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Laboratory note

Synthesis, physicochemical and in vitro pharmacological investigation of new platinum (II) complexes with some cycloalkanespiro-5′-hydantoins

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Abstract

Platinum (II) complexes with cyclobutanespiro-5′-hydantoin and cycloheptanespiro-5′-hydantoin were synthesized and evaluated by means of general physicochemical methods. The data from the elemental analysis, IR and NMR spectra suggested the formation of *cis*-$[Pt(C_6H_8N_2O_2)_2(NH_3)_2](NO_3)_2{\cdot}4H_2O$ (PtCBH), when cyclobutanespiro-5′-hydantoin was used as a ligand and *cis*-$[Pt(C_9H_{14}N_2O_2)(NH_3)_2](NO_3)_2{\cdot}4H_2O$ (PtCHTH), when cycloheptanespiro-5′-hydantoin was used, respectively. The novel complexes exerted cytotoxic effects at micromolar concentrations against a panel of human tumor cell lines. They were found to trigger apoptosis in HL-60 and BV-173 cells as evidenced by DNA-laddering detection. The evaluation of the effects of PtCBH, PtCHTH and the antineoplastic drugs cisplatin and oxaliplatin against cultured murine kidney epithelial cells revealed that the hydantoin complexes were far less nephrotoxic in vitro.

Keywords: Pt(II) complexes; Hydantoins; Cytotoxicity; Nephrotoxicity

1. Introduction

The square planar platinum complex *cis*-dichlorodiammineplatinum (II) (cisplatin) is an important antineoplastic agent, effective for the therapeutic management of various solid malignancies such as testicular teratoma, ovarian, cervical and bladder cancers, malignant melanoma, head and neck cancers, non-small cell lung cancer, endometrial cancer etc. [1–3]. Unfortunately the clinical success of cisplatin is limited by its severe side effects such as nephrotoxicity, cumulative neurotoxicity, ototoxicity, and extreme emetogenic potential [2–4]. Other major factor compromising its clinical usefulness is the development of acquired or primary resistance of malignant cells by virtue of different cellular mechanisms [5–7]. Therefore, much attention has been focused on designing cisplatin analogues with reduced toxicity and/or broader antitumor spectrum [6,8]. In spite of the numerous compounds investigated so far, only few of them are clinically applied as drugs—carboplatin, nedaplatin and oxaliplatin [2,6,8,9]. Despite having some advantages as compared to cisplatin, these agents are by no means ideal substitutes for the prototype since carboplatin is more myelotoxic, whereas oxaliplatin treatment is frequently associated with neurotoxicity [2,3,6].

Some recent advances in platinum-based drug development, however revealed that the structural resemblance to the prototype is not an obligatory requirement for cytotoxic activity [6,8–11]. One of the alternative approaches towards development of non-classic platinum-based antineoplastic agents is focused upon charged species, whereby the platinum atom is bound to more than two donor nitrogen atoms [8]. We have

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previously described three cationic diammine Pt(II) complexes with substituted hydantoin ligands, that proved to exert cytotoxic activity in vitro [12,13]. Although less potent than cisplatin in respect to IC_{50} values these agents exerted considerable cytotoxic effects at higher concentrations and some of them were proved to induce programmed cell death in malignant cells [13].

The present study represents the synthesis, physicochemical evaluation and the pharmacological investigation of Pt(II) complexes with cyclobutane- and cycloheptanespiro-5′-hydantoins as well as the assessment of their nephrotoxic potential in vitro as compared to the clinically applied drugs cisplatin and oxaliplatin.

2. Chemistry

The ligands cyclobutanespiro-5′-hydantoin (1,3-diazaspiro [4,3] octane-2,4-dione) (CBH) and cycloheptanespiro-5′-hydantoin (1,3-diazaspiro [4,6]dodecane-2,4-dione) (CHTH) were synthesized as previously described [14]. *Cis*-dichlorodiammineplatinum (II) utilized for the synthetic procedures was purchased from Merck, Germany. All of the other chemicals were of analytical grade.

3. Pharmacology

In the present study a comparative evaluation of the cytotoxic effects of two newly synthesized platinum (II) complexes and the referent agent cisplatin in a panel of human tumor cell lines, using the standard MTT-dye reduction assay for cell viability was performed. The panel consisted of the acute myeloid leukemia-derived HL-60, the chronic myeloid leukemia-derived CML-T1 and BV-173, the chronic lymphoid leukemia-derived SKW-3, the Hodgkin-lymphoma derived HD-MY-Z and the urinary bladder carcinoma-derived 5637 cell lines.

In order to elucidate some of the mechanistic aspects of the cytotoxicity of the new Pt(II) complexes we carried out DNA-isolation and gel electrophoresis to probe their ability to trigger apoptotic cell death.

In addition, considering the importance of the nephrotoxicity of cisplatin as a major dose-limiting factor for its high-dose clinical use we evaluated the direct cytotoxic potential of the novel complexes and cisplatin against primary cultures of murine kidney epithelial cells.

4. Results and discussion

4.1. Chemistry

On the basis of the data from the elemental analysis for the new complexes the following formulae can be derived: *cis*-$[Pt(C_6H_8N_2O_2)_2(NH_3)_2](NO_3)_2{\cdot}4H_2O$ (PtCBH), when cyclobutanespiro-5′-hydantoin was used as a ligand and *cis*-$[Pt(C_9H_{14}N_2O_2)(NH_3)_2](NO_3)_2{\cdot}4H_2O$ (PtCHTH), when cycloheptanespiro-5′-hydantoin was used, respectively. In order to evaluate the mode of coordination of the ligands to the metal ion, the IR-spectra of the pure ligands as well as of their Pt(II) complexes were recorded. The data are shown on Table 1.

The comparative analysis of the IR-spectra of the free ligand cyclobutanespiro-5′-hydantoin and of the corresponding Pt(II) complex revealed that the bands, characteristic for $\nu^{as}_{C=O}$ and $\nu^{s}_{C=O}$ at 1775 and 1732 cm^{-1}, were unchanged in the complex. This finding is an indication that these groups are not involved in the binding to the metal ion. In the IR-spectrum of the complex the bands, characteristic for the stretching vibrations of the two NH groups, were shifted towards the higher frequencies. The shifting assigned to one of the NH groups was more pronounced in comparison to the other, which is an indication that only one of the NH groups is involved in the complex formation. A new band at 1652 cm^{-1} appeared in the spectrum of the complex, that could be assigned to δ(NH). On the basis of these data it could be concluded that, most probably, the ligand coordinates monodentally to the platinum, through one of the NH groups from the hydantoin ring.

In the IR-spectrum of the Pt(II) complex with cycloheptanespiro-5′-hydantoin, the bands related to the stretching vibrations of the two carbonyl groups at 1771 and 1710 cm^{-1} remained unchanged in the complex. This fact is an evidence that these groups are not involved in the complex formation. The bands, characteristic for the stretching vibrations of the two NH groups were shifted towards the higher frequencies—from 3204 and 3282 cm^{-1} to 3229 and 3304 cm^{-1}, respectively. The shifting was found to be equal for the two NH groups, which shows that they are both involved in the complex formation (bidentate binding). Two new bands at 1610 and 1282 cm^{-1} appeared, respectively, in the spectrum of the complex, that could be assigned to δ(NH).

Additional information, regarding the coordination-mode of the metal ion to the ligand, was retrieved from the ^{1}H- and ^{13}C-NMR spectra, recorded for the free ligands and the corresponding platinum complexes. The data are summarized in Table 2.

The comparative analysis of the ^{1}H-NMR spectra of the cyclobutanespiro-5′-hydantoin and of the corresponding com-

Table 1
IR data for the free ligands CBH, CHTH and for the complexes PtCBH, PtCHTH

Compound	ν(NH)	δ(NH)	ν(C=O)
CBH	3210	–	1775
	3119		1732
PtCBH	3218	1652	1776
	3209		1737
CHTH	3204	–	1771
	3282		1710
PtCHTH	3229	1610	1771
	3304	1282	1714

Table 2
NMR data for the free ligands CBH, CHTH and for the complexes PtCBH, PtCHTH

Compound	^{1}H-NMR				^{13}C-NMR				
	H(6), H(8)	H(7)	H(1)	H(3)	C(7)	C(6), C(8)	C(5)	C(2)	C(4)
CBH	1.64–1.75m	1.85–1.93m	8.14	10.50	37.2	24.8	68.5	179.5	156.5
PtCBH	2.21–2.33m	1.71–1.87m	8.32	10.54	32.6	13.4	61.8	178.8	156.2
	H(6), H(11)	H(7)–H(10)	H(1)	H(3)	C(6), C(11)	C(7)–C(10)	C(5)	C(2)	C(4)
CHTH	1.76–1.83m	1.52–1.64m	8.21	10.45	37.3	22.5–29.2	65.0	162.6	186.5
PtCHTH	2.34m	1.21–1.90m	8.09	–	37.4	22.5–29.1	66.3	156.2	180.1

plex showed that in the spectrum of the complex the signals of the protons from the cyclobutane ring were insignificantly shifted towards the range of 2.33–1.71 ppm. The signals of the protons at the N-atoms from the hydantoin ring were shifted as well. This shift was more pronounced for the proton at N(1) (from 8.14 to 8.32 ppm), while the signal for the proton at N(3) was shifted very slightly (from 10.50 to 10.54 ppm). This fact indicates a monodentate binding of the ligand, through the N(1) atom of the hydantoin ring to the platinum.

In the ^{1}H-NMR spectrum of the Pt(II) with cycloheptanespiro-5′-hydantoin a shifting of the signal for H(1) (from 8.21 to 8.09 ppm) was encountered. The signal at the H(3) was not visible in the spectrum. The signals for the protons of the cycloheptane ring were not affected and they absorbed as a wide multiplet in the range 2.34–1.21 ppm.

The juxtaposition of the ^{13}C-NMR spectra of the free ligand and the corresponding Pt(II) complex with cyclobutanespiro-5′-hydantoin, showed an up field shifting of the signal for the C-atoms from the cyclobutane ring in the ^{13}C-NMR spectrum of the complex. In the spectrum of the ligand the band for C(5) was at 68.5 ppm, while in the complex it was shifted to 61.8 ppm. The signals for the methylene groups from the cyclobutane ring were shifted from 37.2 and 24.8 ppm in the ligand to 32.6 and 13.4 ppm in the complex, respectively. This chemical up field shifting is most probably due to the diamagnetic screening of the platinum ion [13]. The values of the chemical shifting of C=O groups were almost not affected. The shift of the signal for C(2), which is closer to N(1) was much more influenced due to the complex formation.

In the ^{13}C-NMR spectrum of the free ligand cycloheptanespiro-5′-hydantoin the chemical shifts encountered were as follows: 186.5 ppm (C(4)), 162.6 ppm (C(2)), 65.0 ppm (C(5)). In the ^{13}C-NMR spectrum of the corresponding complex a shifting of the signals for C(2) and C(4) (162.6 and 186.5 ppm, respectively) was observed. signal for C(5) (66.3 ppm), was slightly affected, whereas the signals for the carbon atoms from the cycloheptane ring were practically not affected at all.

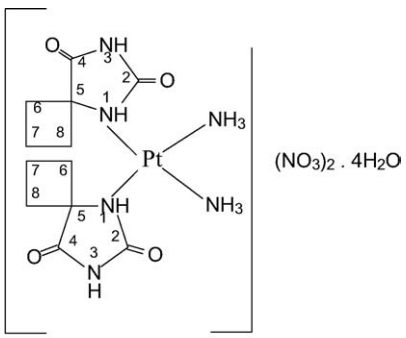
An additional investigation of the ligands and the complexes of Pt(II) in solution was carried out. Solutions (10^{-3} M) in DMSO were prepared and their electro-conductance was evaluated. For cyclobutanespiro-5′-hydantoin a λ_M value of 1.323 μS cm^{-1} was encountered, whereas for the complex the λ_M value was 48.3 μS cm^{-1}. For cycloheptanespiro-5′-hydantoin the value for λ_M was 8.4 and 42.2 μS cm^{-1} for the complex. According to the referent literature data, the newly synthesized complexes could be regarded to the 1:2 type of electrolytes [14].

On the basis of the results from the physicochemical investigations, the following, most probable schematic formulas of the Pt(II) complex with cyclobutanespiro-5′-hydantoin and of the Pt(II) complex with cycloheptanespiro-5′-hydantoin could be proposed (Fig. 1).

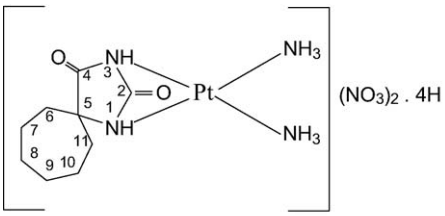
4.2. Pharmacology

4.2.1. In vitro cytotoxicity

The evaluation of the cell viability in a panel of human tumor cell lines, following 48 h treatment revealed that the referent drug and the new Pt(II) complexes exerted cytotoxic effects in a concentration-dependent manner. The concentration–response curves were drawn (Fig. 2) and the IC_{50} values were extrapolated. As evident from the IC_{50} values obtained (Table 3) the new Pt(II) compounds with cycloalkanespiro-



PtCBH



PtCHTH

Fig. 1. Schematic structures of the Pt(II) complexes PtCBH and PtCHTH.

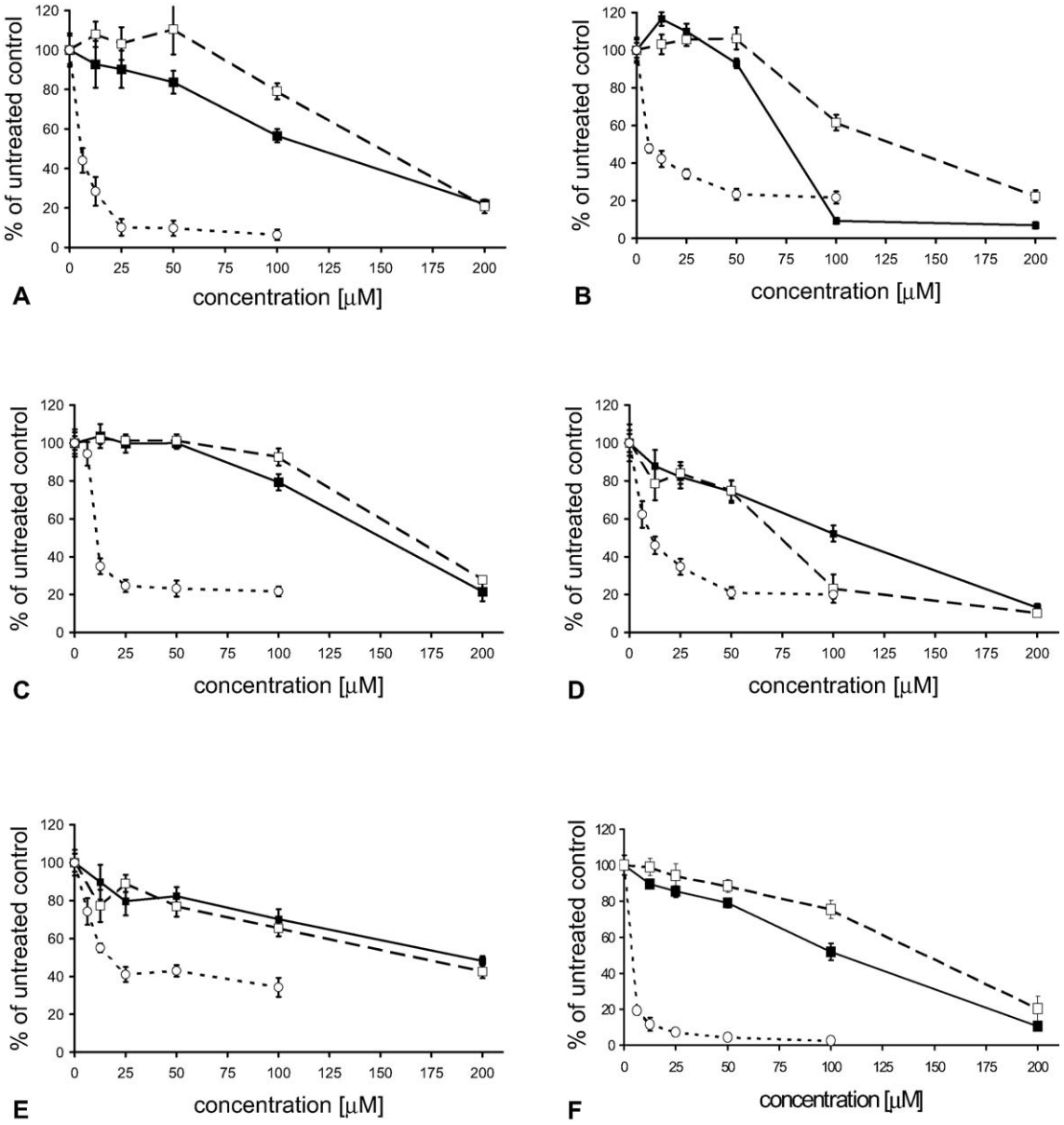


Fig. 2. Cytotoxic effects of cisplatin (---○---), PtCBH (—■—), PtCHTH (– –□– –) as assessed by the MTT-dye reduction assay following 48 h treatment of HL-60 (A), CML-T1 (B), BV-173 (C), SKW-3 (D), HD-MY-Z (E) and 5637 cells (F). Each data point represents the arithmetic mean ± standard deviation of at least eight independent experiments.

Table 3
Relative potency (IC_{50} values) of PtCHTH, PtCBH and cisplatin obtained from the corresponding concentration–response curves

Cell line	Cell type	IC_{50} value (μM)		
		cis-DDP	PtCBH	PtCHTH
HL-60	Acute myeloid leukemia	5.55	119.2	149.9
CML-T1	Chronic myeloid leukemia	6.01	76.66	129.13
BV-173	Pre-B-cell leukemia	10.07	150.73	160.1
SKW-3	Chronic lymphoid leukemia	10.87	105.09	75
HD-MY-Z	Hodgkin-lymphoma	16.87	192.02	167.03
5637	Urinary bladder carcinoma	3.95	104.47	146.01

5′-hydantoins were far less active than the referent drug causing 50% cell growth inhibition at substantially higher concentrations.

The observed difference in the relative potencies between the new Pt(II) complexes and cisplatin are most probably an outcome of their slower ligand exchange rate in comparison to the referent drug; it is well established that cisplatin and its analogues elicit their cytotoxic effects after intracellular conversion to mono- and diaquacomplexes [1,2,6]. These aquated species are extremely electrophilic and cross-link DNA, through covalent bonding, preferably to the N_7 position of guanine [1–6]. However, while cisplatin is prone to prompt ligand exchange its organic analogues, such as carboplatin are more stable, because of the substitution of the chloride

anions to the bidentate cyclobutanedicarboxylate ligand and thus carboplatin exerts its cytotoxic effects at higher concentrations [2,3].

The new Pt(II) compounds are cationic complexes with four donor nitrogen atoms bound to the Pt, and so they actually lack the conventional leaving groups (organic or halogenide anions), present in most of the platinum complexes synthesized so far. Thus, we could suggest, that their lower cytotoxic activity could be explained by slower if at all substitution of the bidentate hydantoin ligand to water molecules, because of the better donor properties of the heterocyclic N-atoms as compared to the chloride anions in cisplatin.

This study was in part conducted in order to elucidate further some of the structure activity relationships (SAR) for the cationic diamminecycloalkanespiro-5′-hydantoin complexes. In accordance with some previous investigations, showing that the increase of the cykloalkane substitute reduces the relative potency of the complexes, herein we encountered that the introduction of the cycloheptane moiety reduced the cytotoxicity of the compound compared to the previously described complex with cyclopentanespiro-5′-hydantoin ligand [12,13]. The data for the cyclobutanespiro-5′-hydantoin complex, however could not be interpreted in respect to SAR elucidation, since the physicochemical characterization of this compound suggested for monodentate binding of two hydantoin ligands, in contrast to the chelate structure of the other cationic platinum complexes described so far.

Despite of their lower relative potencies however, both of the new complexes demonstrated considerable maximal efficacy in the panel investigated, which was generally comparable to that of the referent drug.

4.2.2. DNA-fragmentation analysis

Following 24 h treatment of SKW-3 and BV-173 cells with either PtCBH or PtCHTH, at 100 μM led to an apparent DNA-laddering, that is a major hallmark of programmed cell death and is an outcome of the action of activated nucleases that degrade the higher order chromatin structure of DNA into mono- and oligonucleosomal DNA-fragments during the apoptotic process (Fig. 3).

These data show that the induction of apoptosis at least in part mediates the cytotoxic effects of the new complexes, a feature that is well documented for cisplatin and its clinically applied analogues [4,6].

4.2.3. In vitro nephrotoxicity

The performed 72 h in vitro nephrotoxicity determination revealed that cisplatin was more potent than oxaliplatin in this aspect, whereas the two new compound PtCBH and PtCHTH induced less pronounced toxic effects on these non-malignant cells (Fig. 4). Cisplatin treatment resulted in a significant decrease of the number of viable kidney epithelial cells, even at the lowest concentration of 5 μM. When applied at concentrations higher than 10 μM, it induced more than 50% decrease of the cell survival fraction. The other clinically applied referent drug oxaliplatin caused significant

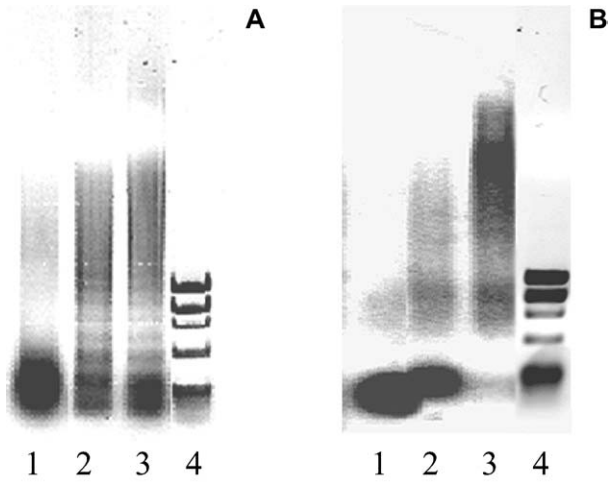


Fig. 3. Gel electrophoresis of DNA, isolated from the cytosolic fraction of SKW-3 (A) or BV-173 (B) cells following 24 h treatment with either PtCBH (lane 2) or PtCHH (lane 3) at 100 μM; untreated controls (lane 3), size marker (line 4).

decrease of the cell survival at 20 μM, whereas at 40 μM it generally reached the maximal effect encountered for cisplatin.

Taking into consideration the fact that the new Pt(II) complexes exerted cytotoxic effects against the tumor cell lines at higher concentrations than cisplatin we evaluated their in vitro nephrotoxic potential at a broader concentration range as well. In contrast to the referent agents PtCBH failed to induce any significant diminishment in cell viability at the lower concentrations evaluated (12.5; 25 and 50 μM). When applied at 100 μM it reduced the percentage of viable cells by ca. 16%, an effect registered with cisplatin at ca. 20-fold lower concentrations. At the highest concentration of PtCBH evaluated (200 μM) the vital cells were reduced to ca. 57%. The PtCHTH was found to be more cytotoxic against the normal kidney epithelium as compared to the other novel compound but as evident from the concentration–response curve depicted on Fig. 4, it was far less active than both the referent drugs under investigation.

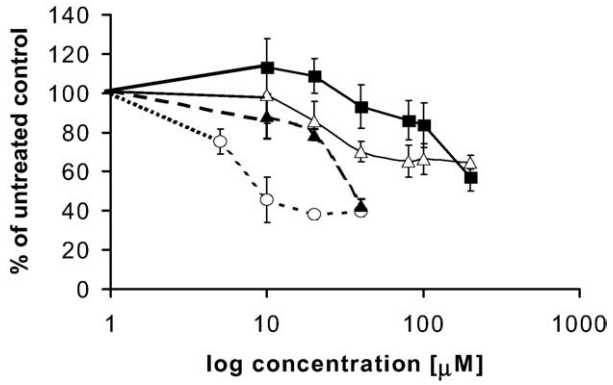


Fig. 4. In vitro nephrotoxic effects of cisplatin (---○---), oxaliplatin (--▲--), PtCBH (—■—), and PtCHTH (—Δ—) on murine kidney epithelial cells as assessed by the MTT-dye reduction assay, following 72 h treatment. Each data point represents the arithmetic mean ± standard deviation of at least eight independent experiments.

The nephrotoxicity of cisplatin is somewhat an unusual side effect which is not commonly exhibited by antineoplastic agents, since kidney epithelial cells represent a quiescent, non-rapidly proliferating cellular population [4,5]. Some recent findings suggest that several mechanisms, other than DNA-crosslinking, are implicated in the cytotoxic effects of cisplatin against the renal epithelial cells [4]. It appears that not cisplatin per se, but its biotransformation products, formed through conjugation to glutathione (GSH) are responsible for the kidney epithelial damage. In renal tissue, the Pt-GSH conjugates are found to be extensively metabolized to yield Pt-Cys conjugates, that could re-enter the proximal tubular cells, where they are finally converted to reactive thiol species triggering cell death [4,7,15].

The lower nephrotoxic potential of both oxaliplatin and especially of the spirohydantoin complexes as compared to cisplatin, that we encountered, is probably due to the presence of organic ligands, which are more stable than the chlorine ligands of cisplatin and so render the compounds less prone to form GSH-adducts.

5. Conclusion

Both the new Pt(II) complexes under investigation exhibited cytotoxic effects in micromolar concentrations although far less pronounced than the referent drug cisplatin. Based on our current data, it is not likely that these complexes could reveal comparable or higher clinical activity than cisplatin. Their ability to induce apoptosis however as well as the observed marginal nephrotoxicity suggest that the cationic diamminecycloalkanespiro-5′-hydantoin Pt(II) complexes as a distinct class of cytotoxic platinum coordination compounds deserve attention for further evaluation as antineoplastic agents.

6. Experimental protocols

6.1. Chemistry

6.1.1. General methods

The new Pt(II) complexes were characterized by elemental analysis, IR, ^{1}H- and ^{13}C-NMR spectra. The carbon, nitrogen and hydrogen content of the compounds were determined by elemental analysis. The elemental analysis was carried out on a 'Carlo Erba' apparatus. The results were within ± 0.5% of the theoretical values.

6.1.1.1. Spectral measurements. The IR-spectra were recorded on Shimadzu FTIR-8101M spectrophotometer in the range of 4500–400 cm^{-1} (nujol). The ^{1}H-NMR and ^{13}C-NMR spectra were registered on a Bruker WM 250 (250 MHz) spectrometer in DMSO-d_6. Corrected melting points were determined, using a Bushi 535 apparatus. The electroconductivity of 10^{-3} M solutions of the complexes in DMSO was measured by means of a Metrohm conductometer 660 (cell constant—9.79 cm^{-1}).

6.1.2. Synthesis of the complexes

6.1.2.1. Synthesis of the Pt(II) complex with cyclobutanespiro-5′-hydantoin. A new Pt(II) complex with cyclobutanespiro-5′-hydantoin was synthesized by means of a previously described method [16]. Briefly, *cis*-$[Pt(NH_3)_2Cl_2]$ (0.1011 g; 0.34 mmol) was added to 10 cm^3 0.07 M water solution of $AgNO_3$. The solution was heated to 50 °C at constant stirring until a precipitate of AgCl was formed and discoloration of the solution was observed. The AgCl precipitate obtained was filtered and washed several times with hot water.

$C_6H_8N_2O_2$ (L) (0.0954 g; 0.68 mmol) was dissolved in the filtrate with stirring. The solution was then evaporated at 50 °C, thus diminishing the volume. Dark-green crystals were formed, then filtered and purified repeatedly via recrystallization from hot water. Yield: ca. 60%, m.p.: >260 °C (dec.). The complex is soluble in hot water and in DMSO. Anal. Found: Pt, 26.79; C, 19.96; H, 3.14; N, 16.47; Calc. for $C_{12}H_{30}N_8O_{14}Pt$: Pt, 27.66; C, 20.43; H, 4.26; N, 15.89.

6.1.2.2. Synthesis of the Pt(II) complex with cycloheptanespiro-5′-hydantoin. The complex was synthesized, using the same method [16]. Yield: ca. 70%, m.p.: 273–276 °C (dec.). The complex is soluble in hot water and in DMSO. Anal. Found: Pt, 31.46; C, 16.26; H, 2.81; N, 13.65; Calc. for $C_9H_{28}N_6O_{12}Pt$: Pt, 32.13; C, 17.79; H, 4.61; N, 13.84.

6.2. Pharmacology

The cell culture maintenance, cellular treatment and viability determination (MTT assay) [17] as well as the DNA-isolation and gel electrophoresis were carried out as described in our previous paper [13].

6.2.1. Preparation of murine renal epithelial cell culture

The procedure was carried out as previously described with some minor modifications [18]. Briefly, kidneys from 10 adult mice where dissected out aseptically and minced until formation of tissue particles approximating 1 mm^3. These were then washed in PBS several times and allowed to sediment between the washing procedures and finally the supernatant (containing cellular debris and blood cells) was discarded. A sufficient amount of warm enzymatic solution (0.1% collagenase/0.1% trypsin) was then added to the tissue mass and it was incubated for 100 min in a water bath shaker at 37 °C. After the digestion completion the enzymatic solution was replaced with FCS supplemented RPMI-1640 medium (without glucose). The tissue suspension yielded was turned in single cell suspension by vigorously driving it through a Pasteur pipette. Finally the isolated cells were transferred in cell culture flasks at a density of 1×10^5 cm^{-2} and incubated at 37 °C for several days to allow the adhesion of the cells to the wall of the flask. Thereafter the cells were harvested via hot trypsina-

tion, counted and transferred in 96-well microplates (100 μl per well) at a density of 1.5–2 × 10^5 cells per ml. Following 24 h incubation in the microplates the kidney epithelial cells were exposed to various concentrations of *cis*-DDP, oxaliplatin, PtCBH or PtCHTH for 72 h The cell viability was thereafter assessed using the MTT-dye reduction assay.

6.2.2. Statistics

The data processing included the Student's *t*-test with $P \leq 0.05$ taken as significance level, using Microsoft EXCEL for PC.

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