Scientific Paper

Syntheses and Cytotoxicity of Pt(II) Complexes with Acyclovir

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Abstract

Acyclovir is an antiviral drug which acts against the viruses, including H*erpes simplex* 1 and 2 and *Varicella-zoster*. The novel *cis*-[PtI_2(3-Hmpy)$_2$](3-Hmpy=3-hydroxymethylpyridine)(**2**) and the water-soluble cationic Pt(II) complexes with acyclovir (acv, (9-[(2-hydroxyetoxy)methyl]guanine)), *cis*-[Pt(acv-*N7*)$_2$(NH_3)$_2$]Cl_2·2H_2O (**1**) and *cis*-[Pt(acv-*N7*)$_2$(3-Hmpy)$_2$](NO_3)$_2$ (**3**), have been prepared and characterized by multinuclear NMR spectroscopy. The results of cytotoxicity assay of **1**, **3** and *cis*-[$PtCl_2$(NH_3)$_2$] (cisplatin, cDDP) performed in malignant melanoma B16 cells *in vitro* provide evidence that complex **1** and **3** are less cytotoxic compared to cisplatin. *In vivo* experiments performed on the same malignant melanoma B16 cells, grown as subcutaneous tumours, demonstrated that antitumour effectiveness of **1** was comparable to that of cisplatin.

Keywords: Pt(II) complexes; acyclovir; cytotoxicity; tumour cells; melanoma

Introduction

Since the discovery of anticancer properties of cis-diamminedichloroplatinum(II) (cisplatin) by Rosenberg at al., numerous other platinum(II) complexes have been prepared in order to reduce side-effects of cisplatin such as nephrotoxicity and drug resistance.[1,2]

It is now generally accepted that the antitumour activity of platinum anticancer drugs is due to the platination of DNA. However, platinum compounds have a very high affinity for sulfur-containing biomolecules (i.e. methionine, cysteine, glutathione) on their pathways to DNA, which leads to nephrotoxicity, inactivation of drugs and drug resistance. A cisplatin analogue carboplatin, *cis*-[Pt(1,1-cyclo butanedicarboxylate)(NH_3)$_2$], as a second generation anticancer drug possesses a slowly aquating bulky 1,1-cyclobutanedicarboxylato group instead of the readily leaving chloro ligands. The activation of the prodrug in the latter case primarily involves the reaction with sulphur-containing bionucleophile.[3] It is less toxic than cisplatin and can be administrated at much higher dose than cisplatin. Unfortunately, carboplatin is active in the same range of tumours with cross-resistance to cisplatin (and it possesses cross-resistance to cisplatin). Replacement of both, the leaving group, *i.e.*, chlorides for oxalate, and the carrier non-leaving group, *i.e.*, ammonia for (*trans*-R, R)-1,2-diaminocyclohexane, resulted in (*trans*-R, R)-1,2-diaminocyclohexane oxalatoplatinum(II), also known as oxaliplatin, which is often effective in cisplatin-resistant cell lines and tumours.[4,5] Recent attention has also focused on a novel sterically hindered classes of antitumour agents, *i.e.* picoline complex *cis*-[$PtCl_2$(NH_3)(2-picoline)] (AMD 473).[6,7] Acyclovir (9-[(2-hydroxyetoxy)methyl]guanine) (Fig.1), a synthetic nucleoside analogue with a linear chain in place of the ring sugar, is active against herpes viruses. The inhibitory activity of acyclovir is due to its affinity for the viral enzyme thymidine kinase which converts acyclovir into acyclovir monophosphate. This nucleotide analogue is further converted by a number of cellular enzymes into acyclovir triphosphate which finally stops replication of herpes viral DNA.[8-11] Complexes of platinum(II) ion with acyclovir can be classified as multifunctional drugs due to two different cytotoxic moieties within the same molecule. Natile et al. reported that [$PtCl(NH_3)_2$(acv)](NO_3) is active against the cisplatin-resistant P388 subline. Its DNA interaction properties are different from those of cisplatin suggesting a different mechanism of action could be involved.[12] Further investigations on this field lead to changing both molecules of ammonia with sterically demanding 1,10-phenanthroline or 2,9-dimethyl-1,10-phenanthroline and the resulting Pt(II) complexes have in some cases even better antiviral activity than free

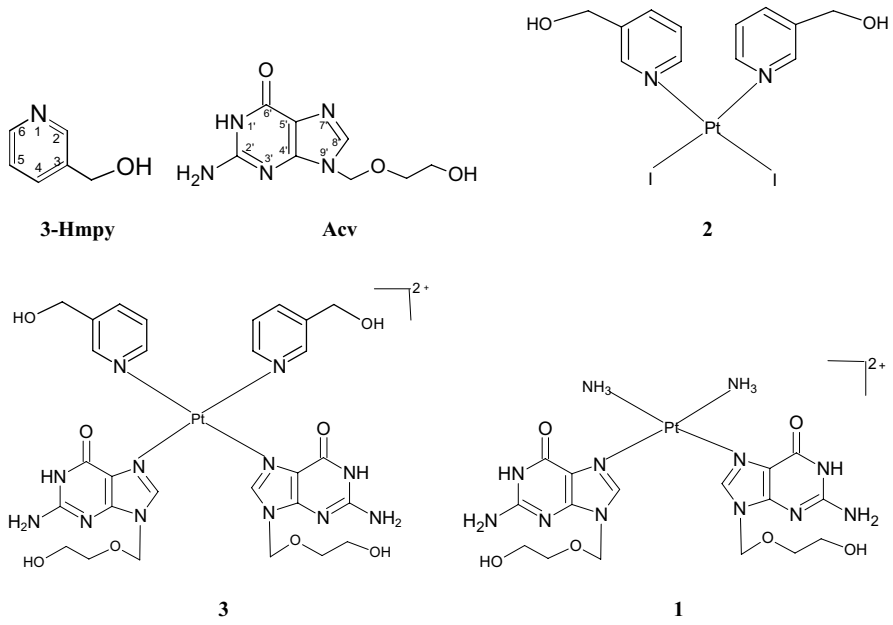

Figure 1. The numbering and chemical structures of **3-Hmpy, Acv** , **2**, **3** and **1**.

acyclovir.[13-16] We have already prepared crystals of Pt(II) bis(acyclovir) complexes $[Pt(acv)_2(NH_3)_2]Cl_2 \cdot 2H_2O$, $[Pt(acv)_2en][PF_6]_{1.5}$ $Cl_{0.5} \cdot 3H_2O$ and $[Pt(acv)_2en]SO_4 \cdot 2.5H_2O$ (en = 1,2diaminoethane).[17-19] Using two different counterions, PF_6^- and SO_4^{2-}, we managed to isolate two different conformers of $[Pt(acv)_2en]^{2+}$ ion with the two guanine moieties in a Head-to-Tail conformation (*HT*) and in a Head-to-Head (*HH*) conformation respectively. Extending the work on the synthesis of acyclovir complexes, we prepared a novel starting Pt(II) complex *cis*-$[PtI_2(3\text{-Hmpy})_2]$ (**2**) with a larger aromatic base, 3-hydroxymethylpyridine (3-Hmpy) (Fig. 1). The ligand with its hydroxo group which is a good hydrogen bond donor or acceptor could additionally stabilize interaction between a Pt(II) complex and DNA through formation of hydrogen bonds. Cytotoxic activities on malignant melanoma B16 cells were examined for cisplatin, complexes *cis*-$[Pt(acv)_2(NH_3)_2]Cl_2 \cdot 2H_2O$ (**1**) and $[Pt(acv)_2(3\text{-Hmpy})_2](NO_3)_2$ (**3**) *in vitro* and *in vivo* experiments.

Experimental

All commercial reagents were Aldrich or Fluka reagent-grade products. Acyclovir was gift from Lek, Slovenia. Elemental analyses for C, H, N were obtained on a Perkin-Elmer CHN Analyzer 2400.

NMR spectroscopy

^1H, ^{13}C and ^{195}Pt NMR spectra were recorded on a Bruker DPX 300 instrument with standard pulse programs at 300.13 (^1H), 76.48 (^{13}C), and 64.35 MHz (^{195}Pt). 2D [^1H,^{13}C] HMQC, spectra were recorded in a gradient-enhanced mode on a Bruker DPX 300 instrument with standard pulse programs at 300.13 (^1H) and 76.48 MHz (^{13}C). ^{195}Pt was recorded with a 32 or 64 kHz spectral width, 1 k time domain, 150 k scans, and line broadening 50 Hz. The chemical shift references were as follows: ^1H (internal), TMS, HOD; ^{195}Pt (external), $Na_2[PtCl_6]$ ($K_2[PtCl_4]$ (in D_2O) adjusted to δ –1625 ppm from $Na_2[PtCl_6]$).

Synthesis of *cis*-$[Pt(acv)_2(NH_3)_2]Cl_2 \cdot 2H_2O$ (1)

99.0 mg *cis*-$[PtCl_2(NH_3)_2]$ (0.333 mmol) and 150 mg (0.666 mmol) of acyclovir were disolved in water preheated to 333 K. The reaction mixture was stirred for two days at 333 K in dark. Colourless prismatic crystals of *cis*-$[Pt(acv)_2(NH_3)_2]Cl_2$ were obtained after slow evaporation at ambient temperature. Yield: 70 %. Found (calc. for $C_{16}Cl_2H_{31}N_{12}O_8Pt$): C 24.1(24.4), N 20.9(21.4), H 3.60(3.94)%. ^1H NMR (300 MHz, D_2O) δ 3.78 (m, 8H, O-CH_2CH2OH), 5.42 (s, 4H, N-CH_2-O), 8.44 (s, 2H, H-8').^{13}C NMR (300 MHz, D_2O) δ 160.0 (C-6'), 158.0 (C-2'), 152.2 (C-4'), 143.0 (C-8'), 115.0 (C-5'), 74.9 (N-CH_2'), 71.9 (OCH_2'C), 61.5 (CH_2'OH).^{195}Pt NMR (64.3 MHz, D_2O) δ –2440.

Synthesis of *cis*-$[PtI_2(3\text{-Hmpy})_2]$ (2)

Compound **2** was prepared according to Dhara's method.[20] A water solution of $K_2[PtCl_4]$ (100 mg, 0.241 mmol) and KI (320 mg, 1.93 mmol) was stirred at room temperature. After 15 minutes, 3-hydroxymethylpyridine (3-Hmpy) (52.4 mg, 0.482 mmol) was added dropwise to the reaction mixture. In a few minutes, a yellow solid deposited. The compound was separated by filtration and washed with cold water and ether. The yield was 60%. Found (calc. for $C_{12}H_{14}$ $I_2N_2O_2Pt$): C 21.2(21.6),

N 4.35(4.20), H 2.31(2.12)%. ^{1}H NMR (300 MHz, acetone-d_6) δ 4.67 (s, 3H, $\underline{CH_2}O\underline{H}$), 7.46 (m, 1H, H-5), 7.92 (d, 1H, J=7.7Hz, H-4), 8.85 (d, 1H, J=5.5Hz, H-6), 8.95 (s, 1H, H-2). ^{13}C NMR (75.5 MHz, acetone-d_6) δ 151.4 (C-6), 151.1(C-2), 141.2 (C-3), 137.2 (C-4), 126.1 (C-5), 60.8 (CH_2). ^{195}Pt NMR (64.3 MHz, acetone-d_6) δ -3164.

Synthesis of $[Pt(acv)_2(3\text{-}Hmpy)_2](NO_3)_2$ (3)

A water suspension of *cis*-$[PtI_2(3\text{-}hmpy)_2]$ (20.0 mg, $3.00\cdot10^{-5}$ mol) was treated with silver nitrate (9.98 mg, $5.88\cdot10^{-5}$ mol) in dark. A precipitate of silver iodide was filtered off and acyclovir (13.5 mg, $6.00\cdot10^{-5}$ mol) was added to the filtrate. Reaction was allowed to proceed for 24 hours at 310 K. The product was obtained from the aqueous solution after evaporation at room temperature. The yield was 70 %. Found (calc. for $C_{18}H_{20}N_2O_6Pt$): C 38.6(38.9), N 4.91(5.04), H 3.50(3.63)%. ^{1}H NMR (300 MHz, D_2O) δ 3.78 (m, 8H, O-CH_2CH2OH (acv)), 4.58 (s, 4H, $\underline{CH_2}O\underline{H}$ (3py), 5.42 (s, 4H, N-CH_2(acv)), 7.45(dd, 2H, J=5.8Hz, J=7.9Hz, H-5), 7.92 (d, 2H, J=8.1Hz, H-4), 8.44 (s, 2H, H-8'), 8.65 (d, 2H, J=5.5Hz, H-6), 8.71 (s, 2H, H-2). ^{13}C NMR (300 MHz, D_2O) δ 158.0 (C-6'), 156.4 (C-2'), 152.6 (C-6), 152.5 (C-4'), 151.8 (C-2), 143.3 (C-8'), 142.0 (C-3), 141.1.9 (C-4), 128.6 (C-5), 115.0 (C-5'), 75.3 (N-CH_2'), 72.2 (OCH_2'C), 61.7 (CH_2OH), 61.6 (CH_2'OH). ^{195}Pt NMR (64.3 MHz, D_2O) δ –2296.

Cytotoxicity assay

Malignant melanoma B16 cells (Royal Marsden Hospital, Cancer Research Institute, Sutton, UK) were grown as monolayer in Eagle`s minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) (Gibco,USA), 10 mML glutamine, penicilin (100 units/ml, streptomycin (100 μg/ml) and gentamycin (11 μg/ml) (Sigma Chemicals, USA). The cells were routinaly subcultured every 5 days, and incubated at 310 K in humidified air with 5% CO_2. To determine the cytotoxic effect of cisplatin, **1** and **3** clonogenic assay was performed. Cells from exponential growth phase were harvested by trypsinization, washed and resuspended in EMEM with 10%FCS. Cisplatin, **1** and **3** were dissolved in EMEM and added to the cell suspensions at different concetrations. Cisplatin concentrations used in experiments were ranging from 0.001 to 1.0 μg/ml, whereas concentrations of **1** were ranging from 2 to 1000 μg/ml and **3** from 1 to 1000 μg/ml. Cell suspensions were mixed and then aliquoted at 4 ml per 60 mm petri dishes (Costar, The Netherlands) in quadruplicates. After 10 days, colonies were fixed, stained with crystal violet (Kemika, Croatia) and counted. Colonies containing less than 50 cells were disregarded. The results were expressed as the percentage of the colonies obtained with untreated control cells. Plating efficiency of control cells was above 70%. The results of cytotoxicity were evaluated with IC_{50}, which is the drug concentration required to reduce cell survival to 50% of the untreated controls.

Animals and tumours

Female C57Bl/6 mice were purchased from Rudjer Bošković Institute, Zagreb Croatia. Animals were maintained at constant room temperature (297 K) at natural day/night light cycle in conventional animal colony. Before experiments, animals were subjected to an adaptation period of at least 10 days. Mice in good condition, without signs of fungal or other infection, 10-12 weeks old, were included in experiments. Subcutaneous tumours were initiated by injection of $1x10^6$ B16 melanoma cells obtained from *in vitro* cell culture. The viability of the cells was determined by Trypan-blue dye exclusion test. When the tumours reached approx. 40 mm^3 in volume, mice were marked individually and randomly divided into 3 groups, consisting of 8 mice. Cisplatin as well as **1** were injected intraperitoneally at a dose of 5 mg/kg. Injection volume was 0.5 ml. Mice in the control group received intraperitoneal injection of 0.9% NaCl.*

* Animal studies were performed with approved protocols, in accordance with the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia and EU commission regulation for the laboratory animals' care.

Assessment of response

Tumour growth was followed by measuring three mutually orthogonal tumour diameters (e_1, e_2, e_3) with vernier caliper gauge each consecutive day. Tumour volumes were calculated by formula $\pi.e_1.e_2.e_3/6$. From the measurements, the mean (AM) and standard errors of the mean (SE) were calculated for each experimental group. Tumour doubling time (DT) was determined for each individual tumour and tumour growth delay (GD) from mean DT of experimental groups.

Each mouse was also weighed 2-3 times/week. The percentage of body weight loss from pretreatment values was calculated. The general condition of the mice was followed throughout the experiments. Statistically the differences between the experimental groups were evaluated by parametric Tukey test, after one way analysis of variance was performed and fulfilled. Levels of P less than 0.05 were taken as statistically significant.

Results and discussion

Recent biological studies on Pt(II) coordination compounds with antivirus drug acyclovir as ligand encouraged us to prepare new complexes **2** and **3** with the larger aromatic base 3-Hmpy and to perform some biological tests in melanoma cells. Acyclovir as a ligand posseses several coordination sites (exocyclic amine group, ring nitrogen atoms

(N1, N7), carbonyl group). The crystal structure of the complex 1 revealed that in solid state two acyclovir molecules were coordinated through N-7 atom to Pt(II) ion with head-to-tail conformation.[17] But under physiological conditions the complex is in a solution where it could exist in several conformers. Restricted rotation about the Pt-N(7) can lead to three different bis(acv) stereoisomers for square-planar complexes having two *cis* ligands with C_2 local symmetry: two head-to-tail (HT) and one head-to-head (HH) species, with H-8s of acv on the opposite or on the same side of the metal coordination plane, respectively. In our NMR experiments only one H-8 resonance was observed for complexes **1** and **3.** This suggests that rotation of acyclovir around the Pt-N7 bond is fast on the NMR timescale in both cases, with NH_3 or bulkier 3-Hmpy in vicinity. Due to deshielding of protons located near the coordination site of ligand, the signal for H-8 has moved downfield by ca. 0.3-0.4 ppm upon binding to Pt(II) ion relative to free acyclovir. One set of resonances for coordinated 3-Hpmy in **3** confirm that neither rotation around Pt–N(3-hmpy) is restricted. Significant downfield shifts for the resonances of H2 and H6 protons of 3-Hmpy (ca. 0.4 ppm) were also observed upon coordination of 3-Hmpy to Pt(II) ion in complex **2**. After substitution of iodo ligand in **2** with acyclovir there was no significant changes in chemical shifts of 3-Hmpy in **3**.

The ^{195}Pt NMR spectroscopy is a very useful tool for determination of donor atoms in the coordination sphere of Pt(II), Pt(III) or Pt(IV) ions, especially when a ligand has several possible coordination sites *i.e.* acyclovir. Complexes **1** and **3** had resonances at –2440 and –2296 ppm respectively which is in the range typical for PtN_4 coordination sphere (two nitrogen atoms from two molecules of ammonia and two nitrogen atoms from two acyclovir molecules) (Fig. 2).[21] The reason for 144 ppm difference in ^{195}Pt chemical shifts is in the nature of the nitrogen donor ligand. 3-Hmpy contains empty π^* orbitals which can form π–bonds with platinum and is therefore capable to accept electron density from the metal. Ammonia can not accept π back-donation so compared to the 3-Hmpy complex, the signals for the ammine complexes occur upfield since the NH_3 is a better donor with no π back-bonding capacity.[22] Complex **2** had a signal at –3164 ppm which is expected range for PtI_2N_2 coordination sphere.[22]

In order to determine the cytotoxic effect of **1** and **3** on tumour cells, colony forming assay was performed in malignant melanoma B16 cells (Fig. 3). The cells were treated with different concentration of **1** and **3** (1-1000 μg/ml). The cytotoxic effect was demonstrated at concentrations above 20 μg/ml. The shape of survival curves was similar to the shape of survival curve of cisplatin treated cells. However, the

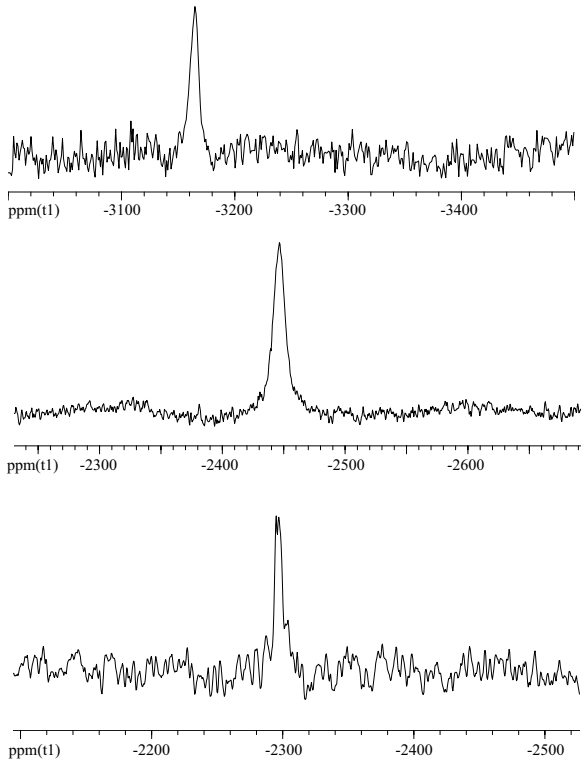

Figure 2. ^{195}Pt NMR spectra of **3** (bottom), **1** (middle) and **2** (top).

B16 melanoma cells were more sensitive to cisplatin than to **1** (1600 times) and **3** (1100 times). The IC_{50} values for cells treated with cisplatin, **1** and **3** were 0.1, 180 and 120 μg/ml respectively. These results indicated that complex **3** with 3-Hmpy ligand is 1.5 times more cytotoxic than complex **1** with ammonia ligand. Both complexes are active at higher concentrations compared to cisplatin in *in vitro* experiments.

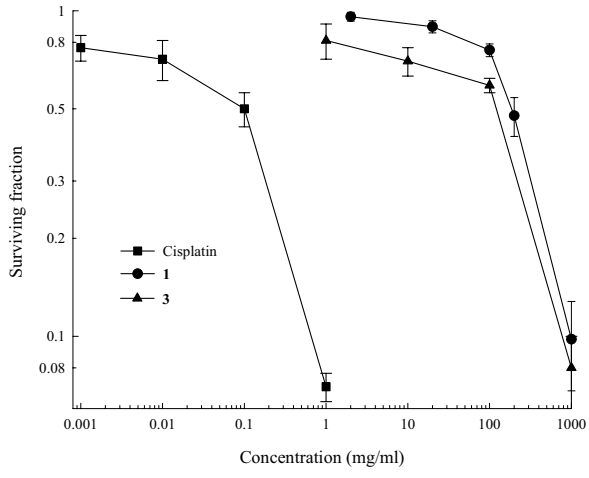

Figure 3. Surviving fraction of B16 melanoma after exposure of cells to different concentration of cisplatin, compound **1** or **3**. Experiments were performed two times using triplicate samples. Each point represents AM±SE.

In vivo experiments were performed on the same malignant melanoma B16 cells, grown as subcutaneous tumours, as used in the *in vitro* experiments. Treatment of tumours with either cisplatin or **1** statistically significantly delayed tumour growth compared to control untreated tumours. Antitumour effectiveness of **1** was comparable to that of cisplatin (Fig. 4). Growth delay of tumours treated with cisplatin was 0.7 days and of tumours treated with **1** was 0.9 days. In addition, animals tolerated the treatment with this relatively low dose of cisplatin (maximal tolerated dose for mice is 15 mg/kg) and **1** well; the treatment did not induce any body weight change.

The reason for the observed difference between *in vitro* and *in vivo* response of **1** compared to cisplatin is currently unknown, but it can be speculated that other mechanisms, such as effect on tumour vasculature or involvement of immune response and transformation leading to active species analogous to those afforded by cisplatin, than direct cytotoxic action on cells might be involved in the antitumour effectivness of **1** *in vivo* .

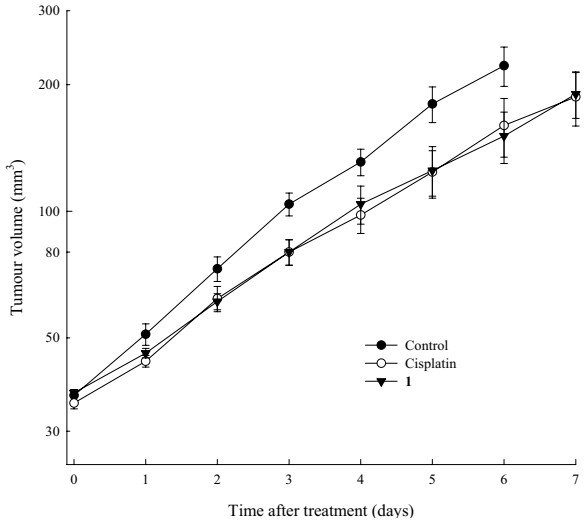

Figure 4. Tumour growth curves of B16 melanoma treated with cisplatin or compound **1** injected intraperitoneally (5 mg/kg). Data are AM±SE pooled from 8 animals per group.

Conclusions

The positively charged Pt(II) complexes **1** and **3** prepared in this study do not match some important structure-activity relationships valid for cisplatin type compounds, *i.e.* good leaving groups (halides, carboxylates), neutrality. Nevertheless, **1** exhibited comparable antitumour effectiveness to that of cisplatin *in vivo* tests on the malignant melanoma B16 cells, grown as subcutaneous tumours. Work is in progress to establish cytotoxic effect *in vivo* for complex **3**.

Acknowledgements

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Povzetek

Sintetizirali smo vodotopna kationska kompleksa Pt(II) iona z aciklovirjem, ki je protivirusno zdravilo in je učinkovit proti virusu *Herpes simplex* tipa 1 in tipa 2 (HSV-1 in HSV-2) ter virusu *Varicella zoster* (VZV). Sestavo spojin smo določili na osnovi multinuklearne NMR spektroskopije. Oba kompleksa imata na Pt(II) ion v *cis* poziciji koordinirana dva aciklovirja prek *N-7*, medtem ko se razlikujeta v preostalih dveh aminskih ligandih, ki sestavljata koordinacijsko sfero kovine – amoniak v kompleksu **1** in 3-hidroksimetilpiridin v kompleksu **3**. Z variacijo aminskega liganda smo želeli ugotoviti, kako večji aromatski ligand s hidroksi skupino vpliva na antitumorsko aktivnost kompleksa. Citotoksične teste smo izvedli *in vitro* in *in vivo* na melanomski celični kulturi (B16) s kompleksoma **1** in **3** ter cisplatinom, ki je eden najbolj uporabljenih kemoterapevtikov pri zdravljenju rakastih obolenj. Rezultati poskusov *in vitro* so pokazali večjo antitumorsko aktivnost kompleksa **3** od kompleksa **1**, vendar bistveno manjšo od cisplatina. Rezultati bioloških testov *in vivo* pa so bili bistveno boljši, saj je bila citotoksičnost kompleksa **1** primerljiva s cisplatinom.