Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 38 (2003) 627–632
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Short communication

Synthesis, physicochemical investigation and cytotoxic activity of new Pt(II) complexes with hydantoin ligands

Adriana Bakalova [a,*], Rossen Buyukliev [b], Iovka Tcholakova [c], Georgi Momekov [d], Spiro Konstantinov [d], Margarita Karaivanova [d]

[a] Department of Chemistry, Faculty of Pharmacy, Medical University, 2 Dunav Street, 1000 Sofia, Bulgaria
[b] Department of Organic Chemistry, Faculty of Pharmacy, Medical University, 2 Dunav Street, 1000 Sofia, Bulgaria
[c] Department of Inorganic Chemistry, University of Chemical Technology and Metallurgy, 8 'Kl. Ochridsky' Boulevard, 1756 Sofia, Bulgaria
[d] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Medical University, 2 Dunav Street, 1000 Sofia, Bulgaria

Received 12 March 2002; received in revised form 3 March 2003; accepted 17 March 2003

Abstract

The interaction of *cis*-dichlorodiaminplatinum(II) (*cis*-DDP) with 2,4-imidazolidenedione-5-methyl-5-phenyl was studied. The method of preparation of the new Pt(II) complex consisted in precipitation of chloride ions from *cis*-DDP via a diaqua complex and reaction with the ligand in water–organic media. On the basis of IR spectra, ^{1}H- and ^{13}C-NMR analysis the coordination mode of the ligand and most fitting structures of two isomeric complexes were proposed. The pharmacological investigations revealed that the new Pt(II) complex with 5-methyl-5-phenylhydantoin (PtMPH) as well as the previously described Pt(II) complexes with cyclopentanespiro-5′-hydantoin and cyclohexanespiro-5′-hydantoin (PtCHH) exerted concentration-dependent cytotoxic effect in a panel of human tumor cell lines. On the basis of the IC_{50} values obtained PtMPH proved to be the most active cytotoxic agent. The other investigated complexes were less active, and among them PtCHH was the least potent antineoplastic agent. The pharmacodynamic investigation of PtMPH showed that this compound induces programmed cell death (apoptosis), as evidenced by the detection of oligonucleosomal DNA fragmentation in HL-60 cells after treatment with PtMPH.

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

1. Introduction

The complexation of transition metals with five-member heterocyclic ligands possessing more than one donor atom, are of great interest in the chemistry of coordination compounds, since they can act either as neutral molecules or as deprotonated anions [1–9].

Furthermore the complexation can lead to mono- and poly-dentate binding. In the second case, a chelate structure or a structure of bridge type can be realised.

It could be expected a complexation between Pt(II) and 5-methyl-5-phenylhydantoin which is analogous to that of spirohydantoins (e.g. cyclopentanespiro-5′-hydantoin) described in previous publications [10,11].

Cisplatin *cis*-dichlorodiaminplatinum(II) (*cis*-DDP) and its analogues constitute a well-established class of antineoplastic drugs. Despite of the high therapeutic activity of *cis*-DDP against ovarian carcinoma, testicular teratoma and other solid malignancies, severe adverse effects, such as nephrotoxicity, ototoxicity, peripheral neuropathy, as well as myelosuppression limit its usefulness [12–14]. Other major disadvantage of *cis*-DDP is the occurrence of primary or acquired resistance [14,15]. In order to ameliorate these disadvantages much effort has been put in the development of new antitumor platinum coordination compounds having superior to *cis*-DDP cytotoxic activity and/or decreased toxicity as well as capability of overcoming resistance to *cis*-DDP [15,16]. Despite the numerous attempts for creating novel platinum anticancer agents, only few complexes have been introduced as antineoplastic drugs worldwide. The 'second generation' plati-

* Corresponding author.
E-mail address: georgi@satline.net (A. Bakalova).

0223-5234/03/$ - see front matter
doi:10.1016/S0223-5234(03)00080-1

num drug carboplatin (*cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II)) is less nephrotoxic than the parent compound, but causes severe thrombocytopenia and has the same spectrum of activity as *cis*-DDP. The 'third generation' compound oxaliplatin (1,2-diaminocyclohexanoxalatoplatinum(II)) has an additional advantage of being effective against *cis*-DDP-resistant tumours, its neurotoxicity, however, is greater than that of *cis*-DDP [14,15].

The rationale for synthesis of cytotoxic platinum species is usually based on the well-established structure-activity relationships for platinating antineoplastic agents [16]. It is claimed, that the cytotoxicity of platinum complexes largely depends on the presence of labile halogenide, carboxylate or other leaving groups (which exchange for water molecules in biologic milieu, thus yielding the reactive diaqua complexes), nitrogen-containing moieties usually designated as carrier ligands, *cis*-configuration of the ligands, planar structure, electroneutrality, etc. [12,15]. However, numerous reports demonstrate that diverse platinum complexes, such as: *trans*-configuration complexes, cationic species, complexes lacking the conventional leaving groups, polynuclear platinum compounds, etc. despite of the lack of structural resemblance to cisplatin, exert significant cytotoxic activity, thus contradicting the conventional structure-activity relationship concept, and thus broadening the possibilities for the search of novel platinum-based antineoplastic agents [15,17].

In the present report we focus upon the synthesis, physicochemical characterisation and the pharmacological investigation of the newly synthesised platinum(II) complex with 5-methyl-5-phenylhydantoine (PtMPH), in comparison to previously described, analogous cationic platinum(II) complexes with cyclopentanespirohydantoin (PtCPH) and cyclohexanespirohydantoin (PtCHH) [10,11].

2. Chemistry

The coordination reaction between *cis*-DDP(II) and the ligand was carried out in water–organic media. The preparation method [18] consists in precipitation of chloride ions from *cis*-DDP(II) via a diaqua complex according to the scheme:

$$cis\text{-}[Pt(NH_3)_2Cl_2] \xrightarrow{+AgNO_3\cdot aq} cis\text{-}[Pt(NH_3)_2(H_2O)_2]^{2+} \times \underset{-AgCl}{\overset{+L}{\rightarrow}} cis\text{-}[Pt(NH_3)_2L]^{2+}$$

where L is the ligand (5-methyl-5-phenylhydantoin).

The compounds used for the preparation of the complexes were of analytical grade *cis*-DDP, $AgNO_3$, and anhydrous ethanol purchased from Fluka. The ligand, 5-methyl-5-phenylhydantoin was synthesised by Idita Vassileva UCTH, Sofia, Bulgaria.

3. Pharmacology

3.1. Cytotoxicity

The cytotoxic activity of the newly synthesised complex PtMPH, as well as that of the previously described complexes of Pt(II) with cyclopentanespiro-5′-hydantoin (PtCPH) and cyclohexanespiro-5′-hydantoin (PtCHH) was assessed in vitro on a panel of human tumor cell lines, using the MTT-dye reduction assay as described by Mosmann [19], with some modifications [20]. The method is based on the ability of vital cells to metabolise the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in violet crystals of formazan. Since the latter is water-insoluble, it should be dissolved using appropriate solvent, and thereafter its concentration is determined colorimetrically. The quantity of formazan is proportional to the number of viable cells.

3.2. DNA isolation and gel electrophoresis

Isolation and gel electrophoresis DNA isolated from cells treated with *cis*-DDP and PtMPH were carried out in order to elucidate the mode of action of the investigated compounds especially their ability to induce oligonucleosomal DNA fragmentation (DNA ladder), which is a characteristic feature of the programmed cell death or apoptosis [20,21]. During the apoptotic process, activated nucleases degrade the higher order chromatin structure of DNA into mono- and oligonucleosomal DNA-fragments, which could be extracted from cells and visualised by consequent horizontal electrophoresis, ethidium bromide staining and UV-transillumination.

4. Results and discussion

4.1. Chemistry

The analytical data obtained indicate the formation of a 1:1 $[Pt(5\text{-methyl-5-phenylhydantoin})(NH_3)_2](NO_3)_2\cdot H_2O$ complex. In order to obtain information about the coordination mode of the ligand, the IR spectra of the complex and the free ligand were recorded. The data obtained are shown in Table 1. It is evident that the C=O group is not involved in the coordination as both of the two ν(C=O) bands present in the spectrum of the complex are not affected.

On the other hand the bands of the complex in the range of stretching NH—ν_s(NH) and ν_{as}(NH) at 2354

Table 1
IR data for the free ligand and for the complex (cm^{-1})

Compound	δ(NH)	ν(NH)	ν(C1–N1)	ν(Pt–N)	ν(C=O)
Ligand	–	2989w	1530w	–	1770m
	–	2389w	–	–	1715m
Complex	1629m	2954w	1523m	497s	1768m
	1585m	2354m	–	488s	1712m

and 2954 cm^{-1} indicate shifting of 15 and 35 cm^{-1}, respectively. Bands in the range of the deformation vibration characteristic for the coordination mode δ(NH) at 1629 and 1585 cm^{-1} appear and for ν(C1–N1) at 1523, 18 cm^{-1} shifting is observed. It is evident that both NH groups of the ligand are involved in the formation of the complex. The ν(Pt–N) bands at 497 and 480 cm^{-1} confirm the *cis*-position of the ligands in the complex.

Additional information was obtained from ^{1}H- and ^{13}C-NMR spectra of the complex (Table 2).

The ^{1}H-NMR data indicate coordination through the NH groups (their protons in the complex are shifted as compared to those of the free ligand with 0.20 and 0.15-ppm for H(2) and H(4), respectively). In the spectra of the complex there are two singlets, characteristic for CH_3 group at C(5) (1.55 and 1.65 ppm), which is an evidence for the existence of Pt–N bond and the presence of two isomeric forms as well.

The ^{13}C-NMR spectral data indicate coordination through Pt–NH groups, as the signals of the *C*=O groups are shifted from 156.30 and 177.10 to 163.50 and 183.80 ppm in the complex, respectively. On the other hand in the spectra of the complex two signals for the CH_3 group at 25.10 and 34.40 ppm are observed, while in the spectra of the ligand only one signal at 25.00 ppm exists.

Taking into account all the analytical data, we propose the possible structures of the two isomers of the Pt(II) complex with 5-methyl-5-phenylhydantoin, as shown on Fig. 1.

4.2. Pharmacology

4.2.1. Cytotoxicity

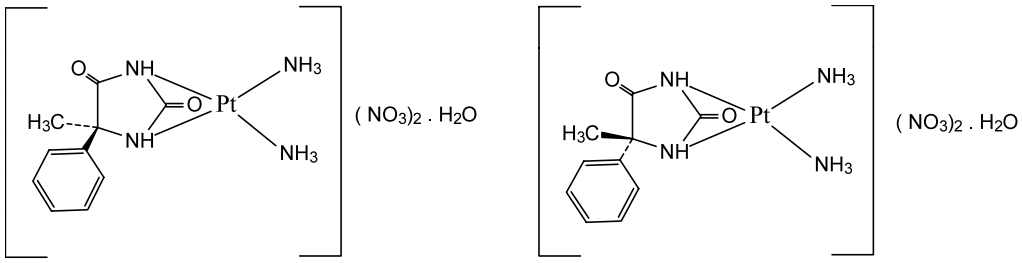
The IC_{50} values for PtMPH, PtCPH and PtCHH, extrapolated from the concentration–response curves are summarised on Table 3. The cytotoxic efficacy of PtMPH was superior to that of both spirohydantoin complexes with the exception of SKW-3 and REH leukemic cells. For all cell lines investigated, lower IC_{50} values were found for PtCPH as compared to those for PtCHH.

Interestingly, the non-Hodgkin lymphoma-derived DOHH-2 cell line, described to express the antiapoptotic protein Bcl-2 was found to be the most sensitive to PtMPH (IC_{50} value of 12.1 μM, which is comparable with that of *cis*-DDP—10.8 μM). PtMPH exerted the strongest growth inhibitory activity on HL-60 and BV-173 leukemic cells with IC_{50} values of 50 and 93.75 μM, respectively. The cytotoxic effects, caused by PtMPH on HL-60 and BV-173 cells were less pronounced than those of *cis*-DDP which IC_{50} values (8.33 μM for HL-60 and 10.42 μM for BV-173), are several times, lower than those obtained for PtMPH. Analogously, the principal platinum agents in clinical use carboplatin and oxaliplatin are more stable than *cis*-DDP and are cytotoxic at higher concentration than *cis*-DDP. The dose schedules for carboplatin and oxaliplatin are usually 5–10 times higher than those for *cis*-DDP. PtMPH, due to its relatively large organic ligand is expected to have similar to that of carboplatin and oxaliplatin toxicological profile; its IC_{50} values are greater than those of *cis*-DDP in a extend comparable to the *cis*-DDP/carboplatin and *cis*-DDP/oxaliplatin IC_{50}/dose ratios. The cytotoxicity of PtMPH on CML-T1, SKW-3 and HD-MY-Z was less pronounced with IC_{50} values over 10 times greater than those of *cis*-DDP. Within the concentration range investigated (0–200 μM) 50% cell growth inhibition could not be reached by PtMPH on the acute lymphocytic leukemia REH cells.

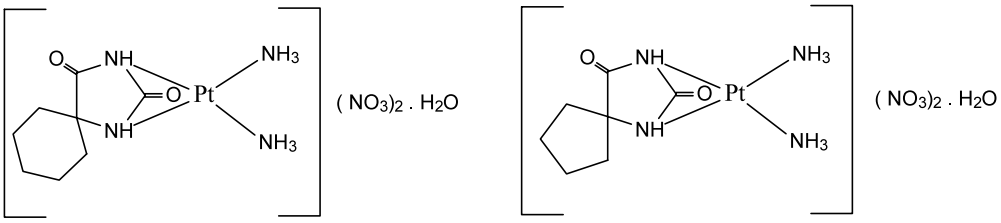
The previously described platinum complex PtCPH demonstrated strong cytotoxic activity in vitro against SKW-3 and DOHH-2 cells (IC_{50} values of 70 and 70.5 μM, respectively), whereas the other investigated cell lines CML-T1, REH and HL-60 were found to be less sensitive to PtCPH ($IC_{50} > 100$ μM). The additional

Table 2
NMR data for the free ligand and for the complex (ppm)

Compound	^{1}H-NMR			^{13}C-NMR		
	CH_3	H(2)	H(4)	CH_3	C(3)=O	C(1)=O
Ligand	1.65s	10.70s	8.60s	25.00	177.10	156.30
Complex	1.55s	10.90s	8.75s	25.10	183.80	163.50
	1.65s	–	–	34.40	–	–



SCHEMATIC STRUCTURES OF TWO ISOMERS OF PtMPH



PtCHH PtCPH

Fig. 1. Chemical structures of the investigated platinum(II) complexes PtMPH (two isomeric forms), PtCPH and PtCHH.

Table 3
IC_{50} values obtained from the concentration–response curves for PtMPH, PtCPH, PtCHH and *cis*-DDP

Cell line	IC_{50} value (μM)			
	cis-DDP	PtMPH	PtCPH	PtCHH
HL-60	8.33	50	143.6	186.7
SKW-3	11.25	163.54	70	172.2
CML-T1	6.2	113	128.15	144.3
BV-173	10.42	93.75	126.9	171.6
REH	4	> 200	147.9	189.6
DOHH-2	10.8	12.1	70.5	160.3
HD-MY-Z	10.4	150.7	> 200	> 200

increase of the molecular weight of the spirohydantoin ligand in PtCHH correlates to a significant loss of antineoplastic activity. PtCPH was found to exert greater antitumor efficacy in comparison to PtCHH against the whole panel of human tumor cell lines, with the exception of HD-MY-Z cells for which IC_{50} could not be reached.

On Fig. 2 a typical dose response curves for *cis*-DDP and PtMPH are shown. Both compounds, despite of the IC_{50} values differences, caused comparable maximal tumor growth inhibition.

4.2.2. DNA isolation and gel electrophoresis

The DNA isolated from the cytosolic fraction of HL-60 cells treated with *cis*-DDP and PtMPH demonstrated oligonucleosomal DNA fragmentation which is a hallmark of the programmed cell death, the so called 'laddering' phenomenon. This DNA fragmentation was more pronounced at the higher concentration used

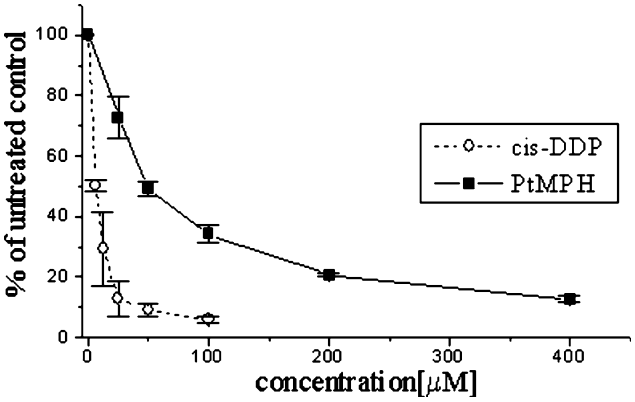


Fig. 2. Cytotoxic activity of PtMPH (■) and *cis*-DDP (○) on the human acute myeloid leukemia-derived HL-60 cell line as assessed by the MTT-dye reduction assay after 48 h treatment. Every data point represents the arithmetic mean of at least eight independent experiments. The error bars represent the S.D. for every data-point.

(Fig. 3). These findings indicate that *cis*-DDP as well as PtMPH induces programmed cell death in HL-60 cells.

5. Conclusion

The data about the in vitro cytotoxicity show that PtMPH, as well as the previously synthesised spirohydantoin compounds PtCPH and PtCHH exert concentration-dependent cytotoxic activity on a panel of tumor cell lines, although less pronounced than that of the referent drug *cis*-DDP. On the basis of the IC_{50} values it can be concluded that PtMPH is the most active cytotoxic agent, whereas PtCPH is less active with the exception of REH and SKW-3 cells. The third com-

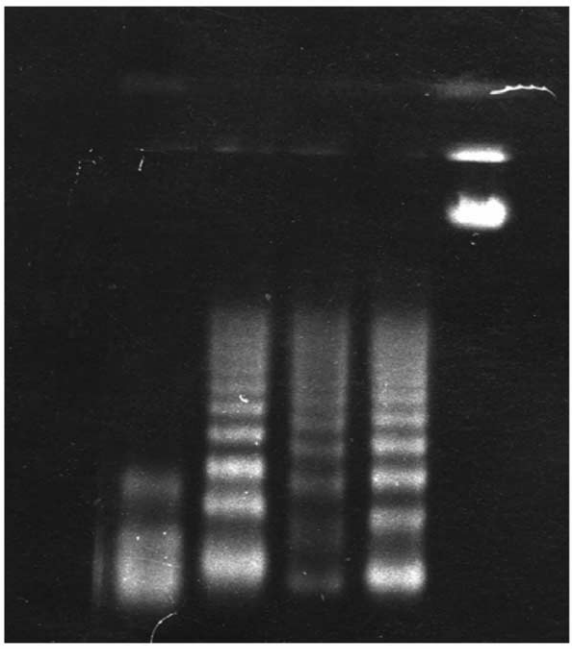


Fig. 3. Gel electrophoresis of DNA isolated from the cytosolic fraction of cells treated with CDDP (100 μM (lane 2)), PtMPH (100 μM (lane 3) and 200 μM (lane 4)) and untreated controls (lane 1).

pound, which contains a larger spiro-cycloalkyl substitute in the hydantoin moiety (PtCHH) is the least active cytotoxic agent. As evidenced by the DNA laddering phenomenon PtMPH induces programmed cell death (apoptosis) in HL-60 cells like *cis*-DDP. These results confirm the assumption that the induction of apoptosis as well as the formation of Pt-DNA cross-links plays an essential role in the cytotoxic mode of action of platinum complexes [20]. The observed cytotoxic activity of PtMPH as well as its ability to trigger programmed cell death condition our interest to further detailed pharmacological and toxicological investigation of this compound. We hope that these compounds possibly have smaller general toxicity and are more specific as antitumor agents. Researches on the in vivo antitumor activity and the pharmacodynamics of PtMPH are currently in progress.

6. Experimental protocols

6.1. Chemistry

6.1.1. General methods

The new Pt(II) complex was characterised by elemental analysis, IR, ^{1}H- and ^{13}C-spectra. The carbon, nitrogen and hydrogen content of the compound was determined by elemental analysis. The results were within $\pm$0.5% of the theoretical values.

6.1.1.1. Spectral measurements. The IR spectra were recorded on a Perkin-Elmer 1600 spectrometer in the range 4000–600 cm^{-1} as tablets of KBr and FT-IR Nicolet spectrometer in the field of 500–140 cm^{-1} as CsI tablets. The ^{1}H-NMR spectra were recorded on Bruker WP 100 (100 MHz) spectrometer in DMSO-d_6. The ^{13}C-NMR spectra were made on Bruker WM 300 (75 MHz) spectrometer with J-coupled spin-echoes.

6.1.2. Synthesis of the complex $[Pt(mphhn)(NH_3)_2](NO_3)_2 \cdot H_2O$

Cis-$[Pt(NH_3)_2Cl_2]$ (0.1173 g, 0.3 mmol) were added to 0.07 M $AgNO_3$, as water solution (10 cm^3). The mixture was heated to 60 °C at constant stirring. The precipitate formed was filtered off. Anhydrous ethanol (4 cm^3) solution of the ligand (0.0656 g, 0.3 mmol) was added to the filtrate and the mixture was evaporated to dryness at 60 °C. The light green amorphous substance was purified repeatedly by recrystallisation from hot water. Yield: 60%, m.p.: $>$160 °C (dec.). Anal. Found: Pt, 34.1; C, 21.1; H, 3.0; N, 15.2; H_2O, 2.9. Calc. for $C_{10}H_{18}N_6O_9Pt$: Pt, 34.8; C, 21.4; H, 3.2; N, 15.2, H_2O, 3.2%.

6.2. Pharmacology

All of the procedures concerning the cell culture maintenance, drug solutions preparation and treatment were carried out in a Heraeus PB16 'Function line' laminar flow cabinet.

6.2.1. Human tumor cell lines and culture conditions

All human tumor cell lines used in this study were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ)—Department of Human and Animal Cell Cultures. In this study the following human tumor cell lines were used: acute myeloid leukemia-derived HL-60 (DSMZ No ACC 3), chronic myeloid leukemia-derived CML-T1(DSMZ No ACC 7), chronic lymphoid leukemia derived SKW-3 (DSMZ No ACC 53), the pre-B cell leukemias BV-173 (DSMZ No ACC 20) and REH, the non-Hodgkin lymphoma-derived cell line DOHH-2 and the Hodgkin lymphoma-derived HD-MY-Z (DSMZ No ACC 346). All cell lines were grown as suspension-type cultures under standard conditions: RPMI 1640 medium (Sigma), supplemented with 10% heat inactivated fetal bovine serum (Sigma) and 2 mM L-glutamine (Sigma), in an 'Heraeus' incubator with humidified atmosphere and 5% carbon dioxide, at 37 °C. In order to maintain the cell growth in log phase, cell suspension was discarded two or three times per week and the cell culture was re-fed with fresh RPMI-1640 aliquots.

6.2.2. Drug solutions and treatment

Stock solutions of PtMPH, PtCPH and PtCHH (20 mM) were freshly prepared in DMSO, and thereafter consequently diluted in RPMI 1640 medium, in order to achieve the final concentrations. At the final dilutions obtained, the concentration of DMSO never exceeded 1%. The stock solution (20 000 μM, in water) of the referent cytotoxic drug CDDP (Platidiam® Lachema, Czech Republic as 10 mg vials for clinical use) was freshly prepared and accordingly diluted in RPMI 1640 medium.

6.2.3. Cytotoxicity determination—MTT-dye reduction assay for cell viability

Exponentially growing cells were seeded in 96-well microplates (100 μL well^{-1}) at a density of 1×10^5 cells mL^{-1} and after 24 h incubation at 37 °C they were exposed to various concentrations of PtMPH and *cis*-DDP for 48 h. For each concentration at least 8 wells were used. After the incubation period with the tested compounds MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) solution (10 mg mL^{-1} in PBS) was added (10 μL well^{-1}). Microplates were further incubated for 4 h at 37 °C and the formazan crystals formed were dissolved by adding 100 μL well^{-1} 5% solution of formic acid in 2-propanol (Merck). The absorption was measured using an ELISA reader (Uniscan Titertec) at wavelength of 580 nm. The blank solution consisted of 100 μL RPMI 1640 medium (Sigma), 10 μL MTT stock and 100 μL 5% formic acid in 2-propanol. Cell survival fractions were calculated as percentage of the untreated control (untreated control = 100%). In addition IC_{50} values were extrapolated from the concentration-effect curves.

6.2.4. DNA isolation and gel electrophoresis

The oligonucleosomal DNA fragmentation, which is a characteristic feature of the programmed cell death, was detected using DNA extraction and horizontal gel electrophoresis [19]. About 5×10^6 HL-60 cells—treated with cisplatin (100 μM) or PtMPH (100 and 200 μM), as well as untreated controls, were washed in PBS. Cell pellets were redissolved in 0.25 mL PBS and lysed through addition of 0.5 mL buffer containing 0.5% Triton X-100, 20 mM Tris–HCl and 1 mM EDTA (pH 7.4). Samples were incubated on ice for 5 min and thereafter spun at 13 000 rpm for 20 min. The supernatants were transferred into fresh 2 mL Eppendorf safe lock tubes and then 0.937 mL 2-propanol as well as 0.187 mL 6 M solution of NaCl were added to each sample. The tubes were gently agitated and incubated at −20 °C for 12 h in order to allow precipitation of the water-soluble DNA. The samples were centrifuged for 20 min at 13 000 rpm, the supernatants were decanted and DNA was washed in 1 mL ice cold 70% ethanol and then air dried. After that DNA was re-dissolved in 20 μL distilled water and analysed by gel electrophoresis in 0.8% agarose gel and then stained with ethidium bromide. Finally, DNA was visualised using an UV-transilluminator and photographed with a fixed digital camera (Bio Doc IT™ system).

6.2.5. Statistics

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

Acknowledgements

This work was supported by a grant from the Medicinal Science Council at the Medical University of Sofia, Bulgaria.

References

[1] W.D. Basson, A.L du Preez, J. Chem. Soc. Dalton Trans. (1974) 1708.
[2] P.P. Singh, A.K. Srivastava, Aust. J. Chem. 27 (1974) 509.
[3] M.R. Udupa, M. Padmanabhan, J. Inorg. Nucl. Chem. 37 (1975) 1808.
[4] P.P. Singh, U.P. Shukla, A.K. Srivastava, Aust. J. Chem. 29 (1976) 79.
[5] P.P. Singh, O.P. Orgawal, A.K. Gupta, Inorg. Chim. Acta 18 (1976) 19.
[6] A.C. Fabretti, G. Peyronel, Trans. Met. Chem. 2 (1977) 207.
[7] A.C. Fabretti, G. Peyronel, G.C. Franchini, Trans. Met. Chem. 3 (1978) 125.
[8] F.A. Devillanova, F. Isaia, G. Verani, J. Inorg. Nucl. Chem. 43 (1981) 2749.
[9] L. Antolini, F. Christiani, F.A Devillanova, A. Diaz, G. Verani, J. Chem. Soc. Dalton Trans. (1983) 1261.
[10] I. Tcholakova, A. Bakalova, M. Karaivanova, S. Konstantinov, B. Alexiev, M. Pankova, Compt. Rend. Acad. Bulg. Sci. 46 (1993) 39–42.
[11] I. Tcholakova, A. Bakalova, B. Alexiev, M. Karaivanova, S. Konstantinov, M. Pankova, M. Parvanova, BG Patent, 61 482 B1, 1993.
[12] D.S. Alberts, H.M. Pinedo, J.M. Schornagel (Eds.), Platinum and Other Coordination Compounds in Cancer Chemotherapy 2, Plenum Press, New York, 1996, pp. 303–309.
[13] P.J. Loehrer, L.H. Einhorn, Ann. Intern. Med. 100 (1984) 704–713.
[14] L.R. Kelland, Crit. Rev. Oncol. Hematol. 15 (1993) 191–219.
[15] L.R. Kelland, J. Inorg. Biochem. 77 (1999) 121–124.
[16] D. Lebwohl, R. Canetta, Eur. J. Cancer 34 (1998) 1522–1534.
[17] M.J. McKeage, J.D. Higgins, III, L.R. Kelland, Br. J. Cancer 64 (1991) 788–792.
[18] M.J. Cleare, J.D. Hoeshele, B. Rosenberg, L.L. Van Camp, US Patent 4 140 707, 1979.
[19] T. Mosmann, J. Immunol. Methods 65 (1983) 55–63.
[20] S.M. Konstantinov, M.R. Berger, Cancer Lett. 144 (1999) 153–160.
[21] P.M. Henkels, J.J. Turchi, Cancer Res. 59 (1999) 3077–3083.