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Pd(II) and Pt(II) Complexes of 2-Phenyl- and 2-Benzyl-imidazoline: Synthesis, Structural Characterization, DNA Modification and *in vitro* Antileukaemic Activity

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In this paper we describe the synthesis and chemical characterization of three new Pd(II)–imidazoline complexes: [$PdCl_2$ (C_6H_5–CH_2–$C_3H_5N_2$)$_2$] (2), [PdCl(SEt_2) (C_6H_4-$C_3H_5N_2$)] (5) and [Pd(C_6H_4-$C_3H_5N_2$) (μ-Br)]$_2$ (6). We have also analyzed the DNA modifications and *in vitro* antileukaemic activity of these compounds and of their previously reported analogs [Pd Cl_2 (C_6H_5–$C_3H_5N_2$)$_2$] (1), [Pd (C_6H_4–$C_3H_5N_2$) (μ-OAc)]$_2$ (3), [Pd (C_6H_4–$C_3H_5N_2$) (μ-Cl)]$_2$ (4) and [Pt(C_6H_4–$C_3H_5N_2$)(μ-Cl] (7). All these compounds modify the DNA secondary structure since they alter the melting temperature (T_m) of the DNA. Circular dichroism spectra indicated, moreover, that compounds 3, 5 and 6 induced higher modification on the double helix than compounds 1, 2 and 4. While compounds 1, 2 and 5 seem to induce slight changes in the electrophoretic mobility of the open and covalently closed circular forms of pUC8 DNA at high r_i (input molar ratio of Pd or Pt to nucleotides), compounds 3, 6 and 7 do not modify at any r_i the tertiary structure of the plasmid DNA. Antileukaemic tests suggest that compounds 1, 4 and 7 exhibit important cytotoxic activity since their IC_{50} values against HL-60 human leukaemic cells were below 10 μg ml^{-1}.

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INTRODUCTION

The antitumour activity of the therapeutic drug *cis*-DDP has been attributed to binding to DNA forming bifunctional lesions, mainly by cross-linking of two adjacent guanines in the same DNA strand.[1] For structural reasons this kind of lesion cannot occur when the *trans*-DDP isomer is used. The classical structure–activity relationships established for platinum antitumor agents have been violated in recent years by some new classes of complexes.[2] Thus, it has been observed that [$Pt(NH_3)_2AmCl]^+$ (where Am is an heterocyclic amine based on pyridine, pyrimidine, purine or piperidine substituents)[3] and platinum complexes containing other intercalating ligands such as anthraquinones[4] or ethidium[5] not only induce monofunctional or intercalative binding but also have interesting antitumor activity. It has been reported that *trans*-platinum complexes with heterocycles such as pyridine, *N*-methylimidazole and quinoline have cytotoxic activity.[6] It has been observed, moreover, that some ortho-palladated complexes may originate intercalative or monofunctional covalent interactions on DNA[7] and that pyridine and quinoline cyclo-

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palladated complexes show cytotoxic effects against tumor cells.[8]

Previous studies from our laboratory using benzoylbenzylidenamine drugs showed that they bind to DNA in a different way from *cis*-DDP and that they have remarkable *in vitro* antitumor activity.[9,10] Thus these studies indicate that suitable cyclometallated complexes (with planar structures), and some coordination complexes containing planar ligands, may induce intercalative lesions on DNA with cytotoxic effects. Based on this rationale we report in the present paper: (1) a study of the DNA interaction of palladium *trans* complexes containing planar ligands and cyclometallated derivatives having three fused rings; and (2) the *in vitro* antileukemic activity of these complexes. In addition, we compare the influence of the metallic center between several cyclometallated complexes as well as that of different kinds of leaving ligands. We have selected the planar 2-phenylimidazoline (Imd) and the 2-benzylimidazoline (Bimd) ligands for their analogy with the DNA nucleobases.

EXPERIMENTAL

The solvents were purified and dried by standard methods.[11] Palladium chloride was purchased from Johnson-Matthey and palladium(II) acetate, 2-phenylimidazoline and 2-benzylimidazoline from Aldrich Chemie. Complexes **1**, **3**, **4** and **7** were prepared as previously described.[12]

Elemental analyses were carried out on a Perkin Elmer elemental analyzer 240B. IR spectra in the 4000–200 cm^{-1} region were recorded as polyethylene and KBr pellets on a Perkin-Elmer 1650 spectrophotometer. NMR spectra were obtained in $CDCl_3$ (TMS as internal reference) and DMSO-d_6 (undeuterated residual DMSO as reference), and were recorded on Bruker AC-200 and AMX-300 spectrometers. NMR spectra were assigned by chemical shift, in ppm, and were assisted with HMQC[13–15] (^{1}H-detected heteronuclear multiple-quantum coherence), HMBC[16] (heteronuclear multiple-bond connectivity) and COSY-45.

Chemical syntheses

Synthesis of [$PdCl_2(C_6H_5–CH_2–C_3H_5N_2)_2$] (2)
To a solution of Li_2PdCl_4 prepared *in situ* from $PdCl_2$ (1 mmol) and LiCl (2 mmol) in 10 ml of methanol, a solution of Bimd (1 mmol) in 5 ml of methanol was added. After stirring for three days at 20 °C, an orange precipitate was formed, filtered off, washed with methanol and dried in vacuum (yield 66.2%). Calcd for $PdCl_2C_{20}H_{24}N_4$: C, 48.26; H, 4.86; N, 11.25. Found: C, 48.18; H, 4.83; N, 11.27%.

Synthesis of [$PdCl(SEt_2)(C_6H_4–C_3H_5N_2)$] (5)
SEt_2 (2 mmol) was added to a dichloromethane suspension of complex **4** (1 mmol). The yellow solution formed immediately was filtered off. Addition of diethyl ether resulted in a precipitate which was filtered off and washed with diethyl ether and dried in vacuum (yield 52.1%). Calcd for $PdClC_{13}H_{19}N_2S$: C, 41.36; H, 5.08; N, 7.43. Found: C, 41.25; H, 4.99; N, 7.42%.

Synthesis of [$Pd(C_6(H_4–C_3H_5N_2)(\mu\text{-}Br)]_2$ (6)
To a stirred solution of complex **3** (0.5 mmol) in acetone (10 ml), an excess of a solution of lithium bromide in water (10^{-2} mol l^{-1}) was added. After stirring for 24 h, the precipitate formed was filtered off, washed with water and acetone and dried in vacuum (yield 59.0%). Calcd for $Pd_2Br_2C_{18}H_{18}N_4$: C, 37.62; H, 3.14; N, 9.75. Found: C, 37.52; H, 3.10; N, 9.68%.

Biological assays

DNA samples
pUC8 DNA was isolated from the JM83 strain of *E. coli* following the alkaline lysis method.[17] Scanning of pUC8 samples after gel electrophoresis indicated that 80% of plasmid DNA was in the supercoiled form (ccc) and 20% was in the open circular form (oc). Calf thymus DNA (CT DNA) was purchased from Sigma Biochemicals.

Formation of drug-DNA complexes
Compounds **1**–**7**, Imd and Bimd were dissolved in 5% DMSO (dimethyl sulfoxide) in water. Stock solutions of the compounds (1 mg ml^{-1}) were prepared immediately before use. Aliquots of the compounds were added to the DNA in TE buffer (10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA). The amount of each compound added to the DNA solution was expressed as r_i (input molar ratio of Pd or Pt to nucleotides).

Melting assays
T_m (melting temperature) in drug–DNA complexes (DNA concentration = 20 μg ml^{-1};

$r_i = 0.05$, 0.1 and 0.25) was determined by differential spectrophotometry in a Beckman Acta cIII spectrophotometer attached to a temperature programmer. The temperature of the drug–DNA complexes was increased from 37 °C to 95 °C at a rate of 1 °C min^{-1}. The maximum value of hyperchromicity in control CT DNA was 33%. The T_m values represent the mean value obtained from three independent experiments.

Circular dichroism studies

Circular dichroism (CD) spectra of drug–CT DNA complexes (DNA concentration = 20 μg ml^{-1}; $r_i = 0.05$, 0.1 and 0.25) were run at 37 °C on a JASCO J-600 spectropolarimeter. The data were expressed as mean residue molecular ellipticity (Θ) in units of deg cm^2 $dmol^{-1} \times 10^3$. The CD spectrum of the drug alone was subtracted from that of the drug–CT DNA complexes by computer software. Each CD spectrum represents the mean of three runs.

Electrophoretic data

DNA aliquots of pUC8 plasmid DNA (50 μg ml^{-1}) were incubated with the drugs ($r_i = 0.05$, 0.1 and 0.25) in TE buffer, pH 7.4, during 24 h at 37 °C. Aliquots (20 μl) of drug–DNA complexes containing 1 μg of DNA were subjected to 1.5% agarose gel electrophoresis for 16 h at 1 V cm^{-1} in 40 mM Tris–acetate and 2 mM EDTA (pH 8.0) buffer. Gels were stained in an aqueous solution of ethidium bromide (0.5 mg ml^{-1}) and photographed with an MP-4 Polaroid camera on a 665 polaroid film using an orange filter.

Antileukemic tests

HL-60 human leukemic cells were cultured in RPMI 1640 medium free of serum and supplemented with 1% antibiotic–antimycotic solution. The replication period of HL-60 cells was 16 h when cultured in this medium, under an atmosphere of 95% air and 5% CO_2, reaching logarithmic growth at 24 h. In order to calculate the IC_{50} (drug concentration that inhibits 50% of cell growth) and LC_{50} (drug concentration that produces 50% of cell killing), 100 μl of cell suspension (2.5×10^5 cells ml^{-1}) were added to 95-well microtiter plates. Cell suspensions were exposed to every compound at concentrations ranging from 0 to 100 μg ml^{-1}. After an incubation period of 48 h cell density was determined both in the controls and in the drug-treated cultures using an MTT colorimetric method.[18] Absorbance in the wells was measured at 540 nm in a Dynatech MR 5000 autoreader. IC_{50} and LC_{50} values were determined from survival curves. All experiments were performed in triplicate.

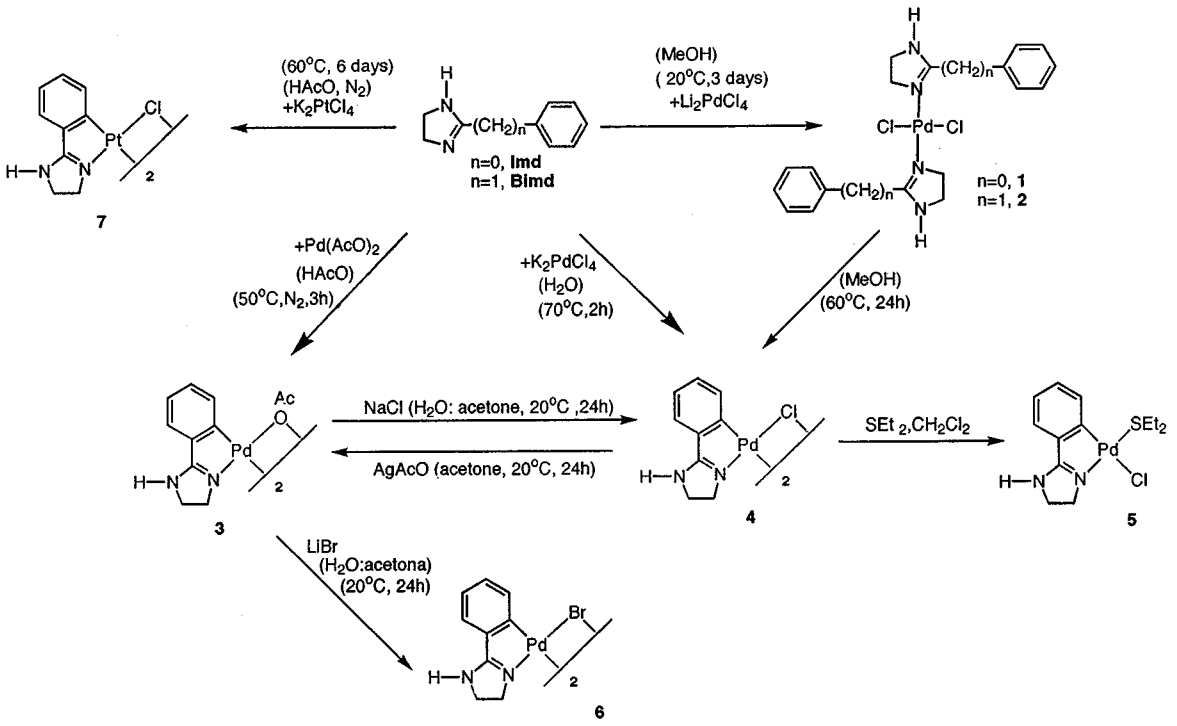
RESULTS AND DISCUSSION

Synthesis and characterization of the compounds

A schematic representation of the routes used for the synthesis of complexes **1**–**7** is given in Scheme 1. The microanalytical data for all the complexes are consistent with the proposed formulae. When the reaction between Li_2PdCl_4 and Imd or Bimd was carried out in methanol under mild conditions, *trans*-palladium(II) coordination complexes were obtained. The Imd ligand undergoes palladation with $Pd(OAc)_2$, Li_2PdCl_4 and K_2PdCl_4 under the conditions summarized in Scheme 1, giving rise to the formation of the cyclopalladated complexes desired. However, cycloplatination of Imd can be obtained only from K_2PtCl_4 in acetic acid.[12] The Bimd ligand could not be metallated, probably due to the low stability of the cyclometallated six-membered ring.

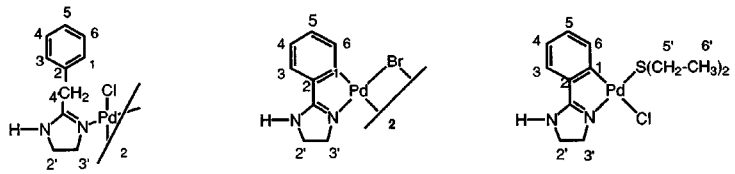
The IR spectrum of complex **2** shows two bands, at 355 and 327 cm^{-1}, assignable to ν_{as}(Pd–Cl) and ν_{as}(Pd–N) stretching vibrations, respectively.[19–21] Thus, this complex has a *trans* disposition analogous to that shown in complex **1**.[12] The ^1H and ^{13}C NMR assignations for the ligands and the complexes are given in Tables 1 and 2. The ^1H and ^{13}C NMR spectra of complex 2 show small variations in relation to those of complex **1**.[12] It is also observed that, similarly to complex **1**,[12] H2′ and H3′, and C2′ and C3′, are not chemically equivalent when coordination to palladium is produced in complex **2**. The signals assigned to H2′ and H3′ appear as an A_2B_2 system and as two triplets in **1** and **2**, respectively. The chemically equivalent carbons C2′ and C3′ observed in the ligands appear as two different signals in complexes **1** and **2**.

The bromo-bridged complex **6** was synthesized since it would allow us to study differences in DNA complexation with different leaving ligands and because bromo-bridged complexes are usually more soluble than chloro-bridged ones having, however, similar structures. The ^1H NMR spectrum shows (Table 1) variations in



Scheme 1. Synthetic routes.

Table 1. ^{1}H NMR Data (ppm) for Imd, Bimd and palladium complexes[a]



2 **5** **6**

	Imd	Bimd	**2**	**5**	**6**
H1	7.83, m	7.27, m	7.57, dd(1.3,7.8)		
H3	7.83, m	7.27, m	7.57, dd(1.3,7.8)	7.21, dd(1.6,7.4)	7.39, m
H4	7.45, m	7.22, m	7.25, m	7.02, dt (1.0,7.5)	7.16, m
H5	7.45, m	7.22, m	7.25, m	7.12, dt (1.6,7.4)	7.16, m
H6	7.45, m	7.22, m	7.25, m	7.48, dd (1.0,7.5)	8.03, d br (7.1)
H2′	3.60, s	3.38, s	3.34, t (10.3)	4.05, t (10.1)	3.78, m
H3′	3.60, s	3.38, s	3.64, t (10.3)	3.72, t (10.1)	3.78, m
H4′		3.42, s	4.09, s		
H5′				3.05, s br	
H6′				1.41, t (7.5)	
NH	n.o.[b]	5.91, s br	7.50, s br	5.50, s br	8.66, s br

[a] The numbers in parentheses correspond to $J(^1H–^1H)$ in Hz. Solvent, DMSO-d_6.
[b] n.o., not observed.

Table 2. ^{13}C NMR data (ppm) for Imd, Bimd and palladium complexes[a]

	Imd	Bimd	**2**	**5**	**6**
C1	127.2	126.4	129.3	151.2	153.4
C2	130.7	137.4	135.5	135.8	135.4
C3	127.2	126.4	129.3	125.0	125.3
C4	128.3	128.3	128.4	124.1	124.0
C5	130.3	128.9	128.4	130.5	130.5
C6	128.3	128.3	128.4	132.8	131.8
C1′	164.0	165.9	168.1	174.0	174.3
C2′	49.7	49.5	43.1	44.7	44.3
C3′	49.7	49.5	53.8	52.0	53.0
C4′		35.4	35.5		
C5′				31.1	
C6′				13.5	

[a] For carbon numbering, see Table 1. Solvent, DMSO-d_6.

both signal multiplicities and chemical shifts in relation to the Imd ligand. H4 protons decrease their chemical shift as these protons are unaffected by steric interactions, indicating Pd–C back-bonding.[22] The variations observed in H3 and H5, *meta* to the Pd–C bond, must be less affected by orthopalladation. However, H3 and H5 show significant variations which could be attributed to orthopalladation and to changes in the ligand conformation.[23] The ^{1}H NMR spectrum of complex **6** is similar to that of complex **4**. Thus, they should have planar structures. The ^{13}C NMR spectrum of complex **6** (Table 2) shows two signals, at 44.3 and 53.0 ppm, assignable to C2′ and C3′, respectively. These signals indicate a Pd–N coordination, and the existence of chemical shift variations analogous to those observed in the acetate- and chloro-bridged complexes **3** and **4** confirms cyclopalladation.[12]

Since it has been shown that platinum complexes can undergo displacement of the S-bound molecules due to the guanosine residue,[24] we thought that the diethyl sulfide monomeric compound could be a complex that would not afford reaction with S-proteins and would be more soluble than dimeric complexes. The ^{1}H NMR spectrum of complex **5** shows (Table 1) unique signals for each proton of the corresponding ligands. This complex should show, therefore, the SEt_2 *trans* to the nitrogen atom by the higher *trans* effect of the carbon atom. The ^{1}H NMR spectrum of complex **5** is similar to the chloro- and bromo-bridged spectrum. The main differences between these complexes, corresponding to the deshielding value of H6, are probably due to electronic effects induced by the different ligands.[23] The ^{13}C NMR spectrum of complex **5** is analogous to that observed for complex **6**.

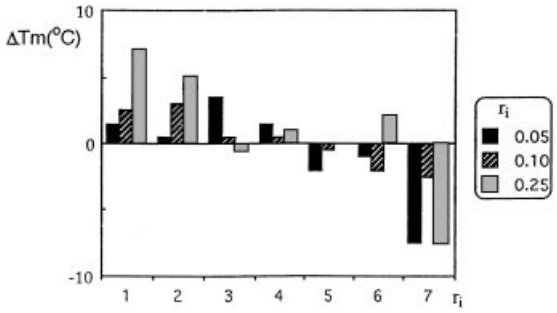


Figure 1 ΔT_m values of CT DNA incubated during 24 h at 37 °C with compounds **1**–**7** at $r_i = 0.05$, 0.1 and 0.25.

T_m modification in drug–DNA complexes

Figure 1 shows the changes in T_m of the CT DNA incubated during 24 h at 37 °C with compounds **1**–**7** ($r_i = 0.05$, 0.1 and 0.25). It may be observed that as the r_i increases, compounds **1** and **2** induce an increase in the T_m of CT DNA. At $r_i = 0.25$ the T_m of compounds **1**–DNA and compound **2**–DNA complexes is 7 °C and 5.5 °C higher than that of control CT DNA, respectively. It seems, thus, that compounds **1** and **2** compact the DNA double helix and that since they have a *trans*-Pd(II) center it is most likely that both drugs produce interstrand crosslinks on the DNA.

On the other hand, compound **3** increases the T_m of CT DNA at $r_i = 0.05$ ($\Delta T_m = 4$ °C). At $r_i = 0.1$ and 0.25 the T_m value does not vary significantly relative to control CT DNA. Considering the structure of compound **3**, it is likely that at low r_i the compound preferentially intercalates within the double helix, producing an increase in base-stacking, while at high r_i the compound preferentially binds monofunctionally to DNA through the Pd center, leading to a decrease in T_m relative to $r_i = 0.05$. Compound **4** does not significantly affect the thermal stability of DNA although it produces a slight increase in T_m (between 0.5 °C and 1.5 °C depending on the r_i tested) probably due to a predominantly intercalative mode of binding to DNA.

Compound **5** produces a decrease of 2 °C in the T_m of CT DNA at $r_i = 0.05$ and of 0.5 °C at $r_i = 0.1$. At $r_i = 0.25$, the T_m of the compound

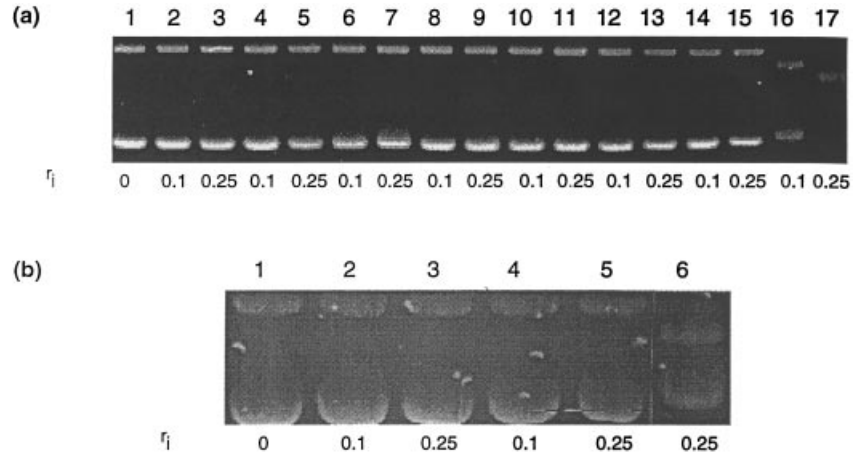


Figure 2 Changes in electrophoretic mobility of pUC8 plasmid DNA after incubation with ligands and complexes **1**–**7**: (a) lane 1, pUC8 DNA; lanes 2 and 3, Imd; lanes 4 and 5, complex **1**; lanes 6 and 7, complex **4**; lanes 8 and 9, complex **3**; lanes 10 and 11, complex **7**; lanes 12 and 13, complex **6**; lanes 14 and 15, complex **5**; lanes 16 and 17, *cis*-DDP. (b) lane 1, pUC8 DNA; lanes 2 and 3, Bimd; lanes 4 and 5, complex **2**; lane 6: *cis*-DDP.

5–CT DNA complex is similar to that of control CT DNA. Thus, it is likely that compound **5** compacts DNA at low r_i by means of an intercalative DNA binding mode, while at higher r_i it may also form monofunctional adducts on DNA through the metallic center. Compound **6** decreases the T_m value of CT DNA at $r_i = 0.05$ and 0.1 ($\Delta T_m = -1$ °C and -2 °C, respectively). In contrast, at $r_i = 0.25$ the compound induces an increase in the T_m value ($\Delta T_m = 2$ °C). As indicated for compounds **3** and **5**, it seems that compound **6** also has different modes of DNA binding. At low r_i, compound **6** may bind preferentially to DNA through the Pd(II) center, forming monofunctional adducts, while at high r_i it may also intercalate within the double helix. Compound **7** induces a slight decrease in T_m at all r_i tested, suggesting that the drug preferentially binds to DNA through the Pt(II) center forming monofunctional adducts.

CD spectra of drug–DNA complexes

Table 3 shows the maximum and minimum values of ellipticity, and the wavelengths at which these values are located in native CT DNA and in CT DNA incubated during 24 h at 37 °C with the cyclometallated compounds and their ligands ($r_i = 0.05$, 0.1 and 0.25). It may be noted that when r_i increases, the synthesized compounds induce a decrease in both the maximum value of the ellipticity of the positive band and the minimum value of the ellipticity of the negative band as well as in the area under those bands. In contrast, ligands Imd and Bimd do not seem to alter significantly the CD spectrum of CT DNA. The largest modifications in the ellipticity of the positive band are induced by compounds **3** and **6**. In compound **6**–DNA and compound **3**–DNA complexes at $r_i = 0.25$, the maximum value of ellipticity of the positive band decreases from 6.11Θ units at 277 nm in native CT DNA to 2.65Θ units at 280 nm, and to 1.90Θ units at 288 nm, respectively. The largest modifications in the ellipticity of the negative band are induced by compounds **3** and **5**. In the DNA complexes formed with compounds **3** and **5** at $r_i = 0.25$, the minimum value of ellipticity of the negative band decreases from -10.52Θ units at 243 nm in native CT DNA to -5.31Θ units at 244 nm in compound **5**–CT DNA complexes and to -1.44 units at 247 nm in compound **3**–CT DNA complexes. The CD data suggest that the binding of the synthesized compounds to DNA induces some opening and rotation of the stacked bases since the conservative nature of the CD spectrum was not altered and because there was a displacement of the CD curve towards higher wavelengths.[25]

Electrophoretic behaviour of drug–DNA complexes

Figure 2 shows the electrophoretic mobility of the ccc and oc forms of pUC8 plasmid DNA after incubation with the complexes, ligands and

cis-DDP at $r_i = 0.1$ and 0.25. It was observed that compounds **1**, **4** and **5** do not alter the mobility of the oc form while at high r_i they induce a slight decrease in the mobility of the ccc forms smaller than that induced by *cis*-DDP at $r_i = 0.1$. The rest of the compounds and of the Imd and Bimd ligands do not alter the mobility of the pUC8 forms at either $r_i = 0.1$ or $r_i = 0.25$, as an indication that these compounds do not modify the DNA tertiary structure.

Antileukemic activity

The IC_{50} and LC_{50} values (in $\mu g\,ml^{-1}$ ml and μM) obtained for the synthesized complexes, their ligands and *cis*-DDP against HL-60 human leukemic cells are shown in Table 4. Since compounds **1**, **4** and **7** have IC_{50} values lower than 10 $\mu g\,ml^{-1}$, it is likely that they exhibit potentially useful antiproliferative activity.[26] It is interesting to note that at a concentration of 10 $\mu g\,ml^{-1}$ the cytotoxic activity of these compounds is similar to that of *cis*-DDP (80% of cell killing). The cytotoxic activity of Imd is substantially increased when it is coordinated to a metal center (Pd or Pt). Thus, compounds **1**, **4** and **7** may be regarded as potential antitumour agents since their antiproliferative and cytotoxic activities are close to those of the therapeutic drug *cis*-DDP and they are, moreover, highly soluble.

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Table 3. CD of ligands and complexes **1**–**7**

Compound	r_i	Θ_{max} (deg cm^2 $dmol^{-1} \times 10^3$)	$\lambda\Theta_{max}$ (nm)	Θ_{min} (deg cm^{-2} $dmol^{-1} \times 10^3$)	$\lambda\Theta_{min}$ (nm)
	CT DNA	6.11	277	−10.52	243
Imd	0.05	6.79	279	−10.24	244
	0.10	6.93	277	−11.40	244
	0.25	6.69	275	−10.49	244
Bimd	0.05	6.52	277	−10.11	244
	0.10	5.86	277	−9.35	244
	0.25	6.62	276	−9.39	244
1	0.05	6.79	280	−8.62	242
	0.10	6.93	281	−9.27	244
	0.25	3.75	280	−7.41	243
2	0.05	5.78	278	−9.57	244
	0.10	5.14	278	−9.21	244
	0.25	3.41	283	−7.41	244
3	0.05	4.18	275	−6.27	243
	0.10	3.51	278	−5.51	247
	0.25	1.90	288	−1.45	247
4	0.05	5.30	279	−8.96	244
	0.10	4.90	280	−8.82	244
	0.25	3.38	282	−8.93	244
5	0.05	6.77	278	−9.08	245
	0.10	5.86	278	−8.06	246
	0.25	4.60	280	−5.31	244
6	0.05	4.21	280	−9.64	246
	0.10	4.10	280	−9.35	245
	0.25	2.65	281	−8.08	245
7	0.05	6.96	274	−11.12	245
	0.10	7.24	274	−11.10	243
	0.25	6.89	274	−10.64	246

Table 4. IC_{50} and LC_{50} values obtained for Imd and complexes **1**, **4** and **7** against HL-60 (2×10^4 cells ml^{-1}) during 72 h

Compound	LC_{50} (μg ml^{-1})	IC_{50} (μg ml^{-1})	LC_{50} (μM)	IC_{50} (μM)
cis-DDP	0.85	0.18	2.57	0.54
Imd	8.0	2.3	54.7	15.7
1	3.5	1.5	7.4	3.1
4	2.3	0.6	4	1.0
7	4.2	2.0	5.6	2.6

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