

Synthesis and Cytotoxicity of Platinum(II) Complexes of 3-Amino-cyclopentanespiro-5-hydantoin and 3-Aminocycloheptanespiro-5-hydantoin

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Four new platinum(II) complexes of 3-aminocyclopentanespiro-5-hydantoin (acpsh) and 3-aminocycloheptanespiro-5-hydantoin (achpsh) were synthesized and characterized by elemental analysis, IR and ¹NMR spectra. The spectral analyses indicated a *cis*-square planar structure of the complexes with ligands coordinated *via* the NH₂ group. The complexes were evaluated for *in vitro* cytotoxicity in murine erythroleukemia (MEL) cells, clone F4N, using cell-growth and macromolecular synthesis assay. The compounds, with exception of [Pt(NH₃)(achpsh)Cl₂] (**IV**), exhibited much lower cytotoxicity than that of cisplatin (DDP). Compound **IV** was nearly as cytotoxic as DDP. The new complexes exerted low antibacterial activity as assessed by seven bacterial strains.

Key words: Platinum(II) Complexes, Cytotoxic Effects, Antibacterial Effect

Introduction

Cis-diamminedichloroplatinum(II) (cisplatin, DDP) is a drug world widely used in clinical treatment of cancer (Loehrer *et al.*, 1984). Despite the success of cisplatin it has several disadvantages that include nephrotoxicity, neurotoxicity, and emetogenesis (De Conti *et al.*, 1973). The development of acquired resistance to DDP (Eastman *et al.*, 1988) and the severity of toxicity (De Conti *et al.*, 1973) associated with this drug encouraged the design of new drugs containing platinum in an attempt to overcome drug resistance while reducing host toxicity.

A noteworthy approach in the design of cisplatin analogues is the use of physiologically active compounds as ligands. In a previous report (Kushev *et al.*, 2002) we have described platinum(II) complexes of 3-aminocyclohexanespiro-5-hydantoin (achsh). The interest in such ligands was provoked by the data that various hydantoins have shown biological activity (Avendano *et al.*, 1985). It has been shown that spirohydantoin aziridine has antitumor effect (Struck *et al.*, 1986). The complexes synthesized by us (Kushev *et al.*, 2002) exerted significantly lower *in vivo* and *in vitro* toxicity compared with those of cisplatin. The com-

plex [Pt(NH₃)(achsh)Cl₂] exhibited antitumor activity against L1210 leukemia, comparable to that of cisplatin. This complex, as well as cisplatin, induced apoptosis in F4N cells and exerted antibacterial activity.

In order to evaluate the effect of the substitution of the cyclohexane ring in 3-aminocyclohexanespiro-5-hydantoin with cyclopentane or cycloheptane on toxicity and activity of platinum complexes, four platinum complexes containing these ligands were synthesized and characterized. Their effect on cell growth and macromolecular (DNA, RNA and protein) synthesis in murine erythroleukemia (MEL) cells, and their antimicrobial activity were examined.

Materials and Methods

Starting materials

Cis-[Pt(NH₃)₂Cl₂] and K₂[PtCl₄] were prepared according to Spassovska *et al.* (1981), K[Pt(NH₃)Cl₃] according to Muir *et al.* (1988). The ligands, 3-aminocyclopentanespiro-5-hydantoin (acpsh) and 3-aminocycloheptanespiro-5-hydantoin (achpsh) were synthesized from cyclopentanone and cycloheptanone to cyclopentanespiro-5-hydantoin and cycloheptanespiro-5-hydantoin,

respectively, following the Bucherer-Lieb reaction (Bucherer *et al.*, 1934). After that cyclopentanespiro-5-hydantoin and cycloheptanespiro-5-hydantoin were transformed into 3-aminocyclopentanespiro-5-hydantoin and 3-aminocycloheptanespiro-5-hydantoin by means of $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$. The remaining reagents and solvents were AR grade products.

Preparation of the complexes

The procedures for preparation of platinum complexes of acpsh and achpsh were analogous to those described by Kushev *et al.* (1999; 2002), which were based on the method of Kharitonov *et al.* (1975).

$[\text{Pt}(\text{acpsh})_2\text{Cl}_2]$ (I). A solution of 0.243 g (1.44 mmol) of acpsh in 15 ml water was added to stirred solution of 0.200 g (0.48 mmol) of $\text{K}_2[\text{PtCl}_4]$ in 5 ml water. The reaction was carried out for 2 days at room temperature. The yellow precipitate was filtered, washed with water and ethanol, and dried *in vacuo*. Yield: 0.100 g (34%). Melting range: 205–210 °C (decomp.).

Elemental analysis: Calculated for $\text{C}_{14}\text{H}_{22}\text{Cl}_2\text{N}_6\text{O}_4\text{Pt}$: C, 27.82; H, 3.67; Cl, 11.73; N, 13.90; Pt, 32.28. Found: C, 27.52; H, 3.52; Cl, 11.34; N, 13.97; Pt, 31.71.

$[\text{Pt}(\text{NH}_3)(\text{acpsh})\text{Cl}_2]$ (II). A solution of 0.379 g (2.24 mmol) of acpsh in 20 ml water was added while stirring to a solution of 0.800 g (2.24 mmol) of $\text{K}[\text{Pt}(\text{NH}_3)\text{Cl}_3]$ in 10 ml water. After 4 day mixing at room temperature the yellow solid was collected, then washed with ethanol, and dried *in vacuo*. Yield: 0.270 g (17%). Melting range: 170–180 °C (decomp.).

Elemental analysis: Calculated for $\text{C}_7\text{H}_{14}\text{Cl}_2\text{N}_4\text{O}_2\text{Pt}$: C, 18.59; H, 3.12; Cl, 15.68; N, 12.39; Pt, 43.14. Found: C, 18.64; H, 3.01; Cl, 15.42; N, 12.45; Pt, 42.66.

$[\text{Pt}(\text{achpsh})_2\text{Cl}_2]$ (III). A solution of 0.284 g (1.44 mmol) of achpsh dissolved in 15 ml water was added to stirred solution of 0.200 g (0.48 mmol) of $\text{K}_2[\text{PtCl}_4]$ in 5 ml water. The reaction was carried out for 2 days at room temperature. The yellow precipitate was filtered, washed with water and methanol, and dried *in vacuo*. Yield: 0.210 g (66%). Melting range: 248–255 °C (decomp.).

Elemental analysis: Calculated for $\text{C}_{18}\text{H}_{30}\text{Cl}_2\text{N}_6\text{O}_4\text{Pt}$: C, 32.73; H, 4.58; Cl, 10.74; N,

12.72; Pt, 29.54. Found: C, 32.30; H, 4.20; Cl, 10.50; N, 12.40; Pt, 29.02.

$[\text{Pt}(\text{NH}_3)(\text{achpsh})\text{Cl}_2]$ (IV). A solution of 0.166 g (0.84 mmol) of achpsh in 7 ml water was added while stirring to a solution of 0.300 g (0.84 mmol) of $\text{K}[\text{Pt}(\text{NH}_3)\text{Cl}_3]$ in 3 ml water. After 3 days mixing at room temperature the yellow solid was filtered. Then washed with water, ethanol, and dried *in vacuo*. Yield: 0.170 g (42%). Melting range: 195–205 °C (decomp.).

Elemental analysis: Calculated for $\text{C}_9\text{H}_{18}\text{Cl}_2\text{N}_4\text{O}_2\text{Pt}$: C, 22.51; H, 3.78; Cl, 14.76; N, 11.67; Pt, 40.62. Found: C, 22.35; H, 4.24; Cl, 15.02; N, 12.11; Pt, 41.10.

Analyses and physical measurements

The elemental analyses were performed by routine micro-analytical procedures (Institute of Organic Chemistry, Bulgarian Academy of Sciences).

Melting ranges were determined with a Boetius heating-plate microscope.

^1H NMR spectra were registered on a Bruker WM 250 spectrometer at 250 MHz in DMSO-d_6 solution using TMS as internal standard. D_2O exchange was applied to confirm the assignment of nitrogen-bound protons.

Infrared spectra were recorded on a Bruker ISF-113V spectrophotometer in CsI disks ($4000\text{--}400\text{ cm}^{-1}$, $(400\text{--}150\text{ cm}^{-1})$).

Incubation with drugs and in vitro cytotoxicity test

MEL cells, clone F4N (virus-transformed murine erythroleukemia precursor cells) (Dube *et al.*, 1975) were cultured in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) (Dulbecco and Freeman, 1959) supplemented with 10% calf serum, under 5% CO_2 atmosphere at 37 °C, and passed every day at a concentration of 5×10^5 cells/ml.

The complexes were dissolved immediately before use in DMSO to obtain stock solutions of different concentrations. Each of these solutions was used at 1% concentration in the experiments with F4N cells. The final concentration of DMSO in the medium did not affect cell growth.

Exponentially growing cells (0.5×10^6 cells/ml) were incubated in triplicate with increasing concentrations of the test complexes in 24-well microtiter plates. After 24, 48 and 72 h of drug treat-

ment, the cells were counted hemocytometrically. The number of dead cells was determined by staining with trypan blue. The mean of triplicate determinations of two independent experiments was calculated. The 50% inhibitory dose (IC₅₀) was defined as drug concentration that reduced the number of living cells by 50%.

Macromolecular synthesis assay

The macromolecular synthesis determination was essentially carried out as described (Grancharov *et al.*, 1988). F4N cells at a concentration of 0.5×10^6 cells/ml were incubated in duplicate for 24 h at 37 °C with incremental concentrations of platinum complexes. Thereafter, [³H]thymidine (0.04 MBq), [¹⁴C]uridine (0.04 MBq) or [¹⁴C]leucine (0.04 MBq) was added and the cells were incubated for an additional 1 h to monitor the precursor incorporation into DNA, RNA or proteins, respectively. Aliquots of cell suspension were pipetted on Whatman filter paper disks, the acid-soluble radioactivity was extracted with cold trichloroacetic acid (5%), and the incorporated activity determined by scintillation counting. The average value of quadruplicate determinations in two independent experiments was calculated.

Antimicrobial assay

Antimicrobial effect was studied against both Gram-positive, Gram-negative bacteria and yeast. The following microbes were used: *Staphylococcus aureus* 209P, *Streptococcus pyogenes* 10935, *Escherichia coli* C 600, *Proteus mirabilis* P52, *Klebsiella pneumoniae* 52146, *Bacillus subtilis* 1ε6, *Candida albicans* 220.

The antimicrobial effect was studied through the minimal inhibitory concentrations (MIC). MIC's were determined by the twofold broth dilution method (Reiner *et al.*, 1982). The tested compounds were prediluted in DMSO so that DMSO/broth ratio was 1:10 v/v. In this concentration DMSO had no visually observable growth inhibiting effect. Nutrient broth (Difco) was added to bacteria and Sabouraud nutrient broth to yeast. In the experiments, compound concentrations ranged from 1.6 mM to 0.1 mM. The microbial inoculum size was 1×10^5 culture forming units per ml. Visual reading was made after 18–20 h incubation at 37 °C to bacteria, and after 48 h incubation at 30 °C to yeast.

Results and Discussion

The complexes are yellow coloured solids that decompose upon heating. They are soluble in DMF or DMSO, and some of them are practically insoluble in water.

Infrared spectra

The IR spectral data of the ligands and complexes are collected in Table I. For interpretation of the IR data, the spectra were divided in two groups. The absorption bands in the range of 4000–600 cm⁻¹ belong to the absorptions of the organic ligand. The absorption bands in the far IR below 600 cm⁻¹ characterize the ligand-metal bonding. The bands in the range of absorptions of the N-H stretching vibrations in the spectra of complexes were shifted (about 30–140 cm⁻¹) to lower frequency as compared to those of the free ligand. This is indicative for a coordination of the ligands to the platinum through the NH₂ group (Kushev *et al.*, 1999). The presence of two absorption bands for the platinum-chlorine stretching vibrations (in the range of 320–340 cm⁻¹) in the far IR spectra is an indication of the *cis*-configuration of the complexes (Kushev *et al.*, 1999, 2002). Some of the weak bands in the range of 553–461 cm⁻¹ in the spectra of the complexes could tentatively be ascribed to platinum-nitrogen stretching (Kharitonov *et al.*, 1975).

¹H NMR spectra

The ¹H NMR spectral data for freshly prepared DMSO-d₆ solutions of the ligands and complexes are presented in Table I. In the spectra of the ligands (3-aminocyclopentanespiro-5-hydantoin and 3-aminocycloheptanespiro-5-hydantoin) it was observed a complicated multiplet in the range of 1.54–1.88 ppm due to the methylene protons at the cycloalkane ring. Signals for NH₂ and NH groups of the ligands were registered at 4.68 ppm and 8.34 ppm for acpsh, and 4.64 and 8.40 for achpsh, respectively. In the spectra of all complexes, the signals of the NH₂ protons were significantly shifted downfields (average 3.46 ppm) in comparison to the free ligand. The signals of NH protons were much less affected (average 0.23 ppm). In our previous works (Kushev *et al.*, 1999, 2002) analogous effects have been described.

Table I. Spectral data for the ligands and complexes.

No Compound	IR spectral data [ν , cm^{-1}]*					^1H NMR spectral data [δ , ppm]**				
	$\nu(\text{NH}_3)$ $\nu(\text{NH}_2)$ $\nu(\text{NH})$	Amide I $\delta(\text{NH}_2)$ $\delta_d(\text{NH}_3)$	$\nu_{\text{as}}(\text{CH}_2)$ $\nu_s(\text{CH}_2)$ $\delta(\text{CH}_2)$	Amide II	$\omega(\text{NH}_2)$ $\delta_s(\text{NH}_3)$	$\nu(\text{Pt-Cl})\text{NH}_3$	CH_2	NH_2	NH	
acpsh	3312 m 3240 m	1653 s 1616 m	2970 m 2873 m 1451 m	1528 m	1334 w 1300 w	—	—	1.59–1.88 m	4.68 s	8.34 s
I [Pt(acpsh) $_2\text{Cl}_2$]	3178 s 3069 m	1669 s 1616 s	2960 s 2873 m 1450 m	1545 m	1325 m	330 m 322 sh	—	1.64–1.88 m	7.58	8.35
II [Pt(NH_3)(acpsh) Cl_2]	3281 s 3191 s	1669 s 1609 s	2960 m 2873 w 1450 m	1542 m	1321 m 1307 m	328 m 321 m	4.68	1.65–1.73 m	7.58	8.49
achpsh	3302 m 3210 m	1646 m 1604 m	2931 m 2862 w 1460 w	1520 w	1325 w 1307 w	—	—	1.54–1.83 m	4.64 s	8.40 s
III [Pt(achpsh) $_2\text{Cl}_2$]	3174 m 3090 m	1623 w	2932 m 2860 m 1465 m	1546 m	1362 w 1312 w	338 s 331 sh	—	1.54–1.87 m	8.34	8.80
IV [Pt(NH_3)(achpsh) Cl_2]	3283 s 3195 m 3090 sh	1609 m	2928 s 2857 m 1459 m	1558 m 1538 m	1361 w 1308 m	340 sh 334 m	4.65 s	1.54–1.88 m	8.43	8.75

* Abbreviations: s, strong; m, medium; w, weak; sh, shoulder.

** Abbreviations: s, singlet; m, multiplet.

Moreover, a downfield shift of the same order has been registered for the proton signals of coordinated NH_2 groups in the spectra of a number of Pt(II) amine complexes (Bitha *et al.*, 1989). That gives us a reason to consider that in the complexes discussed the ligands coordinate to platinum *via* the NH_2 group. The presence of a characteristic NH_3 signal at 4.68 ppm for the complex **II**, and at 4.65 ppm for the complex **IV** confirms their mixed ligand nature (Muir *et al.*, 1988). The signals of NH_3 , NH_2 and NH protons of the studied complexes were accompanied by some weaker peaks. These additional signals can be explained by solvolysis products of the complexes in DMSO (Kerrison *et al.*, 1977). In the spectra of the platinum complexes no significant shift of the signals due to the cycloalkane residue was registered. Similar IR and ^1H NMR spectral data for platinum complexes in our previous papers (Kushev *et al.*, 1999, 2002) have been reported.

The elemental analysis and spectroscopic data confirm the structures of the complexes shown in Fig. 1.

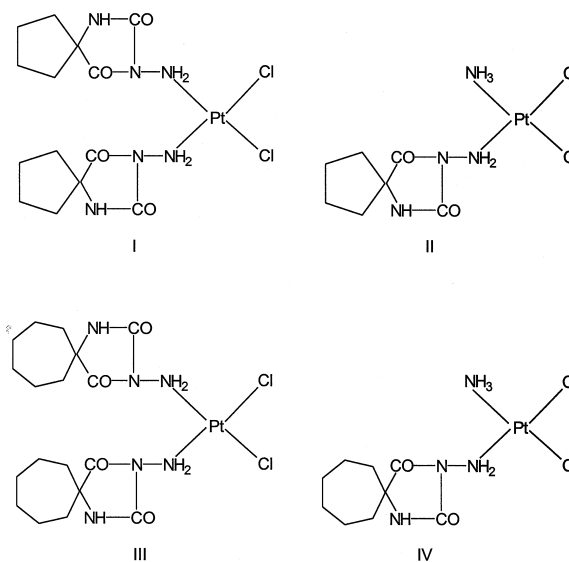


Fig. 1. Chemical structures of the complexes.

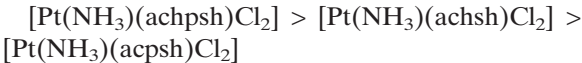
I: *cis*-bis(3-aminocyclopentanespiro-5-hydantoin)-dichloroplatinum(II)**II:** *cis*-ammine(3-aminocyclopentanespiro-5-hydantoin)dichloroplatinum(II)**III:** *cis*-bis(3-aminocycloheptanespiro-5-hydantoin)-dichloroplatinum(II)**IV:** *cis*-ammine(3-aminocycloheptanespiro-5-hydantoin)dichloroplatinum(II).

Cytotoxic effects

The growth-inhibitory effects of the ligands acpsh and achpsh, and their platinum complexes **I–IV** on F4N cells in culture were examined using cisplatin as a positive control. The results, as expressed by IC₅₀ values for various times of drug treatment, are presented in Table II. Complexes **I** and **III** proved to be low inhibitory. A temporary cell-growth retardation upon 48 h treatment was registered (with IC₅₀ of 260 μM and 280 μM, respectively) followed by a recovery of the cells. Unlike these compounds, complexes **II** and **IV** containing an ammine ligand, showed significant differences in their toxicity. While complex **II** was low cytotoxic, with IC₅₀ of 189 μM after 72 h incubation, complex **IV** exhibited cytotoxicity comparable with that of cisplatin. The ligands acpsh and achpsh did not inhibit cell growth at concentrations up to 200 μM.

The most active complexes in this series, **II** and **IV**, were further tested for their inhibitory effect on macromolecular synthesis in F4N cells. Cells were treated for 24 h with the compounds, and thereafter were additionally incubated with [³H]thymidine, [¹⁴C]uridine and [¹⁴C]leucine to monitor precursor incorporation into DNA, RNA or proteins, respectively. Drug concentrations reducing by 50% precursor incorporation into mac-

romolecular material are presented in Table III. The trend of inhibition followed that of the growth-inhibitory assay: a moderate inhibitory activity of complex **II** (IC₅₀, 82 μM for DNA synthesis), and a much higher inhibitory potency of compound **IV** (IC₅₀, 4.75 μM for DNA synthesis), comparable with that of cisplatin. In both cases, DNA synthesis was more affected than those of RNA or protein. When comparing the cytotoxic effects of complexes **II** and **IV** with those of complex [Pt(NH₃)(achsh)Cl₂], containing 3-aminocyclohexanespiro-5-hydantoin as a ligand (Kushev *et al.*, 2002), it is evident that the cytotoxicity of the complexes increases with the increase of the number of atoms in the carbon ring:



Antimicrobial activity

In recent years the interest in the antibacterial activity of platinum group metal complexes increased. The observed effect of platinum species on *Escherichia coli* produced by platinum electrodes in the growth medium (Rosenberg *et al.*, 1967) led to the exploitation of platinum complexes as potential anticancer drugs. However, most of earlier work suffered from a lack of structure-activity studies which proved to be essential in the development of anticancer platinum complexes (Ferguson *et al.*, 1979). Now the continuing design of new platinum complexes revived the interest into the antimicrobial effect of these complexes (Bunker *et al.*, 1989).

Table II. Cytotoxicity of platinum complexes in F4N cells.

No	Compound	IC ₅₀ ^a [μM]		
		24 h ^b	48 h	72 h
	acpsh		c	
I	[Pt(acpsh) ₂ Cl ₂]	d	260	e
II	[Pt(NH ₃)(acpsh)Cl ₂]	c	138, 145	186
	achpsh		c	
III	[Pt(achpsh) ₂ Cl ₂]	c	280	c
IV	[Pt(NH ₃)(achpsh)Cl ₂]	7	10	14
	Cisplatin	5.8	4.9	3.9

^a Drug concentration reducing the number of living cells by 50%.
^b Time of drug treatment.
^c No inhibition of cell growth at concentrations up to 200 μM.
^d Less than 20% inhibition at concentrations up to 200 μM.
^e 30% inhibition of cell growth at 200 μM concentration. Values are mean of triplicate determinations in two independent experiments.

Table III. Effect of platinum complexes on macromolecular synthesis in F4N cells.

No	Complex	IC ₅₀ ^a [μM]		
		DNA	RNA	Protein
II	[Pt(NH ₃)(acpsh)Cl ₂]	82	b	b
IV	[Pt(NH ₃)(achpsh)Cl ₂]	4.75	24	9.75
	Cisplatin	1.20	8.50	5.10

^a Drug concentration that reduces by 50% the incorporation of [³H]thymidine, [¹⁴C]uridine and [¹⁴C]leucine into DNA, RNA and protein, respectively.
^b Less than 20% inhibition of macromolecular synthesis at concentrations up to 150 μM. Values are mean of quadruplicate determinations in two independent experiments.

In the present study the antimicrobial activity of the new platinum(II) complexes in 7 bacterial strains was studied. As a whole, the complexes exhibited a low antibacterial effect against the tested microorganisms.

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