.

There are some reasons which can explain why this kind of compounds present activity. It is believed that cisplatin antitumour activity is mediated by cellular proteins that bind very specifically to its bifunctional adducts

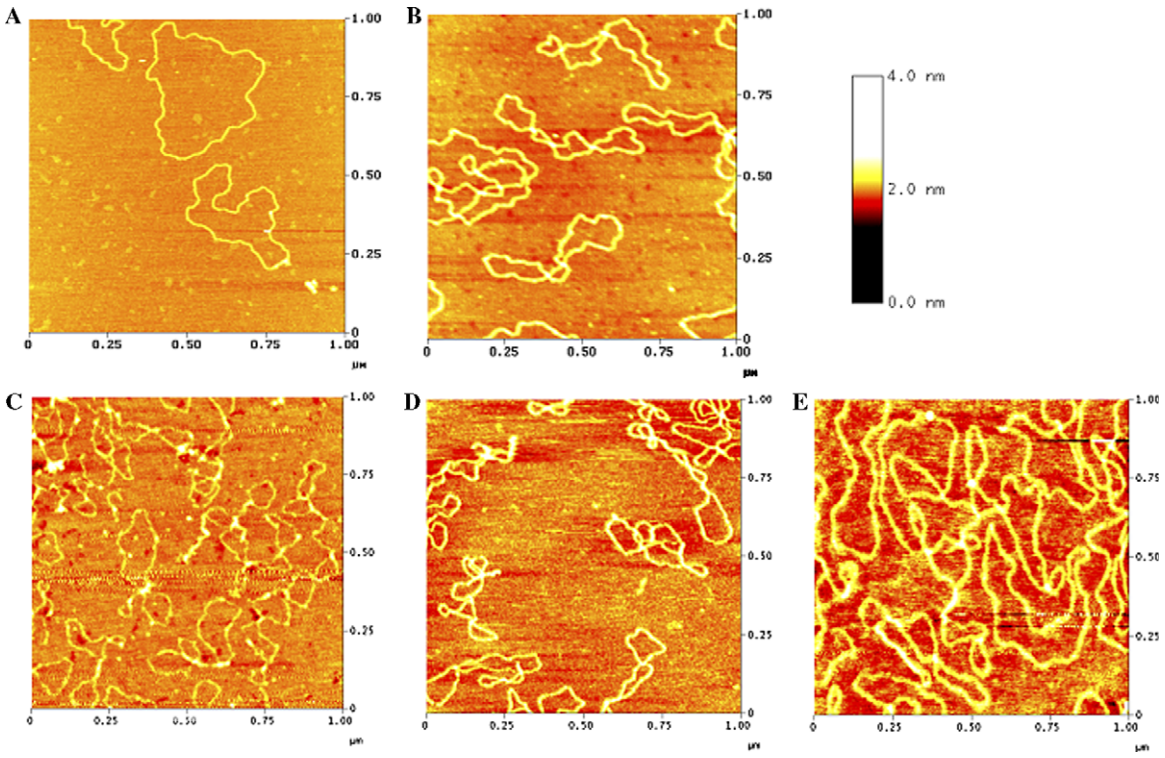

Figure 4. TMAFM images of (A) DNA pBR322, (B) DNA with cisplatin, (C) DNA with complex **3**, (D) DNA with complex **2** and (E) DNA with complex **1**.

Table 4. Cellular DNA platination (by ICP-MS) in HL-60 cells treated with cisplatin and metal complexes **1** and **2**

	5 μM		25 μM	
	6 h	24 h	6 h	24 h
Cisplatin	14.63*	36.28	73.45	471.83
Complex-**3**	27.16	10.07	561.38	120.66
Complex-**2**	6.57	18.07	40.77	23.69
Complex-**1**	7.81	10.97	39.41	18.39

* All data expressed in ng Pt/mg DNA.

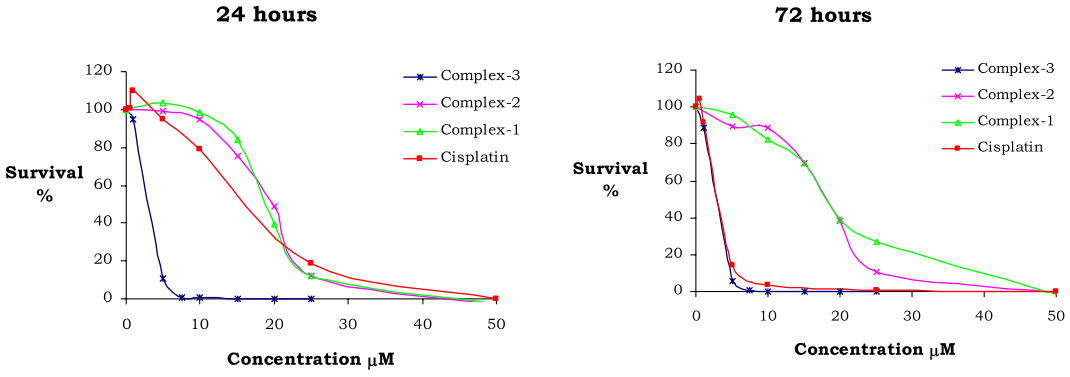

Figure 5. Percentage of survival of HL-60 cells after treatment with cisplatin and the *trans* complexes **1** and **2**.

but not to monofunctional ones of cisplatin or transplatin.[38] Some studies have proved that HMGB1 proteins bind to monofunctional adducts of some *trans* platinum compounds, as *trans*-Pt-thiazole, with the same affinity as to bifunctional adducts of cisplatin.[7] Then, HMGB1 and other proteins, such as transcription factors (which have very important roles in cells), can bind to Pt–DNA adducts and this fact not only makes proteins lose their activity, but it also blocks these damaged areas from reparation, so these adducts may persist and potentiate

Table 5. IC_{50} values of *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)], complexes **1** and **2**, and cisplatin against HL-60 cells

Metal complex	IC_{50} (μM) 24 h	IC_{50} (μM) 72 h
3	3 ± 1	3 ± 1
2	19 ± 2	18 ± 2
1	19 ± 1	18 ± 2
CDDP	16 ± 2	2 ± 1

the cytotoxicity. Finally, the fact that *trans* platinum(with planar amines)-DNA adducts are recognised by cellular proteins allows that monofunctional adducts can persist and not react with other cellular sulfur compounds, in contrast to transplatin monofunctional adducts, which is considered to be one of the most important resistance mechanisms in cells.

These phenomena could explain the cytotoxic activity of some *trans* complexes, and also for our three isomers presented in this work: *trans*-[$PtCl_2NH_3$(2-hydroxymethylpyridine)] (**1**), *trans*-[$PtCl_2NH_3$(3-hydroxymethyl pyridine)] (**2**) and *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] (**3**). The accepted hypothesis for this behaviour is that monofunctional adducts of this kind of *trans* compounds distort DNA in a similar way to 1,2-intrastrand cross link of cisplatin with a similar unwind of the double helix due to the *cis* position of the planar ligand with reference to the binding site which can produce a perturbation on DNA. It means that planar ligand interacts or stacks with bases of DNA, while Pt is monofunctional but covalently bonded, producing a 'pseudo' bifunctional interaction.[39]

Beyond hypothesis, Brabec et al.[22] were able to prove that complex **3** inhibits binding of active protein p53 to DNA similarly to cisplatin and is more pronounced

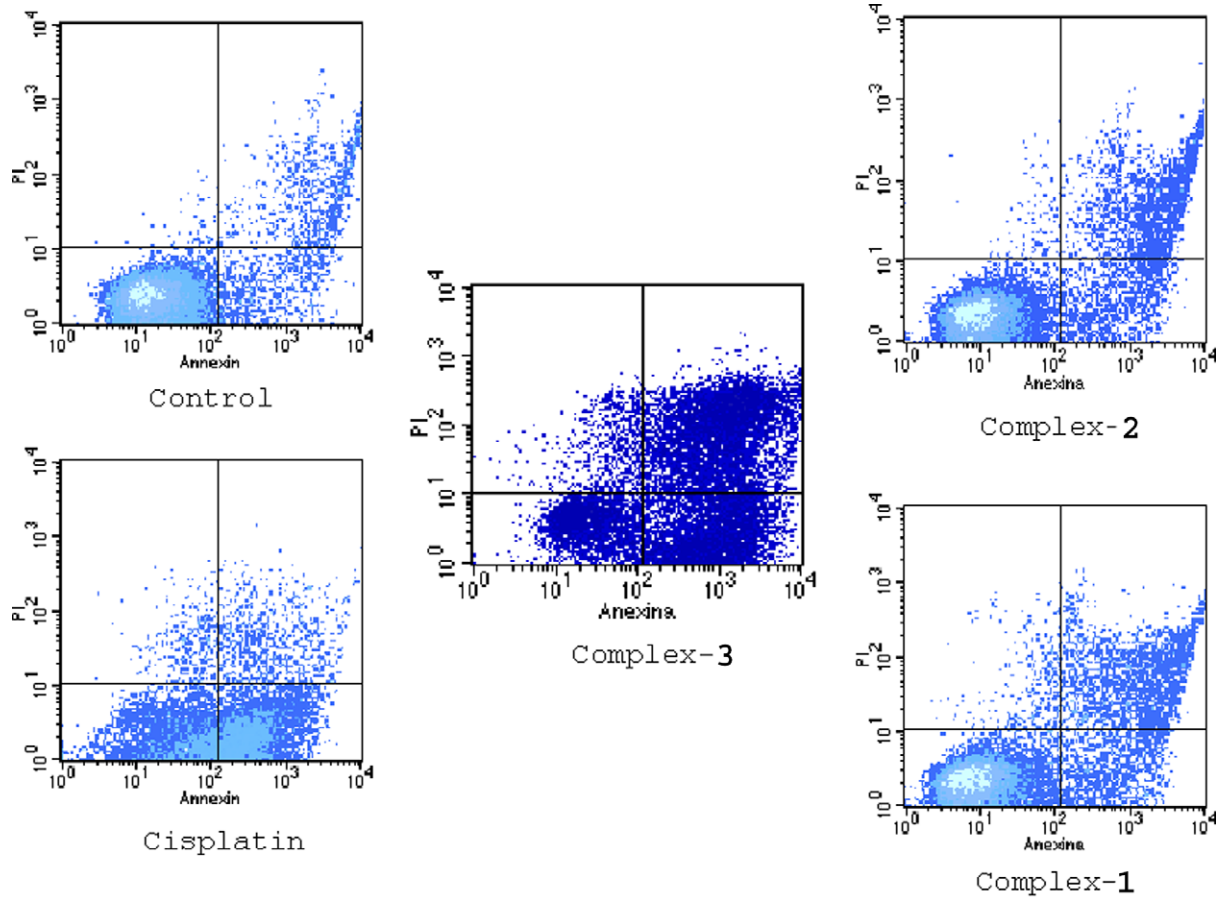

Figure 6. Flow cytometry schemes for HL-60 control cells, and HL-60 cells incubated with cisplatin, complexes **3**, **2** and **1** at a concentration equal to their respective IC_{50} values.

Table 6. Percentage of HL-60 cells in each state after treatment with metal complexes at IC_{50} concentration for 24 h of incubation

Metal complex (μM)	Vital cells An −/ PI −	Early apoptosis An +/PI −	Late apoptosis An +/ PI +	Nechrosis An −/ PI +
Control cells	89.05	4.91	5.68	0.36
Cisplatin (15.6)	46.81	47.25	4.26	1.67
Complex-**3** (3.5)	16.27	61.58	19.06	3.10
Complex-**2** (19.0)	78.00	8.56	12.84	0.60
Complex-**1** (18.85)	75.59	9.30	13.41	1.70

for adducts of **3** than the adducts of transplatin. p53 is a potent mediator of cellular responses against genotoxic aggressions including the treatment with antitumour platinum drugs. Their results also exhibit that in DNA treated with complex **3** there are only a 10% of remaining monofunctional adducts after 48 h of incubation at a drug to nucleotide molar ratio of $r_i = 0{,}05$. The rest of adducts are 26% interstrand and 64% intrastrand, in comparison with the 40% of monofunctional adducts of transplatin at the same conditions. Interestingly, the unwinding angle produced by **3** (28°) is considerably higher than that produced by bifunctional cisplatin.[22] Similar higher unwinding angles in the range of 17–30° were obtained by transplatin analogues where one NH_3 group was replaced by heterocyclic planar or non-planar ligand as piperidine, piperazine, 4-picoline, thiazole or quinoline.[40,41] Such a great value can be explained by the contribution to unwinding associated to the interaction of the planar ligand with the double strand of DNA. The replacement of NH_3 by 4-hydroxymethylpyridine ligand allows positioning of the planar moiety favourable for interaction with double helix ('pseudo' bifunctional adducts). Importantly, It has been observed that complexes with intercalating ligands induce alterations in DNA which are more difficult to repair in comparison to those complexes which interact only covalently.[42] All these data could obviously explain the different behaviour with reference to transplatin.

More difficult is to understand why complexes **1** and **2** are not so active. Apart from all resistance mechanisms which could affect differently for all three complexes,[43] there are several differences between the three *trans* platinum which could give some clues. Cellular uptake of the drug is one of those differences. Despite a direct relationship between cellular uptake and activity not always expected, it is known that a low uptake is one of the reasons for inactivity of platinum complexes. Knowing this, the observed differences in the uptake of complexes **3**, **2** and **1** may explain the different cytotoxicity of all three metal complexes. The structural settings may play a fundamental role since they are responsible for the capacity of the metal complex to go through the cell membrane, and responsible for the kind of interaction between Pt drug and DNA. As we can see in Figure 7, the very different values of the dihedral angles in all three compounds could give us an idea about general differences in the structure, mainly caused by the position of the hydroxymethyl group. These differences might determine the rate of entrance to cells and the chance to interact or stack between planar ligand and nucleobases in DNA. If 'pseudo' bifunctional adducts do not appear there is no recognition by cell proteins as HMGB1 and Pt compound may lose its activity. It is seen by CD, electrophoretic mobility and AFM that modifications of DNA (secondary and tertiary structures) originated by complexes **3** and **2** are in the same direction but much more intensive in the case of the last one. It is also seen by flow cytometry that all of them are able to induce apoptosis, though more efficiently in the case of complex **3**. These results suggest that metal complexes **3** and **2** could interact with DNA in a similar way and the lower rate of platination for **2** could decrease its activity.

In addition, some hydrolysis studies show that substituents in meta position can alter the electronic structure of the molecule and partially deactivate substitution reactions in Pt.[5] This factor could also help to understand the less activity of **2** compared to **3**.

On the other hand, complex **1** has a total different behaviour. Complex **1** is the metal complex which alters less effectively DNA at all levels (secondary and tertiary structures), as it was observed by CD, EF and AFM, and it is also the metal complex which less reaches the DNA and which less induces apoptosis. The ortho position of the hydroxymethyl substituent generates a high steric hindrance which makes difficult the interaction with DNA. It is well known that this hindrance has the effect of destabilizing the expected trigonal bipyramidal transition state in substitution reactions of square planar Pt(II).[5] In addition, the ortho position of the hydroxymethyl group gives the complex some geometric features (dihedral angle of about 85°) which do not allow the metal complex to reach the cell nucleus efficiently and probably formation of the "pseudo" bifunctional adducts does not take place.

3. Experimental

3.1. Chemistry

trans-[$PtCl_2NH_3$(2-hydroxymethylpyridine)] (**1**)

cis-DDP (0.5 mmol) (cisplatin) was suspended in 20 mL H_2O. This suspension was treated with a slight excess of 2-hydroxymethylpyridine (1.03 mmol). The mixture was stirred for 8 h and 80 °C. The solution came colourless, then 5 mL of concentrate HCl was added and after 10 h at 80 °C the solution took a yellow colour. It was concentrated up to 10 mL and left at room temperature until crystals appear. Crystals were filtered off and dried in

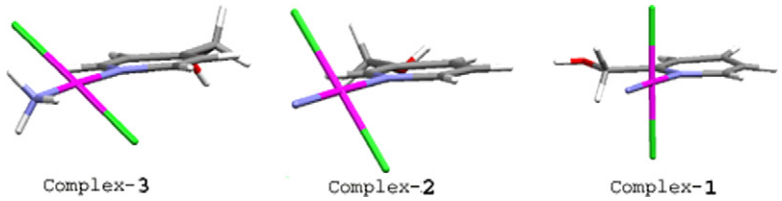

Figure 7. Three-dimensional view of metal complexes **3**, **2** and **1**.

air. Yield: 20%. Calculated for $C_6H_{10}N_2OCl_2Pt$: C, 18.36; N, 7.14; H, 2.57. Found: C, 18.23; N, 6.85; H, 2.93.

Complex **3** *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine) was prepared as indicated in Ref. 21 has been previously described by our group.[21] The synthesis of complex **2** *trans*-[$PtCl_2NH_3$(3-hydroxymethylpyridine) is analogous to that of complex **3**. Calculated for $C_6H_{10}N_2OCl_2Pt$: C, 18.36; N, 7.14; H, 2.57. Found: C, 18.40; N, 7.02; H, 2.63, for complex **2**.

3.2. X-ray diffraction

A prismatic crystal ($0.1 \times 0.1 \times 0.2$ mm) of the complex *trans*-[$PtCl_2NH_3$(3-hydroxymethylpyridine)] (**2**) was selected and mounted on a MAR345 diffractometer with an image plate detector. Unit-cell parameters were determined from 27106 reflections ($3 < \theta < 31°$) and refined by least-squares method. Intensities were collected with graphite monochromatized Mo Kα radiation. 57,331 reflections were measured in the range $1.44 \leqslant \theta \leqslant 31.66$. 8696 of which were non-equivalent by symmetry (R_{int}(on I) = 0.037). 7736 reflections were assumed as observed applying the condition $I > 2\sigma(I)$. Lorentz-polarization but no absorption corrections were made.

The structure was solved by Patterson synthesis, using SHELXS computer program and refined by full-matrix least-squares method with SHELX97 computer program,[44] using 8696 reflections (very negative intensities were not assumed). The function minimized was $\sum w||F_o|^2 - |F_c|^2|^2$, where $w = [\sigma^2(I)]+(0.0507P)^2 + 6.9340P^{-1}]$, and $P = (|F_o|^2 + 2|F_c|^2)/3$, f, f' and f'' were taken from International Tables of X-Ray Crystallography.[45] All H atoms were computed and refined, using a riding model, with an isotropic temperature factor equal to 1.2 times the equivalent temperature factor of the atom which are linked. The final R(on F) factor was 0.038, wR(on $|F|^2$) = 0.109 and goodness of fit = 1.202 for all observed reflections. Number of refined parameters was 325. Max. shift/esd = 0.00, mean shift/esd = 0.00. Max. and min. peaks in final difference synthesis were 0.730 and -0.696 eÅ^{-3}, respectively.

A prismatic crystal ($0.1 \times 0.1 \times 0.2$ mm)of the complex *trans*-[$PtCl_2NH_3$(2-hydroxymethylpyridine)] (**1**) was selected and mounted on a MAR345 diffractometer with an image plate detector. Unit-cell parameters were determined from 9313 reflections ($3 < \theta < 21°$) and refined by least-squares method. Intensities were collected with graphite monochromatized Mo Kα radiation. 8021 reflections were measured in the range $2.01 \leqslant \theta \leqslant 33.20$, 4208 of which were non-equivalent by symmetry (R_{int}(on I) = 0.038). 3329 reflections were assumed as observed applying the condition $I > 2\sigma(I)$. Lorentz-polarization but no absorption corrections were made.

The structure was solved by direct methods, using SHELXS computer program[44] and refined by full-matrix least-squares method with SHELX97 computer program,[44] using 4208 reflections (very negative intensities were not assumed). The function minimized was $\sum w \ ||F_o|^2 - |F_c|^2|^2$, where $w = [\sigma^2(I) + (0.0683\ P)^2]^{-1}$ and $P = (|F_o|^2 + 2|F_c|^2)/3$, f, f' and f'' were taken from International Tables of X-Ray Crystallography.[31] All H atoms were computed and refined, using a riding model, with an isotropic temperature factor equal to 1.2 times the equivalent temperature factor of the atoms which are linked. The final R(on F) factor was 0.035, wR(on $|F|^2$) = 0.098 and goodness of fit = 1.008 for all observed reflections. Number of refined parameters was 217. Max. shift/esd = 0.00, mean shift/esd = 0.00. Max. and min. peaks in final difference synthesis were 0.606 and -0.676 eÅ^{-3}, respectively.

CCDC 289529 and 289530 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

3.3. Biological assays

3.3.1. Formation of drug–DNA complexes. Stock solutions of each compound (1 mg/mL) in TE 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 and 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.

3.3.2. Circular dichroism (CD) spectroscopy. The CD spectra of the complex-DNA compounds (DNA concentration 20 mg/mL, r_i = 0.1, 0.3 and 0.5) were recorded at room temperature on a JASCO J720 spectropolarimeter with a 450W xenon lamp using a computer for spectral subtraction and noise reduction. Each sample was scanned twice in a range of wavelengths between 220 and 330 nm. The CD spectra drawn are means of three independent scans. The data are expressed as mean residue molecular ellipticity (θ) in ° cm^2/dmol.

3.3.3. Electrophoretic mobility in agarose gel. pBR322 plasmid DNA 0.25 μg/μL was used for the experiments. Four microlitres of charge maker was added to aliquot parts of 20 μL of the adducts complex: DNA previously incubated at 37 °C for 24 h. The mixtures were electrophoretised in agarose gel (1% in TBE buffer) for 5 h at 1.5 V/cm. Afterwards, the DNA was dyed with ethidium bromide solution (0.5 μg/mL in TBE) for 20 min. Samples of DNA and adduct cisplatin:DNA were used as control. The experiment was carried out in an ECOGEN horizontal tank connected to a PHARMACIA GPS 200/400 variable potential power supply.

3.3.4. Atomic force microscopy (TMAFM). DNA pBR322 was heated at 60° for 10 min to obtain OC form. Stock solution was 1 mg/mL in a buffer solution of HEPES. Each sample contained 1 μL DNA

pBR322 of concentration 0.25 μg/μL for a final volume of 40 μL. The amount of drug added is expressed as r_i, and it is calculated with formula:

$$r_i = \frac{m \times \text{Mnucl} \times \text{Am}}{C \times \text{Mr} \times V}$$

Images were obtained with a NANOSCOPE III MULTIMODE AFM, of Digital Instruments Inc. operating in tapping mode.

3.4. Determination of platinum binding to DNA in Culture Cells

Culture plates containing exponentially growing HL-60 cells in RPMI medium (cell density = 3×10^5 cells/mL) were exposed to 5 and 25 μM of the platinum drugs dissolved in RPMI. The plates were incubated for several periods of time (6 and 24 h) under the conditions described above. Following drug incubation, culture medium was removed by centrifugation and the cell pellets were washed with PBS. Subsequently, the cells were lysed with 1 mL of a buffer solution containing 0.15 M Tris–HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 0.1 M NaCl and 0.5% SDS, incubated for 15 min on ice and centrifuged at 12,000 rpm for 15 min. Supernatants were treated for 3 h at 37 °C with 100 μg of proteinase K/mL. Afterward, supernatants were incubated for 16 h at 37 °C with 4 μL RNase A at the concentration of 1 mg/mL. Finally, DNA was extracted with a volume of phenol–chloroform–isoamyl alcohol (50:49:1), precipitated with 2.5 volumes of cold ethanol and 0.1 volumes of 3 M sodium acetate, washed with 70% ethanol, dried and resuspended in 0.5 mL of water. The DNA content in each sample was measured by UV spectrophotometry at 260 nm in a spectrophotometer (Eppendorf BioPhotometer) and platinum bound to DNA was determined by ICP-MS in a Perkin Elmer ELAN 500 Spectrometer. The data were obtained from four independent experiments. Instrumental settings were optimised in order to yield maximum sensitivity for platinum. For quantitative, the most abundant isotopes of platinum and rhodium (used as internal standard) were measured at *m*/*z* 195 and 103, respectively.

All samples were diluted 1:10 with HNO_3 1% and a 2% of rhodium solution (also in HNO_3 1%) was added. A treatment in an ultrasonic bath was done when necessary and finally all samples were analysed for total platinum. Concentration values were corrected with respect to rhodium signal.

3.5. Tumour cell lines and culture conditions

The cell line used in this experiment was the human acute promyelocytic leukaemia cell line HL-60 (American Type Culture Collection (ATCC)). Cells were routinely maintained in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated foetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco-BRL, Invitrogen Corporation, Netherlands) in a highly humidified atmosphere of 95% air with 5% CO_2 at 37 °C.

3.5.1. Cytotoxicity assay. Growth inhibitory effect of platinum complexes on the leukaemia HL-60 cell line was measured by the microculture tetrazolium,[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT] assay.[46] 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. For each of the variants tested, four wells were used. Aliquots of 20 μL of MTT solution were then added to each well. After 3 h, the colour formed was quantitated by a spectrophotometric plate reader 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 (drug concentration at which 50% of the cells are viable relative to the control) were obtained by GraphPad Prism software, version 4.0.

3.5.2. In vitro apoptosis assay. Induction of apoptosis in vitro by platinum compounds was determined by a flow cytometric assay with Annexin V-FITC by using an Annexin V-FITC Apoptosis Detection Kit (Roche).[47] Exponentially growing HL-60 cells in 6-well plates (5×10^5 cells/well) were exposed to concentrations equal to the IC_{50} of the platinum drugs for 24 h. After the cells were subjected to staining with the Annexin V-FITC and propidium iodide, the amount of apoptotic cells was analysed by flow cytometry (BD FACSCalibur).

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References and notes

1. Wang, D.; Lippard, S. *Nat. Rev.* **2005**, *4*, 307–320.
2. Farrell, N.; Ha, T. T. B.; Souchard, J. P.; Wimmer, F. L.; Cros, S.; Johnson, N. P. *J. Med. Chem.* **1989**, *32*, 2240–2241.
3. Natile, G.; Coluccia, M. *Coord. Chem. Rev.* **2001**, *216*, 383–410.
4. Pérez, J. M.; Fuertes, M. A.; Alonso, C.; Navarro-Ranninger, C. *Crit. Rev. Oncol. Hematol.* **2000**, *35*, 109–120.
5. McGowan, G.; Parsons, S.; Sadler, P. J. *Inorg. Chem.* **2005**, *44*, 7459–7467.
6. Boccarelli, A.; Intini, F. P.; Sasanelli, R.; Sivo, M. F.; Coluccia, M.; Natile, G. *J. Med. Chem.* **2006**, *49*, 829–837.
7. Kasparkova, J.; Novakova, O.; Farrell, N.; Brabec, V. *Biochemistry* **2003**, *42*, 792–800.
8. Kasparkova, J.; Novakova, O.; Marini, V.; Najajreh, Y.; Gibson, D.; Pérez, J. M.; Brabec, V. *J. Biol. Chem.* **2003**, *278*, 47516–47525.
9. Eastman, A.; Barry, M. A. *Biochemistry* **1987**, *26*, 3303–3307.

10. Zakovska, A.; Novakova, A.; Balcarova, Z.; Bierbach, U.; Farrell, N.; Brabec, V. *Eur. J. Biochem.* **1998**, *254*, 547–557.
11. Brabec, V.; Neplechova, K.; Kasparkova, J.; Farrell, N. *J. Biol. Inorg. Chem.* **2000**, *5*, 364–368.
12. Bierbach, U.; Qu, V.; Hambley, T. W.; Peroutka, J.; Nguyen, H. L.; Doedee, M.; Farrell, N. *Inorg. Chem.* **1999**, *38*, 3535–3542.
13. Brabec, V.; Kleinwachter, V.; Butour, J. L.; Johnson, N. P. *Biophys. Chem.* **1990**, *35*, 129–141.
14. Holford, J.; Raynaud, F.; Murrer, B. A.; Grimaldi, K.; Hartley, J. A.; Abrams, M.; Kelland, L. R. *Anti-Cancer Drug Des.* **1998**, *13*, 1–18.
15. McGowan, G.; Parsons, S.; Sadler, P. J. *Chem. Eur. J.* **2005**, *11*, 4396–4404.
16. Chen, Y.; Guo, Z.; Parson, S.; Sadler, P. J. *Chem. Eur. J.* **1998**, *4*, 672–676.
17. Kai Yan, Y.; Melchart, M.; Habtemarian, A.; Sadler, P. J. *Chem. Commun.* **2005**, 4764–4776.
18. Fernández, R.; Melchart, M.; Habtermariam, A.; Parsons, S.; Sadler, P. J. *Chem. Eur. J.* **2004**, *10*, 5173–5179.
19. Peacock, A. F. A.; Habtermariam, A.; Fernández, R.; Walland, V.; Fabbiani, F. P. A.; Parsons, S.; Aird, R. E.; Jodrell, D. I.; Sadler, P. J. *J. Am. Chem. Soc.* **2006**, *128*, 1739.
20. Ramos-Lima, F. J.; Quiroga, A. G.; Pérez, J. M.; Navarro-Ranninger, C. *Polyhedron* **2003**, *22*, 3379–3381.
21. Martínez, A.; Lorenzo, J.; Prieto, M. J.; Llorens, R.; Font-Bardía, M.; Solans, X.; Avilés, F. X.; Moreno, V. *ChemBioChem* **2005**, *6*, 1–11.
22. Stehlikova, K.; Kasparkova, J.; Novakova, O.; Martínez, A.; Moreno, V.; Brabec, V. *The FEBS J.* **2006**, *273*, 301–314.
23. Yamauchi, O. *Pure Appl. Chem.* **1995**, *67*, 297–304.
24. Drougge, L.; Elding, L. *Inorg. Chim. Acta* **1986**, *121*, 175–183.
25. *The Biochemistry of the Nucleic Acids*, 11th ed. (Eds.: R.L.P. Adams, J. T. Knowler, D. P. Leadre), Chapman Hall, London, UK, **1992**.
26. Johnson, A.; Qu, Y.; Van Hounten, B.; Farrell, N. *Nucleic Acids Res.* **1992**, *20*, 1697–1703.
27. Johnson, W. C.; Itzwotz, M. S.; Tinoco, Y. *Biopolymers* **1972**, *11*, 225–234.
28. Ghezzi, A. R.; Aceto, M.; Casino, C.; Gabano, E.; Osella, D. *J. Inorg. Biochem.* **2004**, *98*, 73–78.
29. Janovska, E.; Novakova, O.; Natile, G.; Brabec, V. *J. Inorg. Biochem.* **2002**, *90*, 155–158.
30. Fuertes, M. A.; Alonso, C.; Pérez, J. M. *Chem. Rev.* **2003**, *103*, 645–662.
31. Hollis, L. S.; Sunquist, W. I.; Surstyn, J. N.; Heiger-Bernays, W. J.; Bellon, S. F.; Ahmed, K. J.; Amundsen, A. R.; Stern, E. W.; Lippard, S. J. *Cancer Res.* **1991**, *51*, 1866–1875.
32. Cini, R.; Caputo, P. A.; Intini, F. P.; Natile, G. *Inorg. Chem.* **1995**, *34*, 1130–1137.
33. Casas, J. M.; Chisholm, M. H.; Sicilia, M. V.; Streib, W. E. *Polyhedron* **1991**, *10*, 1573–1578.
34. Coluccia, M.; Boccarelli, A.; Mariggiò, M. A.; Cardellicchio, N.; Caputo, P.; Intini, F. P.; Natile, G. *Chem.-Biol. Interact.* **1995**, *98*, 251–266.
35. Fanizzi, F.; Intini, F. P.; Natile, G. *J. Chem. Soc. Dalton Trans.* **1989**, 947–951.
36. Coluccia, M.; Nassi, A.; Loseto, F.; Boccarelli, A.; Mariggio, M. A.; Giordano, D.; Intini, F. P.; Caputo, P.; Natile, G. *J. Med. Chem.* **1993**, *36*, 510–512.
37. Leng, M.; Locker, D.; Giraud-Panis, M. J.; Schwartz, A.; Intini, F. P.; Natile, G.; Pisano, C.; Boccarelli, A.; Giordano, D.; Coluccia, M. *Mol. Pharmacol.* **2000**, *58*, 1525–1535.
38. Brabec, V.; Kasparkova, J. *Drug Resistance Updates* **2002**, *5*, 147–161.
39. Keck, M. V.; Lippard, S. J. *J. Am. Chem. Soc.* **1992**, *114*, 3386–3390.
40. Kasparkova, J.; Marini, V.; Najajreh, Y.; Gibson, D.; Brabec, V. *Biochemistry* **2003**, *42*, 6321–6332.
41. Zakovska, A.; Novakova, O.; Balcarova, Z.; Bierbach, U.; Farrell, N.; Brabec, V. *Eur. J. Biochem.* **1998**, *254*, 547–557.
42. Novakova, O.; Kasparkova, J.; Bursova, V.; Hofr, C.; Vojtiskova, M.; Chen, H.; Sadler, P. J.; Brabec, V. *Chem. Biol.* **2005**, *12*, 121–129.
43. Reedijk, J. *Chem. Rev.* **1999**, *99*, 2499–2510.
44. Sheldrick, G. M. *A Computer Program for Determination of Crystal Structure*; University of Göttingen: Germany, 1977.
45. *International Tables of X-Ray Crystallography*, Vol. IV, Kynoch, Birmingham, 1974, pp. 99–100 and 149.
46. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.
47. Vermes, I.; Haanen, C.; Steffens-Nakken, H.; Reutelingsperger, C. *J. Immunol. Methods* **1995**, *184*, 39–51.