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Short communication

Preclinical studies with new pyrrolidine platinum(II) compounds

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Summary — Four new platinum compounds containing different pyrrolidines as ligands are reported. *In vitro* tests show damage to bacterial DNA and participation of RecA protein in the repair system. *In vitro* tests using leukemia L1210 cells show growth inhibition by the new compounds within the range of cisplatin and carboplatin. *In vivo* tests using leukemia L1210 cells on male BDF1 show that these compounds are significantly active.

platinum(II) compound / pyrrolidine / viability / growth / inhibition / antitumoral activity / cancer treatment / platinum(II)-DNA damage / L1210 leukemia

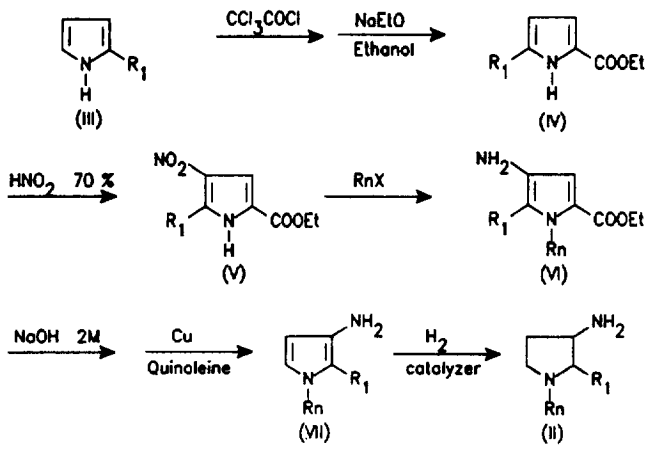
Introduction

The excellent results shown by cisplatin and carboplatin as antitumoral agents, alone or in combination with other cytostatics, in cancer chemotherapy during the last 20 years has promoted research in the area of coordination chemistry, mainly in platinum complexes. *cis*-Dichlorodiammineplatinum(II) (**1**) (cisplatin, CDDP) has become one of the most widely used cancer chemotherapeutic drugs in clinical practice. However, undesiderable effects, such as nephrotoxicity, ototoxicity, peripheral neuropathy, nausea and vomiting, are the greatest problems for the patients treated with cisplatin [1]. *cis*-Diammine-cyclobutane-1,1-dicarboxylatoplatinum(II) (**2**) (carboplatin, CBDCA) is an analogue of cisplatin with significantly reduced side effects, although thrombocytopenia continues to be the most prominent feature [2–4].

Progress has been made in understanding the interactions of platinum complexes at specific sites on DNA, including the effects of hydrogen bonding and interaction with phosphate groups in the DNA double helix. The preferred site of interaction on DNA is the N-7 atom of guanine, while binding to the O-6 atom does not appear to be a determinant factor [5]. The most common and stable binding mode of cisplatin and its analogues with DNA involves substitution of two Cl^- or leaving ligands and formation of two Pt-N bonds to the N-7 atoms of the two guanines on the same strand (intrastrand) or on opposite strands (interstrand) [6, 7]. These results could be useful in understanding complex *in vitro* and *in vivo* systems and there is the possibility of extrapolation in clinical trials.

New studies with carboplatin analogues and malonatoplatinum(II) complexes containing sulfide and phosphine ligands have recently been reported [8, 9]. The intensive program of synthesis initiated after the discovery of cisplatin and more recently carboplatin has led to clinical trials of a number of new generation complexes.

The neutral amine complexes are clearly the most active species in the spectrum of platinum complexes which demonstrate antitumor activity in animal models. There is thus a scope for development as well as understanding of their mechanism of action, especially when these differ from cisplatin. The basic structure based on the *cis*-$\{Pt(amin)_2\}$ unit gives the most active species and a few closely related second-generation analogues have been found to have greater activity than cisplatin in a range of murine tumors.



Scheme 1. Synthesis of pyrrolidine ligands.

Over the last five years, a new method to synthesize new pyrrolidine platinum(II) compounds has been developed by our group and the results of the preclinical studies are reported in this paper.

Results and discussion

Synthesis of ligands and platinum complexes

Pyrrolidinic ligands were synthesized by catalytic hydrogenation of the corresponding 1,2-dialkyl-3-nitropyrroles, following the previously described method [10, 11] (scheme 1). Platinum complexes were obtained by treatment of an aqueous solution of potassium tetrachloroplatinate with the corresponding pyrrolidine ligand as described in the literature [12] (scheme 2). Four new platinum compounds have been characterized by elemental analysis (C, H, N), IR and ^{1}H-NMR spectra. IR spectra show the characteristic appearances of Pt-Cl stretching band according to the following sequence: 312 (**4**) → 315 (**2**) → 318 → (**3**) → 320 cm^{-1} (**1**). ^{1}H-NMR spectra are in agreement with the proposed structure (see *Experimental protocols* for details of coupling constants).

In vitro tests

In order to evaluate the ability of the compounds to interact with DNA and to perturbate its repair system, the survival of different strains of either wild-type or mutant *Escherichia coli* treated with different platinum complexes has been measured [8, 13].

Sudies on bacteria cells are an effective approach to determine the toxicity and the activity on the DNA of these new compounds. It has been shown that **1** produces DNA damage and induces the *RecA* gene-dependent system of DNA repair [14]. Survival against **1** or any DNA-damaging agent is dramatically lower in *RecA*$^-$ than in *RecA*$^+$ strains [15].

Two *E coli* strains, wild-type (AB1157) and *RecA* 13 mutant (AB2463) were treated with various pyrrolidine platinum(II) complexes for 2 h at 37°C. The lower viability of the mutant *RecA*$^-$ (AB2463) compared with that of the wild-type *RecA*$^+$ (AB1157) strain after treatment with four new pyrrolidine platinum(II) complexes suggests that gene *RecA* participates in the tolerance or repair of the DNA damage produced by these compounds by a similar mechanism to that of cisplatin and carboplatin.

The results obtained (table I) show that this new series of pyrrolidine complexes require lower doses **1**,

Compound	R_c	R_n	*Mw (g)*	*Systematic name*
1	CH_3	CH_3	380.16	*cis*-(3-Amino-1,2-dimethylpyrrolidine) dichloroplatinum(II)
2	CH_3	CH_2CH_3	394.21	*cis*-(3-Amino-1-ethyl-2-methylpyrrolidine) dichloroplatinum(II)
3	CH_2CH_3	CH_3	394.21	*cis*-(3-Amino-1-methyl-2-methylpyrrolidine) dichloroplatinum(II)
4	CH_2CH_3	CH_2CH_3	408.25	*cis*-(3-Amino-1,2-diethylpyrrolidine) dichloroplatinum(II)

Scheme 2. The structure and synthesis of pyrrolidine-Pt(II) compounds.

Table I. *In vitro* studies with *E coli RecA*$^+$ and *RecA*$^-$ strains.

Compound	*E coli (AB1157: RecA*$^+$*)*			*E coli (AB2463: RecA*$^-$*)*		
	LD_{10}[a]	LD_{50}[a]	LD_{90}[a]	LD_{10}[a]	LD_{50}[a]	LD_{90}[a]
Cisplatin	12	81	287	5	14	35
1	109	428	> 1315	1	6	13
2	729	1649	2357	10	57	298
3	45	268	> 1268	11	18	36
4	101	673	> 1224	19	36	72
Carboplatin	381	> 2693	> 2693	82	422	599

[a]Dose in concentration (μm).

Table II. Growth inhibition by platinum complexes using L1210 leukemia cells.

Compound	IC_{50} *(μM)*
Cisplatin	23
1	78
2	91
3	98
4	113
Carboplatin	142

similar doses **3** and higher doses **2** and **4** than cisplatin, in order to produce the same mortality in the *RecA*$^-$ mutant (AB2463) strains. However, all four products need higher doses in the wild-type (AB1157). It is noteworthy that when these results are compared with carboplatin, the four products require lower doses to produce an equivalent mortality in both wild-type (AB1157) and *RecA*$^-$ mutant (AB2463).

In order to evaluate the growth inhibitory effect of the above-mentioned products, an MTT colorimetric assay [16, 17] was used to calculate IC_{50} values in L1210 cells (table II). The pattern of the IC_{50} values of these products suggests a perfect structure–activity relationship depending on the position and length of the R_n and R_c groups. For **2** and **3**, the IC_{50} values are similar, meaning that permutation of a methyl group by an ethyl group does not affect the growth inhibition in a direct manner. Nevertheless, a significant difference was observed between **1** and **4**, meaning that substitution of a methyl group by an ethyl group affects the IC_{50} values. These results are in agreement with other previously reported observations [18].

In vivo tests

According to the results shown in table III, the four products tested could be considered as drugs with a significant antitumor activity because their values of % T/C are greater than 125 (the minimal value required for this purpose) [19, 21]. The excellent structure–toxicity relationship between the LD_{50} of the four products according to the sequence **1** = **2** < **3** = **4** is also remarkable in this case.

The % T/C values of this new series of platinum complexes are much better than those of other analogues previously reported by our group [22]. The structure–activity relationships suggest a good correlation between LD_{50} and % T/C values; in the case of **3** and **4** these are very similar, but higher than those of **1** and **2**. This fact is not surprising, but the causes of this increase are not known at present but may involve the position and length of the R_n and R_c groups.

Many cisplatin analogues have been synthesized since the discovery of cisplatin but only a few have entered the stage of clinical evaluation. Development of structurally novel complexes of platinum is important, not only from a mechanistic point of view, but also because new complexes may have a different spectrum of activity, a different grade of toxicity and possibly activity against cisplatin-resistant tumors.

The identification of DNA as a primary target of metal-based drugs with all the important consequences for gene expression and replication has focused special attention on the interaction of platinum complexes with DNA, oligonucleotide sequences and constituent bases. This can help obtain a detailed description of the binding and contribute to the understanding of the molecular mechanism of action of cisplatin and its derivatives.

The main disadvantage of cisplatin in clinical use is its broad spectrum of severe side effects. Recent studies with different platinum complexes revealed that some of these side effects can be modulated by varying the neutral ligands and/or leaving groups [23]. Most new platinum complexes thus have a relatively

Table III. LD_{50} and % T/C values of platinum complexes taken from the tests *in vivo* on BDF_1 mice using L1210 cells.

Compound	LD_{50} *(mg/kg)*	*% T/C*
Cisplatin	17.8	200[a], 165[b]
1	58.0	160–170[b]
2	64.0	167
3	82.7	186
4	117.4	182
Carboplatin	118.0	148[a]

[a]Result consistent with the % T/C values of 164–229 and 130–150 described in ref [18]. [b]Result obtained by our group.

similar spectrum of therapeutic activity but differ in their toxicity. Of all the cisplatin analogues, carboplatin is the most extensively studied drug in the clinic. Carboplatin is the preferred drug for treating patients with renal failure, peripheral neuropathy, hearing loss or severe nausea and vomiting; the dose-limiting toxicity of this agent is its myelosuppression, predominantly thrombocytopenia [4].

Understanding how cisplatin and carboplatin interact with DNA has become a central question in some of the most active research in chemistry, molecular biology and medicine. Studies carried out with three active antitumor agents, cisplatin, $Pt(en)Cl_2$ and $Pt(cyclohexanediamine)Cl_2$, show strong evidence implicating DNA as the principal target of cisplatin in the biological system. An interesting feature in this case is the nature of the affinity of Pt-amine complexes toward DNA. In a similar manner, the 1,2-dialkyl-3-aminopyrrolidine platinum(II) compounds are Pt-amine complexes and the results obtained both *in vitro* and *in vivo* indicated that these novel platinum derivatives have a spectrum of activity similar if not better than cisplatin. We hope that their toxicity, especially nephrotoxicity, will be minimal due to their special structure.

More studies are presently in progress with similar platinum complexes where the R_n and R_c are groups containing 4–12 carbons in order to elucidate the correct structure–activity relationship.

Experimental protocols

Synthesis of platinum complexes

The compounds were synthesized following the method described by Green [10], which was recently modified by our group [11]. All the compounds synthesized have been characterized by elemental analysis (C, H, N), IR and ^{1}H-NMR spectra.

IR spectra were recorded on a Nicolet 5PC FTIR spectrophotometer. ^{1}H-NMR spectra were recorded at 80 MHz on a Bruker WP 80 SY spectrometer. Chemical analysis of C, H and N were carried out by Centre d'Investigació i Desenvolupament, CSIC of Barcelona, Spain. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values.

cis-Dichloro (3-amino-1,2-dimethylpyrrolidine) platinum(II) 1
To a solution containing 1.2 g (0.01 mol) of 3-amino-1,2-dimethylpyrrolidine under an argon atmosphere was added a solution of K_2PtCl_4 (2.1 g, 0.01 mol) in water (50 ml). After stirring for 24 h at room temperature under an inert atmosphere, in order to avoid a decomposition of the amine, the solution was acidified to pH 1.2 by adding 2 M HCl. The solvent was removed by evaporation *in vacuo* and the residue was concentrated to dryness and crystallized with 0.1 M HCl. The resulting yellow crystals were filtered yielding pure **1** (2.0 g, 52%), mp 244–246°C (dec). IR (KBr) cm^{-1}: 3246 s, 3202 s, 3130 s, 2880 s, 1578 m, 1450 m, 1301 w, 1252 m, 1159 s, 947 m, 634 w, 335 m, 320 s. ^{1}H-NMR (D_2O): δ 1.2–1.4 (m, 3H), 2.3–2.6 (m, 2H), 2.9 (s, 3H), 3.1–3.5 (m, 4H). Anal: $C_6H_{14}N_2Cl_2Pt$ (C, H, N).

cis-Dichloro (3-amino-1-ethyl-2-methylpyrrolidine) platinum(II) 2
To a solution of 3-amino-1-ethyl-2-methyl pyrrolidine (1.3 g, 0.01 mol) in an argon atmosphere was added a solution of K_2PtCl_4 (2.1 g, 0.01 mol) in water (75 ml). The mixture was maintained at 60°C for 2 h with stirring and the precipitation of a yellow-green solid was observed. The solvent was removed by evaporation at 50°C. The solid residue is dissolved in 0.1 M HCl at 60°C and filtered through a 0.45 μm millipore membrane. The filtrate was concentrated to 50–60 ml by evaporation *in vacuo* and the solid residue obtained was crystallized with 0.1 M HCl. The resulting yellow crystals were filtered yielding pure **2** (2.6 g, 66%), mp 254–256°C (dec). IR (KBr) cm^{-1}: 3211 s, 3136 s, 2976 s, 1595 m, 1446 m, 1175 s, 336 m, 315 s. ^{1}H-NMR (D_2O) δ: 1.3 (t, 3H; J = 7 Hz), 1.5 (m, 3H), 1.9–2.6 (m, 4H), 3.0–3.9 (m, 6H). Anal: $C_7H_{16}N_2Cl_2Pt$ (C, H, N).

cis-Dichloro (3-amino-2-ethyl-1-methylpyrrolidine) platinum(II) 3
To a solution of 3-amino-2-ethyl-1-methylpyrrolidine (0.4 g, 0.003 mol) under argon atmosphere was added a solution of K_2PtCl_4 (1.21 g, 0.003 mol) in water (50 ml). After stirring for 24 h to room temperature under inert atmosphere, the solution was acidified to pH 1.2 by adding 2 M HCl. The solvent was removed by evaporation *in vacuo* and the residue was concentrated to dryness and crystallized with 0.1 M HCl. The resulting yellow crystals were filtered yielding pure **3** (0.54 g, 44%), mp 244–246°C (dec). IR (KBr) cm^{-1}: 3246 s, 3204 s, 3130 m, 2974 m, 2880 m, 1593 m, 1450 m, 1304 w, 1159 s, 946 m, 634 w, 333 m, 318 s. ^{1}H-NMR (D_2O) δ: 1.2–1.5 (m, 5H), 2.3–2.6 (m, 2H), 2.9 (s, 3H), 3.1–3.5 (m, 4H). Anal: $C_7H_{16}N_2Cl_2Pt$ (C, H, N).

cis-Dichloro (3-amino-1,2-diethylpyrrolidine) platinum(II) 4
To a solution of 3-amino-1,2-diethylpyrrolidine (0.55 g, 0.004 mol) under inert atmosphere was added a solution of K_2PtCl_4 (1.6 g, 0.004 mol) in water (75 ml). After stirring for 24 h at room temperature under argon atmosphere the solution was acidified to pH 1.2 by adding 2 M HCl. The solvent was removed by evaporation *in vacuo* and the residue was concentrated to dryness and crystallized with 0.1 M HCl. The resulting yellow crystals were filtered yielding pure **4** (0.71 g, 46%), mp 215°C. IR (KBr) cm^{-1}: 3263 s, 3208 s, 3134 s, 2968 s, 2883 m, 1600 m, 1460 s, 1368 w, 1255 w, 1122 s, 958 w, 634 w, 325 m, 312 s. ^{1}H-NMR (D_2O) δ: 1.0–1.3 (m, 5H), 1.6 (t, 3H; J = 7 Hz), 2.0–2.7 (m, 2H), 3.2–3.8 (m, 6H). Anal: $C_8H_{18}N_2Cl_2Pt$ (C, H, N).

In vitro studies with E coli RecA$^+$ and RecA$^-$ strains

The *E coli* K-12 strains AB1157 (*RecA*$^+$) and AB2463 (*RecA*$^-$) were obtained from M Blanco. Bacteria were grown in LB medium at 37°C with aeration. Aliquots were distributed into tubes containing different doses of the compound to be tested (each dose was assayed by duplicate). After 2 h of incubation at 37°C with aeration samples were plated in LB medium supplement with streptomycin (75 μg/ml). Cell survival at different doses of platinum derivatives was expressed as a percentage with respect to untreated cultures. The results correspond to three independent experiments.

In vitro growth inhibition

L1210 leukemia cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) containing 10% fetal calf serum (FCS) supplemented with 1 mM L-glutamine and

antibiotic (250 ng/ml gentamicin). Cells were maintained in an exponential growth phase by periodic dilutions with fresh medium at 37°C in a 5% CO_2 humidified atmosphere. A MTT previously described colorimetric assay was used to determine growth inhibition [16, 17]. Growth inhibition was estimated as follows.

Day 0

L1210 murine leukemia cells were seeded in 96-well round-bottomed plates (Greiner), at a density of 10 000 cells/100 µl/well. The cells were left to recover for 2–4 h at 37°C and 5% CO_2. The drugs were then added in 100 µl, in a concentration range from 5 x 10^{-4} M to 10^{-7} M and incubated for 24 h at 37°C and 5% CO_2.

Day 1

MTT (20 µl, 5 mg/ml)was added per well, and incubated for 2–4 h at 37°C. The plates were then centrifuged for 5 min at 1200 rpm in a table centrifuge. Subsequently, the medium was removed very carefully with a pipette. Freshly prepared DMSO-glycin buffer (150 µl) was added to each well. After mixing for 10 min, the optical density was determined at 540 nm within 1 h. The IC_{50} was defined as the concentration of drug resulting in 50% of the optical density of control cultures without drugs.

Antitumor activity in vivo

In vivo antitumor activity was determined as follows. Dilute ascitic fluid (0.2 ml) containing 1.0 x 10^6 leukemia L1210 cells was implanted ip into male BDF_1 mice (Charles River, France) on day 0. The animals were randomized in groups of six. The therapy began 24 h after inoculation (day 1) with ip application of a suspension of the corresponding compound in 0.25% agar (Difco, cat 0140-02) at different doses.

For the evaluation of the % T/C value, the median survival time of the treated animals was compared with that of the untreated control animals and calculated using the following formula:

$$\% \text{ T/C} = \frac{\text{median survival of treated animals}}{\text{median survival of control animals}} \times 100$$

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