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DNA Binding and *In Vitro* Antileukemic Activity of Dimeric and Tetrameric Platinated Complexes Derived from *p*-Isopropylbenzaldehyde Thiosemicarbazone

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***p*-Isopropylbenzaldehyde thiosemicarbazone (*p*-is.TSCN) (1) reacts with $[Pt(\mu\text{-}Cl)(\eta^3\text{-}C_4H_7)]_2$ to form a dinuclear $[Pt(\mu\text{-}Cl)(p\text{-is.TSCN})]_2$ complex (2) and a cyclometallated cluster $[Pt(p\text{-is.TSCN})]_4$ (3). Biological testing of these complexes against HL-60 and U-937 human leukemic cells suggest that complexes 2 and 3 may be endowed with important cytotoxic activity properties since they exhibit IC_{50} values (50% inhibition of cell growth) in the micromolar range, as does the clinically used drug cisplatin (*cis*-DDP). Analysis of the interaction of compounds 2 and 3 with DNA indicates that the kinetics of DNA platination due to compounds 2 and 3 is faster than that of cisplatin and that after 24 h of incubation most of the platinum centers are bound to DNA. Thus, it is likely that the cytotoxic activity displayed by compounds 2 and 3 may be correlated with their high level of DNA platination.** © **1998 John Wiley & Sons, Ltd.**

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INTRODUCTION

Interest in the coordination chemistry of TSCNs (thiosemicarbazones) has increased in recent years since it has been shown that their biological activity is related to their parent aldehyde or ketone functions and to the ability of TSCNs to form complexes with metals.[1,2] A large number of studies describing metal complexes of these compounds have been published in the last few years. These studies have demonstrated that TSCN can act as *S*-monodentate,[3,4] as chelate,[5,6] or even as tridentate *NNS*-ligands[7–15] or *ONS*-ligands.[16] Moreover, we have recently reported that TSCN molecules may act as tridentate *CNS*-ligands in cyclometallated complexes.[17]

It is well known that several factors influence the antitumor activity of metal complexes,[18,19] among which are (i) the nature of the ligand, which may modify the electrophilic properties of the metal; (ii) the stereochemistry of the complex;[20] and (iii) the nature of the leaving group.[21] Thus, as we have previously shown, some cyclometallated complexes can exhibit cytotoxic activity.[17,22] Taking into account the results obtained with two platinum complexes of *p*-isopropylbenzaldehyde thiosemicarbazone, namely the dimeric compound $[Pt(\mu\text{-}Cl)(p\text{-is.TSCN})]_2$ (complex **2**) and its tetrameric cyclometallated derivative $[Pt(p\text{-is.TSCN})]_4$ (complex **3**),[17,22] we have extended our studies with these complexes. Both compounds exhibit important antileukemic activity *in vitro* since they show IC_{50} values in the micro-molar range similar to those of the antitumor drug cisplatin, *cis*-diamminedichloroplatinum(II). Analysis of the interaction of compounds **2** and **3** with DNA indicates that

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platinum binding to DNA follows fast kinetics and that DNA platination is accomplished after 24 h of incubation. Moreover, these compounds do not alter the electrophoretic mobility of plasmid DNA, supporting previously reported data that indicated that compounds **2** and **3** form DNA interhelical crosslinks.[23,24]

EXPERIMENTAL

Materials

Standard methods were used for the preparation of the *p*-is.TSCN[25] ligand and [Pt(μ-Cl)(η^3-C_4H_7)]$_2$.[26] All the solvents were purified prior to use, by standard methods.[27]

Although the synthesis of compounds [Pt(μ-Cl)(*p*-is.TSCN)]$_2$ (**2**) and [Pt(*p*-is.TSCN)]$_4$ (**3**) by reaction of K_2PtCl_4 with *p*-is.TSCN has been previously reported,[24] complex **3** may be prepared by the following alternative method. To a solution of *p*-is.TSCN (0.12 g, 0.52 mmol) in 4 ml of acetone, [Pt(μ-Cl)(η^3-C_4H_7)]$_2$ (0.15 g, 0.26 mmol) was added and stirred at reflux for three days. An unidentified pale-gray solid was collected by filtration. The solvent was removed from the solution filtrate by rotatory evaporation. The residue was dispersed in Celite and purified by chromatography on a silica-gel column. The products were eluted stepwise in the following order: the starting materials, the dinuclear complex (CH_2Cl_2) **2** (5%) and finally the tetranuclear complex **3** (40%) (CH_2Cl_2/EtOH, 100:1). Analysis: Calcd. ($C_{44}H_{52}N_{12}S_4Pt_4$): C, 31.88; H, 3.16; N, 10.14; S, 7.74. Found: C, 31.67; H, 3.30; N, 9.95; S, 7.52%. IR: ν_{max} 3433, 3373, 1611, 1583, 700 cm^{-1}. FAB MS: 1657.7 (M^+), 1243.4 ($M^+ - PtC_{11}H_{13}N_3S$), 1015.9 ($M^+ - PtC_{11}H_{13}N_3S - PtS$).

Cell lines and culture conditions

HL-60 (human promyelocytic leukemia line) and U-937 (human promonocytic leukemia line) were cultured in RPMI (Rosenthal Park Memorial Institute) 1640 medium supplemented with 10% FCS (fetal calf serum), 2 mM glutamine, 100 units ml^{-1} penicillin and 100 mg ml^{-1} streptomycin at 37°C in an atmosphere of 5% CO_2. HL-60 and U-937 leukemic cells were passaged three times per week showing a doubling time between 16 and 24 h depending on the cell line.

Cytotoxicity of the drugs

Cell proliferation was evaluated by a system based on the tetrazolium compound MTT which is reduced by living cells to yield a soluble formazan product that can be assayed colorimetrically.[28] The cells were plated in 96-well sterile plates at a density of 10^4 cells/well in 100 μl of medium and incubated for 3–4 h. The compounds were added to final concentrations ranging from 0 to 100 μM in a volume of 100 μl/well. After an incubation period of 20 h, 50 μl of a freshly diluted MTT solution (1:5 in culture medium) was added at a concentration of 1 mg ml^{-1} to each well. Then the plates were incubated for 5 h at 37°C in a humidified 5% CO_2 atmosphere. Cell survival was evaluated by measuring the absorbance at 520 nm, using a Whittaker Microplate Reader 2001. The IC_{50} values were calculated from curves constructed by plotting cell survival (%) versus compound concentration (μM). All experiments were done in quadruplicate.

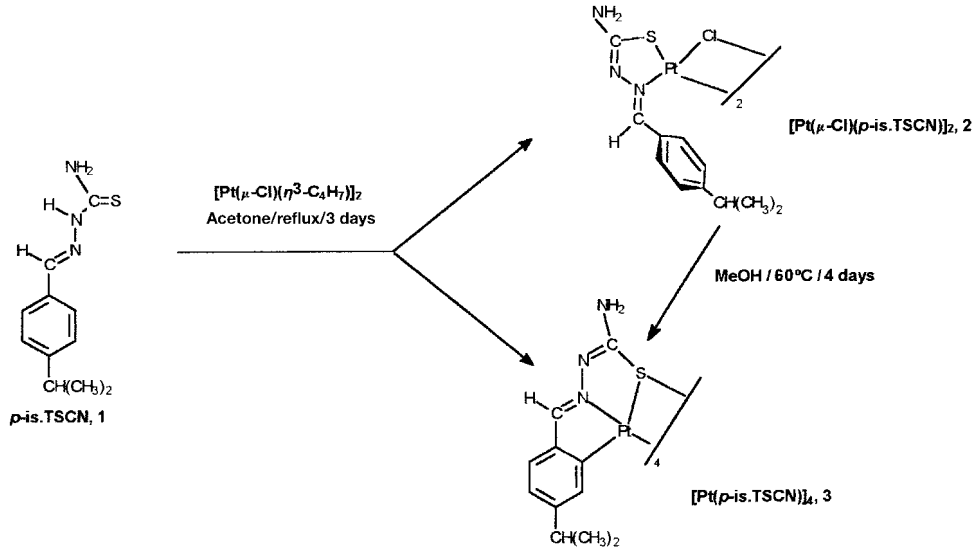
Formation of drug–DNA complexes

cis-DDP [*cis*-diamminedichloroplatinum(II)] was dissolved in PBS (phosphate-buffered saline)and compounds **2** and **3** were disolved in ethanol. Stock solutions (1mg ml^{-1}) were freshly prepared before use.

The drug–DNA complexes were formed by addition to CT DNA or PBR322 DNA of aliquots of each of the compounds at different concentrations in TE buffer (10 mM Tris·HCl, 0.1 mM EDTA, pH 7.4). The amount of each compound added to the DNA solution was expressed as r_i (the input molar ratio of Pt to nucleotides). The mixture was incubated at 37°C for the various periods of time indicated below. The unreacted compounds were separated from the mixture by precipitation of the DNA with 2.5 volumes of ethanol and 0.3 M sodium acetate (NaOAc), pH 4.8.

Quantification of platinum binding to DNA

A 20 μg ml^{-1} solution of CT DNA in TE buffer was incubated at 37°C with the platinum compounds at $r_i = 0.1$. Aliquots of 250 μl were collected at 15 min, 1 h, 5 h, 16 h and 24 h. The DNA was afterwards precipitated twice with 2.5 volumes of cold ethanol and 0.1 volume of 3M NaOAc, pH 4.8. The DNA was washed with 70% ethanol and resuspended in 1 ml of TE buffer. The amount of



Scheme 1 Synthesis route for compounds **2** and **3** from compound **1.**

DNA in each sample was measured by UV spectrophotometry (Beckman Acta cIII) at 260 nm. Platinum bound to DNA was determined by total X-ray fluorescence (TXRF) using a Seifert EXTRA-II apparatus. The platinum background level was also determined on DNA that had not been incubated with the drugs and on DNA–drug mixtures precipitated by ethanol immediately after addition of the compounds. The assays were done in triplicate.

Gel electrophoresis of drug–pBR322 complexes

pBR322 DNA aliquots (50 μg ml^{-1}) were incubated with the platinum compounds in a buffer solution containing 50 mM NaCl, 10 mM Tris·HCl, pH 7.4, and 0.1 mM EDTA at several values of r_i. Incubations were performed in the dark at 37°C, then 20 μl aliquots of the drug–DNA complexes containing 1 μg of DNA were subjected to 1.5% agarose gel electrophoresis for 16 h at 25 V in TAE buffer (Tris–acetate 40 mM, 2 mM, EDTA pH 8.0). The DNA was stained in the same buffer but containing ethidium bromide (0.5 μg ml^{-1}). The gels were photographed with an MP-4 Polaroid camera using a 665 Polaroid film and an orange filter.

Table 1 IC_{50} values obtained for compounds **1, 2** and **3** and *cis*-DDP against HL-60 and U-937 human leukemic cells

	$IC_{50} \pm$ SD (μM)	
Compound	HL-60	U-937
1	87 ± 6	94 ± 4
2	30 ± 4	45 ± 7
3	20 ± 2	24 ± 3
CDDP	7 ± 1	22 ± 3

RESULTS AND DISCUSSION

Synthesis of the complexes

The route of synthesis of platinum complexes **2** and **3** from the *p*-is.TSCN (**1**) ligand is depicted in Scheme 1. The IR and ^{1}H and ^{13}C NMR spectra from the complexes obtained in the reaction of $[Pt(\mu\text{-}Cl)(\eta^3\text{-}C_4H_7)]_2$ and 2 equiv. of *p*-is.TSCN are identical to those previously reported.[23] We have observed that treatment of complex **2** in MeOH at 60°C for four days produces complex **3,** indicating that complex **2** is an intermediate in the orthometallation reaction which results in the formation of complex **3**.

Antileukemic activity *in vitro*

Table 1 shows the IC_{50} values of compound **2,** complex **3,** *cis*-DDP and *p*-is.TSCN (compound **1**)

Table 2 Percentage of platinum bound to DNA after several periods of incubation with compounds **2** and **3** and *cis*-DDP at $r_i = 0.1$ as measured by TXRF

	Platinum bound to DNA (%)[a]				
Compound	1 h	5 h	10 h	16 h	24 h
2	25	30	59	95	96
3	27	32	62	96	98
CDDP	15	25	48	90	98

[a] Percentages represent the mean from three independent experiments. Standard deviations $\leq 5\%$ of the mean values.

against HL60 and U937 human leukemic cells. It can be observed that compound **3** has a cytotoxic activity slightly higher than that of compound **2** (IC_{50} values of 20μM and 24 μM versus 30 μM and 45 μM, respectively). Moreover, the, cytotoxic activity of compounds **2** and **3** is similar than that of *cis*-DDP (IC_{50} values of 7 μM and 22 μM). In contrast, it is interesting to note that compound **1** has poor cytotoxic activity in these cell lines (IC_{50} values of 87 μM and 94 μM). Thus, these results suggest that the coordination of the *p*-is.TSCN ligand to the Pt(II) atom enhances the biological activity of the thiosemicarbazone.

Platinum binding to DNA

Table 2 shows the percentage of platinum bound to DNA after several periods of incubation with compounds **2, 3** and *cis*-DDP at $r_i = 0.1$. It was observed that after 24 h the level of DNA platination produced by compounds **2** and **3** was similar to that produced by *cis*-DDP (96, 98 and 98%, respectively) indicating that most of the platinum centers were bound to DNA. Moreover, platinum binding to DNA reaches a plateau between 16 and 24 h of incubation with the compounds. A simple calculation indicates that in these conditions one platinum atom binds every 10 nucleotides. It was also observed that the kinetics of DNA platination due to compounds **2** and **3** is faster than that of cisplatin. In fact, after 1 h of incubation with compounds **2** and **3** the amount bound to DNA is 25 and 27%, respectively, in contrast with 15% for *cis*-DDP. We think that the cytotoxic activity displayed by compounds **2** and **3** may be correlated with the high level of DNA binding shown by these compounds DNA does not necessarily cause cell death from MTT assay.

Interaction with supercoiled DNA

The effect of the binding of compounds **2** and **3** on DNA tertiary structure was determined by their ability to shift the electrophoretic mobility of the covalently closed circular (ccc) and open circular (oc) forms of pBR322 plasmid DNA. Figure 1 shows the electrophoretic mobility pattern of native pBR322 plasmid DNA and of PBR322 plasmid DNA incubated with compounds **2** and **3** at $r_i = 0.1$ and 0.2 and with *p*-is.TSCN and *cis*-DDP at $r_i = 0.2$. It may be observed that compounds **2** and **3** do not vary the mobility of the ccc and oc forms of pBR322 DNA at both r_i values (lanes 2, 3, 4 and 5). In the same way. the *p*-is.TSCN ligand does not seem to alter the mobility of the ccc and oc forms of pBR322 DNA (lane 7). In contrast, incubation of pBR322 DNA with *cis*-DDP at $r_i = 0.2$ leads to a decrease in the electrophoretic mobility of the ccc forms and to an increase in the mobility of the oc form (lane 6). It has been previously reported that *cis*-DDP binding to ccc forms induces uncoiling of the DNA superhelix, resulting in a loss of negative supercoils, and therefore a decrease in electrophoretic mobility. Moreover, binding of *cis*-DDP to oc forms induces a DNA 'shortening' effect, leading to an increase in electrophoretic mobility.[29] Thus, the fact that binding of compounds **2** and **3** to pBR322 DNA does not change the electrophoretic mobility of either oc nor ccc forms of pBR322 DNA indicates that compound **2**–DNA and compound **3**–DNA adducts may stabilize, rather than unwinding plasmid DNA. Thus, the absence of an unwinding effect after binding of compounds **2** and **3** to supercoiled DNA supports the results pre-

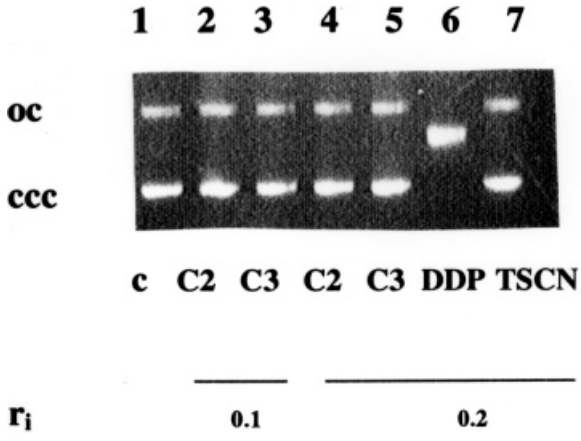


Figure 1 Changes in the electrophoretic mobility of the ccc and oc forms of pBR322 plasmid DNA after 24 h of incubation with compounds **2** (c2) and **3** (c3) at $r_i = 0.1$ and 0.2, and with TSCN and *cis*-DDP at $r_i = 0.2$ (oc, open circular form; ccc, covalently closed circular form; c, unmodified control DNA).

viously reported in linear plasmid DNA indicating that compounds **2** and **3** form DNA interhelical crosslinks located at twists of the superhelix.[17,23]

In summary, the facts that compounds **2** and **3** bind to the same extent to DNA and, moreover, that they show similar cytotoxic activity indicate that the cyclometallation process maintains the biochemical properties observed in the M–Cl bridged arrangement.

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