Cytotoxicity and Antitumor Activity of Platinum(II) Complexes of Aromatic and Cycloalkanecarboxylic Acid Hydrazides

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Platinum(II) Complexes, Cytotoxicity, Antitumor Activity, Macromolecular Synthesis Inhibition

New platinum(II) complexes of cyclohexanecarboxylic acid hydrazide *(chcah)* were synthesized and characterized by elemental analysis, IR, and ^{1}H NMR spectra. Their inhibitory effects on cell growth and macromolecular synthesis of Friend leukemia cells in culture as well as the *in vivo* antitumor activity towards L1210 leukemia in mice were compared with those of complexes containing differently substituted aromatic acid hydrazides. Some of the complexes exhibited antineoplastic activity. No correlation between the *in vitro* cytotoxicity and the *in vivo* antitumor activity was found. However, there was a relationship between the *in vitro* macromolecular synthesis inhibition profile and the *in vivo* antineoplastic effect, similar to that of cisplatin. On the other hand, only agents containing one ammine ligand were active *in vivo.* The substitution of the aromatic ring by a cycloalkane residue increased significantly the antitumor effect, with $[Pt(NH_3)(chcah)Cl_2]$ being the most active compound in this study.

Introduction

The considerable success of *cis*-diamminedichloroplatinum(II) (cisplatin, DDP) in clinical oncology has stimulated the synthesis and screening of a great variety of platinum coordination compounds as potential antitumor agents.

We recently described a series of new platinum(II) complexes of benzoic- (bah), 3-methoxybenzoic (mbah) acid hydrazides (Dodoff *et al.,* 1994), and of 4-methoxy- (pmbah) and 4-chlorobenzoic (pcbah) acid hydrazides (Dodoff *et al.,* 1995). The interest in such ligands was provoked by the data about various biological activities, including antitumor effect (Rutner *et al.,* 1974).

In order to evaluate the effect of the substitution of the aromatic acid hydrazides by cycloalkane ligands on the activity of platinum complexes, a series of platinum coordination compounds with the hydrazide of cyclohexanecarboxylic acid was synthesized and characterized. Such ligands are known to exert biological activity (Offe *et al.,* 1952; Yale *et al.,* 1953). The cytotoxicity of these compounds on Friend leukemia (FL) cells in culture was compared with that of related complexes, previously reported (Dodoff *et al.,* 1994; 1995). Moreover, all platinum complexes containing chlorine anion ligands, were further characterized by their effect on macromolecular (DNA, RNA and protein) synthesis in FL cells, and tested for *in vivo* antitumor activity against L1210 leukemia.

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Materials and Methods

Chemicals

$K_2[PtCl_4]$ and *cis*-$[Pt(NH_3)_2Cl_2]$ (DDP) were prepared according to Spassovska *et al.* (1981). DDP was synthesized by a 6-stage process beginning with the dissolution of platinum to H_2PtCl_6, with a subsequent conversion into $K_2[PtCl_6]$. The reduction to $K_2[PtCl_4]$ was carried out at very mild conditions. $K_2[PtCl_4]$ was converted into $K_2[PtI_4]$ which was reacted with ammonia to give *cis*-$[Pt(NH_3)_2I_2]$. By reaction of this complex with $AgNO_3$ was obtained *cis*-diamminediaquaplatinum(II) from which upon interaction with

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KCl the final product DDP was obtained. $K[Pt(NH_3)Cl_3]$ was prepared according to Muir *et al.* (1988). The hydrazide of cyclohexanecarboxylic acid *(chcah)* was synthesized following Olsen *et al.* (1948). The remaining chemicals were AR grade products.

Synthesis

The procedures for preparation of platinum complexes of *chcah* were analogous to those described in Dodoff *et al.* (1994), which were based on the method of Kharitonov *et al.* (1975).

$[Pt(chcah)_2Cl_2]$ (I). A solution of 0.256 g (1.80 mmol of *chcah* in 30 ml water was added to stirred solution of 0.250 g (0.60 mmol) of $K_2[PtCl_4]$ in 10 ml water. Cream yellow precipitate began to appear about 15 min after mixing of the reagents. The reaction was carried out for five hours at room temperature, thereafter the precipitate was filtered, washed with water and methanol, and dried *in vacuo.* Yield: 0.241 g (73%), m.r. 245–250 °C (dec.).

Anal. Calcd. for $C_{14}H_{28}Cl_2N_4O_2Pt$: C, 30.55; H, 5.13; N, 10.18; Cl, 12.88; Pt, 35.44. Found: C, 30.28; H, 4.98; N, 10.31; Cl, 12.89; Pt, 35.96.

$[Pt(NH_3)(chcah)Cl_2]$ (II). A solution of 0.311 g (2.19 mmol) of *chcah* in 15 ml water was added while stirring to a solution of 0.800 g (2.24 mmol) of $K[Pt(NH_3)Cl_3]$ in 7 ml water. After 6 h mixing at room temperature the light yellow precipitate was filtered, washed with water and cold methanol. The complex was recrystallized from methanol. Yield: 0.730 g (78%), m.r. 155–162 °C (dec.).

Anal. Calcd. for $C_7H_{17}Cl_2N_3OPt$: C, 19.77; H, 4.03; N, 9.88; Cl, 16.68; Pt, 45.88. Found: C, 20.21; H, 4.00; N, 9.72; Cl, 16.20; Pt, 45.25.

$[Pt(chcah)_2X_2]$, X=Br (III), X=I (IV). The solutions of $K_2[PtBr_4]$ and $K_2[PtI_4]$ were obtained *in situ* according to Kharitonov *et al.* (1975). Solutions of 0.250 g (0.60 mmol) of $K_2[PtCl_4]$ in 5 ml water and 1.5 g (12.60 mmol) KBr in 3 ml water (respectively 0.418 g (2.52 mmol) of Kl in 2 ml water) were mixed and heated on a water bath (20 min at 60–70 °C for KBr solution and 20 min at 35–40 °C for Kl solution). A solution of 0.256 g (1.80 mmol) of *chcah* in 15 ml water was added while stirring to a solution of the corresponding $K_2[PtX_4]$. A light yellow precipitate for the bromo-complex, or a dark yellow precipitate for the iodo-complex were immediately formed. After 3 h they were filtered, washed with water and ethanol. The complexes were recrystallized from methanol: tetrahydrofuran 1:5. Yields: III – 0.310 g (80%); IV – 0.360 g (81%), m.r. for III 235–240 °C (dec.); m.r. for IV 215–225 °C (dec.).

Anal. Calc. for $C_{14}H_{28}Br_2N_4O_2Pt$: C, 26.30; H, 4.41; N, 8.76; Br, 25.00; Pt, 30.52. Found: C, 26,76; H, 4.54; N, 8.53; Br, 24.53; Pt, 30.87.

Anal. Calc. for $C_{14}H_{28}I_2N_4O_2Pt$: C, 22.93; H, 3.85; N, 7.64; Pt, 26.60. Found: C, 22.68; H, 4.06; N, 7.72; Pt, 26.16.

Analyses

The elemental analyses were performed by routine microanalytical 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 (4000–400 cm^{-1}), (400–150 cm^{-1}).

In vitro cytotoxic assay

FL cells (clone F4N) were grown in Dulbecco's modified Eagle medium, supplemented with 10% calf serum, under 5% CO_2 atmosphere at 37 °C. The cultures were passed every day at a concentration of 5×10^5 cells/ml.

The compounds studied were dissolved immediately before use in DMSO to obtain stock solution of various concentrations. Each of these solutions was then used at 1% concentration in the experiments. Control cells were incubated in the presence of 1% DMSO. The final concentration of DMSO in the medium did not affect the viability of the cells.

Exponentially growing cells were incubated in triplicate with increasing concentrations of test compounds for 24, 48 and 72 h, and counted thereafter hemocytometrically. The number of dead cells was determined by staining with trypan blue. The mean of two independent determinations was

calculated. The 50% inhibitory dose (IC_{50}) 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).

FL 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, [^{3}H]thymidine (0.02 MBq), [^{14}C]uridine (0.02 MBq) or [^{14}C]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 two independent experiments was calculated.

Table I. Spectral data for the ligand and complexes.

No	Compound	1. IR spectral data [ν, cm^{-1}]*					
		ν(NH_2) ν(NH) ν(NH_3)	Amide I δ(NH_2) δ_d(NH_3)	ν_{as}(CH_2) ν_s(CH_2) δ(CH_2)	Amide II	ω(NH_2) δ_s(NH_3)	ν(Pt-X)
	chcah	3311 s 3207 w 3045 w	1627 s	2931 s 2854 m 1450 w	1535 m	1342 w 1328 w	–
I	[Pt(chcah)$_2$Cl$_2$]	3270 s 3211 m 3156 m	1658 s 1585 m	2932 s 2856 m 1450 m	1557 m 1510 m	1343 w 1329 w	331 m 323 m,sh
II	[Pt(NH_3)(chcah)Cl$_2$]	3275 s 3175 s	1657 s 1600 m	2929 s 2853 m 1448 m	1562 m 1518 m,sh	1331 m 1318 m 1300 m	329 m,sh 322 m,sh
III	[Pt(chcah)$_2$Br$_2$]	3249 s 3193 s 3139 m,sh	1656 s 1592 m	2929 s 2852 m 1446 m	1562 m 1534 m	1335 m 1320 m	246 m 242 m
IV	[Pt(chcah)$_2$I$_2$]	3231 s 3105 m	1647 s	2929 s 2851 m 1442 m	1565 s 1512 m	1334 w 1320 w	206 m 204 m

		2. ^{1}H NMR spectral data (δ, ppm) for DMSO-d_6 solutions**			
		NH_3	CH_2 α-CH	NH_2	NH
	chcah	–	1.10–1.41 m 1.59–1.70 m 1.98–2.09 m	4.10 s	8.85 s
I	[Pt(chcah)$_2$Cl$_2$]	–	1.15–1.39 m 1.63–1.71 m 2.17–2.22 m	7.63	9.68
II	[Pt(NH_3)(chcah)Cl$_2$]	4.26 br	1.16–1.35 m 1.59–1.71 m 2.15–2.28 m	7.57	9.63
III	[Pt(chcah)$_2$Br$_2$]	–	1.18–1.40 m 1.66–1.80 m 2.14–2.24 m	7.50	9.65
IV	[Pt(chcah)$_2$I$_2$]	–	1.16–1.37 m 1.65–1.78 m 2.15–2.25 m	7.47	9.85

* Abbreviations: s, strong; m, medium; w, weak; sh, shoulder.
** Abbreviations: s, singlet; m, multiplet; br, broad.

In vivo antitumor evaluation

The method of European Organisation Research of Treatment of Cancer (EORTC) screening group (1972) was used to determine the toxicity and therapeutic doses of the compounds.

The complexes were tested against L1210 leukemia. BDF_1 mice (20–22 g) were given i.p. L1210 cells (1×10^5, suspended in 0.1 ml of saline). After 24 h (day 1) of tumor transplantation the compounds were administered i.p. as saline suspensions at doses and schedules shown in Table IV.

The antitumor activity was defined as percent increased survival time determined by dividing the mean survival time of treated animals by that of control animals (% T/C).

The statistical analysis was performed using the Student's t-test.

Results and Discussion

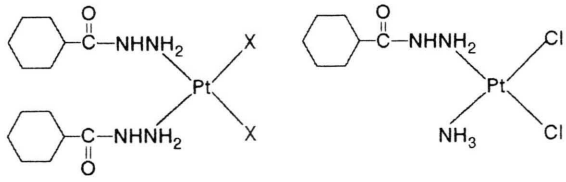
The formulae, analytical and some physical data of the newly synthesized complexes are given in the section Materials and Methods. The complexes are yellow-coloured solids that decompose upon heating. They are soluble in DMF or DMSO, and are practically insoluble in water.

Infrared spectral data for the ligand and complexes as CsI disks are collected in Table I.1. The bands in the range of absorptions of the $\nu(NH_2)$ and $\nu(NH)$ stretching vibrations in the spectra of the complexes were shifted to lower frequency as compared to those of the free ligand. This is indicative of a coordination of the hydrazide to the platinum *via* the NH_2 group. In the far IR range of the spectra were registered two bands which are indicative of a *cis*-configuration of the complexes. Some of the weak bands in the range of 565–486 cm^{-1} in the spectra of the complexes could tentatively be ascribed to Pt–N stretchings.

The ^1H NMR spectral data (DMSO-d_6) of the free ligand and the respective platinum complexes are presented in Table I.2. In the spectra of the hydrazide of the cyclohexanecarboxylic acid, a complicated multiplet was observed, due to the methylene protons at the cyclohexane ring. A clear triplet of triplets was registered in the range of 1.98–2.09 ppm because of the existing α-CH proton of the cyclohexane residue. Signals for NH_2 and NH groups were observed at 4.10 and 8.85 ppm, respectively. For all complexes, the signal of the NH_2 protons was significantly shifted downfields, and those of NH protons were much less moved in comparison with the free ligand. This shows that *chcah* coordinate to platinum *via* the NH_2 group. In the spectra of the platinum complexes no significant shift of the signals due to the cyclohexyl residue was registered.

Similar IR and ^1H NMR spectral data have been reported in our previous papers for platinum complexes of differently substituted benzoic acid hydrazides (Dodoff *et al.*, 1994; 1995).

The elemental analysis and spectroscopic data are in accordance with the structures for the complexes shown in Fig. 1.



I X=Cl
III X=Br
IV X=I

II

In Table II, the data of growth-inhibitory effect of 24, 48 and 72 h incubation of FL cells with platinum complexes of cyclohexanecarboxylic acid hydrazide were contrasted with those of complexes with benzoic acid hydrazide as a ligand (Dodoff *et al.*, 1994). Comparing the IC_{50} values, it could

Table II. Cytotoxicity of platinum complexes containing hydrazides of benzoic or cycloalkanecarboxylic acids on Friend leukemia cells.

No	Compound	IC_{50}[a] [μM]		
		24 h[b]	48 h	72 h
	chcah		[c]	
I	$[Pt(chcah)_2Cl_2]$	13.75	11.10	12.75
II	$[Pt(NH_3)(chcah)Cl_2]$	13.00	4.25	8.60
III	$[Pt(chcah)_2Br_2]$	5.75	3.55	13.00
IV	$[Pt(chcah)_2I_2]$	26.50	9.75	24.30
	bah		[c]	
V	$[Pt(bah)_2Cl_2]$	8.8	5.3	3.8
VI	$[Pt(NH_3)(bah)Cl_2]$	9.2	5.6	4.4
VII	$[Pt(bah)_2Br_2]$	8.5	5.4	2.7
VIII	$[Pt(bah)_2I_2]$	7.6	5.6	4.2
	Cisplatin	5.8	4.9	3.9

[a] Drug concentration that reduces the number of living cells by 50%; [b] Time of drug treatment; [c] No inhibition of cell growth at concentration up to 100 μM.
Values are mean of triplicate determinations in two independent experiments.

be concluded that the complexes with cycloalkane ligands are less toxic than platinum compounds with aromatic ligands. Thus, after 72 h incubation with the new complexes the FL cells seemed to recover from their inhibitory activity.

Further, we examined the macromolecular synthesis inhibition in FL cells and the *in vivo* antitumor activity against L1210 leukemia of selected new as well as previously synthesized platinum coordination compounds (Dodoff *et al.*, 1994; 1995) containing chlorine as acid ligands. Complexes with bromine or iodine anions were not included since they were inactive against experimental tumors (unpublished results).

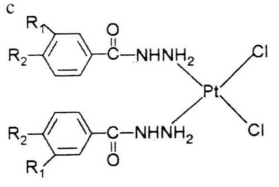
FL cells were incubated for 24 h with the compounds, and thereafter [^{3}H]thymidine, [^{14}C]uridine or [^{14}C]leucine was added to follow DNA, RNA or protein biosynthesis, respectively. Drug concentrations, required to reduce by 50% precursor incorporation into FL cells, are presented in Table III. As a whole, the macromolecular synthesis inhibition profile followed that obtained by the cell-growth assay (Table II; see also Dodoff *et al.*, 1994). However, some agents (**II, XII**) inhibited specifically DNA synthesis, similar to cisplatin, and less those of RNA or protein.

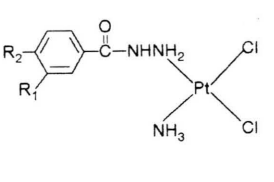
Table III. Effect of platinum complexes on macromolecular synthesis in FL cells.

No	Complex	IC_{50}[a] [μM]		
		DNA	RNA	Protein
I	$[Pt(chcah)_2Cl_2]$	10.25	16.8	9.4
II	$[Pt(NH_3)(chcah)Cl_2]$	8.0	[b]	[b]
V	$[Pt(bah)_2Cl_2]$[c]	5.2	7.4	5.0
VI	$[Pt(NH_3)(bah)Cl_2]$[c]	4.4	6.6	2.3
IX	$[Pt(mbah)_2Cl_2]$[c]	1.9	3.9	2.2
X	$[Pt(NH_3)(mbah)Cl_2]$[c]	11.6	10.5	6.4
XI	$[Pt(pmbah)_2Cl_2]$[c]	1.6	5.0	4.0
XII	$[Pt(NH_3)(pmbah)Cl_2]$[c]	4.0	10.0	8.1
XIII	$[Pt(pcbah)_2Cl_2]$[c]	1.0	5.1	1.8
XIV	$[Pt(NH_3)(pcbah)Cl_2]$[c]	1.2	5.0	3.9
	Cisplatin	1.2	8.5	5.1

[a] Drug concentration that reduces by 50% the incorporation of [^{3}H]thymidine, [^{14}C]uridine and [^{14}C]leucine into DNA, RNA and protein, respectively;
[b] No inhibition of macromolecular synthesis at concentration up to 25 μM.
Values are mean of duplicate determinations in two independent experiments.

c





V $R_1=R_2=H$
IX $R_1=OCH_3$; $R_2=H$
XI $R_1=H$; $R_2=OCH_3$
XIII $R_1=H$; $R_2=Cl$

VI $R_1=R_2=H$
X $R_1=OCH_3$; $R_2=H$
XII $R_1=H$; $R_2=OCH_3$
XIV $R_1=H$; $R_2=Cl$

It was of interest to compare these results with the *in vivo* antitumor activity of the compounds tested. L1210-leukemia-bearing mice were treated intraperitoneally with platinum complexes at doses and schedules shown in Table IV.

The agents $[Pt(NH_3)(chcah)Cl_2]$ (**II**), $[Pt(NH_3)(pmbah)Cl_2]$ (**XII**), and $[Pt(NH_3)(pcbah)Cl_2]$ (**XIV**) administered 3 times on days 1, 5, 9 after tumor transplantation, exhibited antineoplastic activity, with complex **II** being most active. The introduction of **II** on days 1, 3, 5 did not change significantly the antitumor effect.

Comparing the data of Tables II, III and IV, and those reported by us previously (Dodoff *et al.*,

Table IV. Antitumor activity of platinum(II) complexes in L1210 leukemia.

No	Complex	Dose [mg/kg]	Schedule[a]	% T/C[b]
I	$[Pt(chcah)_2Cl_2]$	21×3	1, 5, 9	105
II	$[Pt(NH_3)(chcah)Cl_2]$	21×3	1, 5, 9	137[c], 131, 137[c]
		21×3	1, 3, 5	144[c]
		32×3	1, 5, 9	152[c], 158[c]
		32×3	1, 3, 5	156[c], 164[c]
		42×3	1, 5, 9	toxic
V	$[Pt(bah)_2Cl_2]$	10×3	1, 5, 9	108
VI	$[Pt(NH_3)(bah)Cl_2]$	17×3	1, 5, 9	119
IX	$[Pt(mbah)_2Cl_2]$	10×3	1, 5, 9	104
X	$[Pt(NH_3)(mbah)Cl_2]$	21×3	1, 5, 9	117
XI	$[Pt(pmbah)_2Cl_2]$	52×3	1, 5, 9	109
XII	$[Pt(NH_3)(pmbah)Cl_2]$	84×3	1, 5, 9	137[c]
XIII	$[Pt(pcbah)_2Cl_2]$	42×3	1, 5, 9	101
XIV	$[Pt(NH_3)(pcbah)Cl_2]$	42×3	1, 5, 9	125
	Cisplatin	5×3	1, 5, 9	240[c]

[a] The dose was administered intraperitoneally on day 1, one day after tumor transplantation, and second and third doses on days 5 and 9, respectively. The compound **II** was also given on days 1, 3 and 5.
[b] The mean survival time of treated animals (T) divided by the mean survival time of control animals (C) and then multiplied by 100.
[c] $p<0.05$ vs. saline.

1994; 1995), some important conclusions could be drawn:

First, there is no correlation between the *in vitro* cytotoxic effect and the *in vivo* antitumor activity. Thus, agent **II** exhibiting the highest antitumor activity, showed a moderate cytotoxicity against FL cells in culture, comparable with that of the inactive compound **I**.

Secondly, there is a relationship between the *in vitro* inhibition profile of DNA, RNA and protein synthesis, and the antineoplastic activity of the compounds. Agents with activity towards L1210 leukemia displayed a macromolecular synthesis inhibition pattern similar to that of cisplatin.

Thirdly, regarding the structure-activity relationship, it is evident that only agents containing an ammine ligand exhibited antitumor activity. This finding is in accordance with the observed requirement of at least one ammine ligand for the proper binding of a clinically active platinum agent to DNA, as reported recently by Takahara *et al.* (1995). The substitution of the aromatic ring in the complexes by a cycloalkane residue increased significantly the antineoplastic activity. Thus, complex **II** with chcah as a ligand produced the highest effect with L1210 leukemia, while the relative agent **VI** was inactive.

Acknowledgements

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