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INFLUENCE OF TWO Pt(IV) COMPLEXES ON VIABILITY, APOPTOSIS AND CELL CYCLE OF B16 MOUSE MELANOMA TUMORS

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Several platinum(IV) complexes are showing considerable promise in initial trials, producing reactive intermediates that then interact with DNA. ***Aim:*** **To perform *in vitro* study of two new platinum(IV) complexes cytotoxic effect on B16 mouse melanoma cells.** ***Methods:*** **$PtCl_4(dbtp)_2$ and $PtCl_2(6mp)_2$ complexes were prepared. $PtCl_4(dbtp)_2$ was created as modification of $PtCl_4(dmtp)$ test previously. Apoptosis and necrosis were examined using flow cytometry, upon Annexin V/PI staining.** ***Results*****: LC_{10}, LC_{50} and LC_{90} parameters established for $PtCl_4(dbtp)_2$ were as following: 2.6, 17.0, 58.0 μmol/L. However LC_{10} and LC_{50} established for $PtCl_2(6mp)_2$ were 1.2 and 14.0 μmol/l respectively. The both complexes induced apoptosis. $PtCl_2(6mp)_2$ induced cell cycle arrest in G0/G1, while $PtCl_4(dbtp)_2$ — in S-phase.** ***Conclusions:*** **$PtCl_4(dbtp)_2$ appeared to be more cytotoxic against B16 cells than $PtCl_2(6mp)_2$. Apoptosis was the main mechanism of cell loss in cultures incubated with both tested complexes.**
Key Words: **platinum complexes, cytotoxicity *in vitro*, apoptosis, cell cycle, mouse melanoma model.**

Five-year survival of patients with advanced melanoma is estimated to be 6%. Partial responses were observed only for 10% of patients with advanced melanoma treated with cisplatinum. Radiotherapy and chemotherapy are inefficient, due to radioprotective and antioxidant properties of melanines. Immunotherapy, gene-therapy and other forms of chemotherapy are promising, but still not satisfactory [1, 2]. Therefore much attention has been paid on design of new platinum compounds with improved pharmacological properties against melanoma cells [3]. Consequently new platinum drugs with equal or better antitumor activity but less toxicity have been developed by modifying the pharmacokinetics of cisplatin, replacing the chloride labile and stable amine ligands with other leaving groups. Another way to design new anti-tumor pro-drugs related to *cis*platin is to change the nature of the central ion and used a six-coordinate octahedral platinum(IV) derivatives. Several platinum(IV) complexes are showing considerable promise in initial trials, with functionality thought to depend on the *in vivo* reduction of Pt(IV) to Pt(II), producing reactive intermediates that then interact with DNA. In our previous study we found that $PtCl_4(dmtp)_2$ had a good cytotoxic properties, but the solubility was still low [4, 5]. Therefore intention of our research was to test a new platinum(IV) compounds with difference heterocycle ligands which reveal better solubility. For our study we choose $PtCl_4(dbtp)_2$ (Fig. 1, *a*), with bulky tertbutyl group. Probably presence of the tertbutyl in heterocyle ligands is a key to interaction of the platinum complexes with cellular components. In addition, our earlier study show that the platinum(II) and platinum(IV) presented high antitumor activity against human tumour cells (SW707 rectal adenocarcinoma, T47D breast cancer and HCV29T bladder cancer). In this paper we present *in vitro* cytotoxic effect of two new platinum(IV) complexes; $PtCl_4(dbtp)_2$ and $PtCl_2(6mp)_2$ (Fig. 1, *b*) against B16 mouse melanoma cells. Apoptotic response as a strong predictor for drug effectiveness against neoplasmatic cells was examined as well. Cell cycle was analyzed additionally, to find, if there are specific disturbances of the cell cycle.

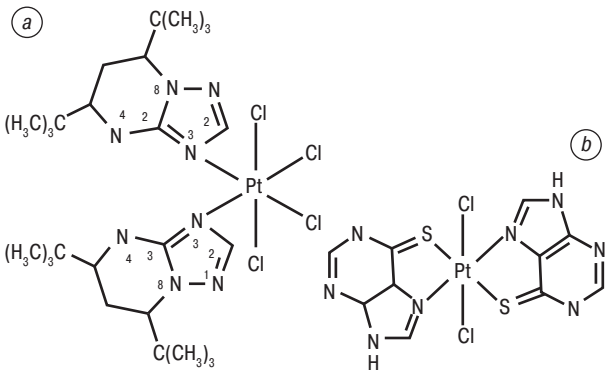

Fig. 1. Schematic molecular representation of $PtCl_4(dbtp)_2$ (*a*) and $PtCl_2(6mp)_2$ (*b*)

Compounds able to trigger apoptosis within target cells are effective anticancer drugs. Cells resistant to chemotherapy are often characterized by the lack of apoptotic response during systemic treatment. Apoptosis can be evoked during the whole cell cycle, but the most suspicious are check points G1/S and G2/M [6].

MATERIAL AND METHODS

Preparation of platinum(IV) complexes. $PtCl_4(dbtp)_2$ was obtained by reaction of H_2PtCl_6 with dbtp in molar ratio M : L=1 : 2 [7]. $PtCl_2(6\text{-}mp)_2$ was obtained by the reaction of 330 μl (0.1 mmol) of H_2PtCl_6, dissolved in 25 cm^3 of 2 M HCl, with 0.340 g (2 mmol) of 6-Hmp · H_2O in 25 cm^3 of methanol. The reaction mixture was heated to 60 °C and stirred for 3 h. The yellow precipitate was filtered, washed with methanol, diethyl ether and dried in vacuum.

Mouse Melanoma B16 cell line. B16 cells were propagated from tumor localized in peritoneum of C57Bl/6J mouse. Cells were cultivated in complete DMEM (Sigma)

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Abbreviations used: B16 – mouse melanoma cells; C1S91 – Cloudman S91 mouse melanoma cells; DMSO – dimethyl sulfoxide; LC – lethal concentration; PBS – phosphate buffered saline; PI – propidium iodide; Pt (IV) – platinum (IV) complexes; Pt (II) – platinum (II) complexes; SD – standard deviation.

medium supplemented with 10% fetal bovine serum (Sigma, USA) and antibiotics as previously described [8].

Cytotoxicity studies. 24-well plates (Nunc, USA) were used in cytotoxic studies. B16 cells were seeded in each well in amount of 10^5 and covered with fresh culture medium for 24 h. Then culture medium was removed and tested platinum compounds were added in appropriate concentrations, dissolved in 0.1% DMSO. Each solution was prepared immediately before use. Cells were incubated with platinum complexes for 6 h. Then cultures were rinsed with PBS and cells were detached with 0.05% trypsin. Cells were centrifuged 5 min at 900 g. Cells in suspensions were stained with trypan blue and counted in Neubauer chamber under magnification of 100 X. Cytotoxicity of tested compounds was calculated as the number of viable B16 cells in relation to control. Our previous study proved that 0.1% DMSO does not affect on viability of B16 cells. Melanoma cells viability was estimated using the regression curves. Regression curves equations were used to calculate LC_{10}, LC_{50}, LC_{90} (lethal concentration) values. Correlation coefficients were calculated and checked by Pearson test. $p < 0.05$ was statistically significant.

Apoptosis and necrosis detection using flow cytometry. To detect apoptotic and necrotic cells Annexin V conjugated with fluorescein izothiocyanate (Annexin V-FITC, Immunotech) and propidium iodide (PI), (Immunotech, USA) were used [5]. Melanoma cells were cultured in 50 mm diameter Petri dishes. Each dish contained 10^5 cells in 5 ml of medium. Platinum complexes were added to each culture after 24 h preincubation. Apoptotic B16 cells were counted after 6 h and 12 h incubation with both complexes. Cells incubated with PBS were used as control samples. 10^5 of B16 cells were suspended in 0.5 ml of binding buffer containing Annexin V-FITC and propidium iodide (Immunotech). Then probes were shaken 5 s in automatic stirrer (Coulter, Miami, FL, USA), and incubated 10 min in darkness at 4 °C. Analysis was performed using flow cytometr EPICS XL (Coulter) with System 2 Software Version 1.0. Three separate measurements were done for each experiment. Resulted were presented as mean +/– standard deviation (SD). The probability between differences of means in groups of apoptotic cells was evaluated with t-Student test. $p < 0.05$ was statistically significant.

Cell cycle analysis. DNA content and the phase of cell cycle was measured after staining with PI. The procedure was done after permeabilization of the cell membranes. B16 cells were incubated with platinum complexes. Control cells were incubated only with PBS. After 6, 12, 24 or 48 h incubation with tested platinum complexes the medium was removed and the cells (10^5) were suspended in 0.1 ml of PBS. 0.05 ml of cell suspension was transferred to the test-tube containing 0.05 ml of cell membrane permeabilizing agent DNA-prep LPR (Immunotech). Tubes were put in automatic stirrer for 8 s. Then 1.0 ml of DNA staining reagent (DNA-prep Stain, Immunotech) was added to each probe. The tubes were stirred again and left for 20 min in room temperature and darkness. Analysis was performed in EPICX XL flow cytometr, programmed by MultiCycle software (Phoenix Flow System Inc., San Diego, CA, USA). Each value represents the mean from the three determinations. Resulted were presented as means with standard deviations (SD). Comparative analysis was performed.

RESULTS

Cytotoxicity activity of $PtCl_4(dbtp)_2$ and $PtCl_2(6mp)_2$. Experimental cytotoxic concentrations of $PtCl_4(dbtp)_2$ ranged from 1.2 to 124.8 μM. B16 cells viability curve after 6 h incubation with $PtCl_4(dbtp)_2$ was presented on Fig. 2, *a*. Toxic concentration for 10% of cells (LC_{10}) was established as 2.6 μM. LC_{50} and LC_{90} were 17.0 and 58.0 μM, respectively (see Fig. 2, *a*). Experimental cytotoxic concentrations of $PtCl_2(6mp)_2$ were in the range 1.2–175.3 μM. LC_{10} and LC_{50} were 1.2 and 14.0 μM, respectively (Fig. 2, *b*). It was impossible to reach LC_{90} value in experimental conditions, due to low solubility of $PtCl_2(6mp)_2$, and precipitation of this compound in concentrations above 180.0 μM, hence LC_{90} was only a calculated value. LC_{90} and for $PtCl_2(6mp)_2$ was 6 much higher the LC_{90} for $PtCl_4(dbtp)_2$.

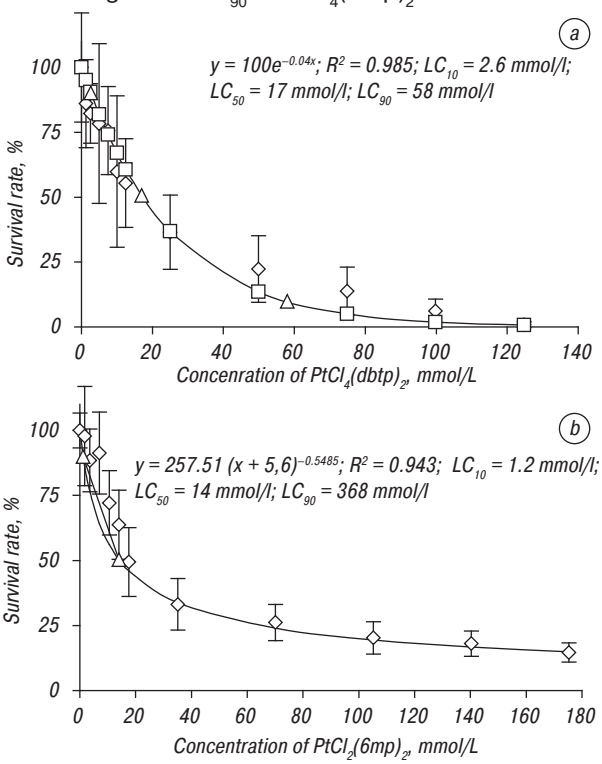

Fig. 2. Regression curves presenting B16 cells viability after 6 h of incubation with $PtCl_4(dbtp)_2$ (*a*) and $PtCl_2(6mp)_2$ (*b*)

Influence of $PtCl_4(dbtp)_2$ and $PtCl_2(6mp)_2$ on apoptosis in B16 cells. Percentage of B16 apoptotic cells after 6 h and 12 h incubations with $PtCl_4(dbtp)_2$ is shown on Fig. 3, *a*. High correlation was found between percentage of apoptotic cells and $PtCl_4(dbtp)_2$ concentrations. Correlation coefficients for 6 and 12 h incubation times were r = 0.97 ($p < 0.05$) and r = 0.99 ($p < 0.01$), respectively. 25% of cells were found to be in early stages of apoptosis in concentration corresponding to LC_{50}. High negative correlations were found between viable cells and concentrations during 6 h and 12 h assays, r = –0.96 ($p < 0.05$) and r = –0.99 ($p < 0.02$).

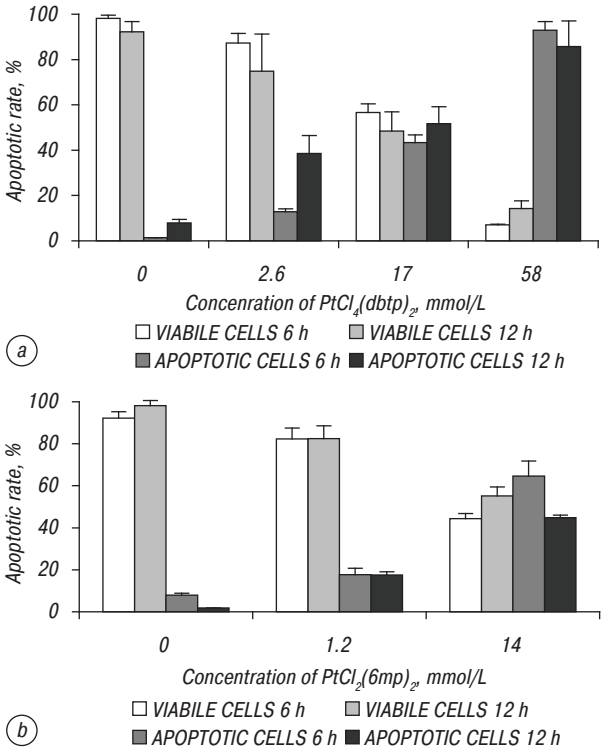

Fig. 3. Percentage of the apoptotic cells after exposure to $PtCl_4(dbtp)_2$ (*a*) and on $PtCl_2(6mp)_2$ (*b*)

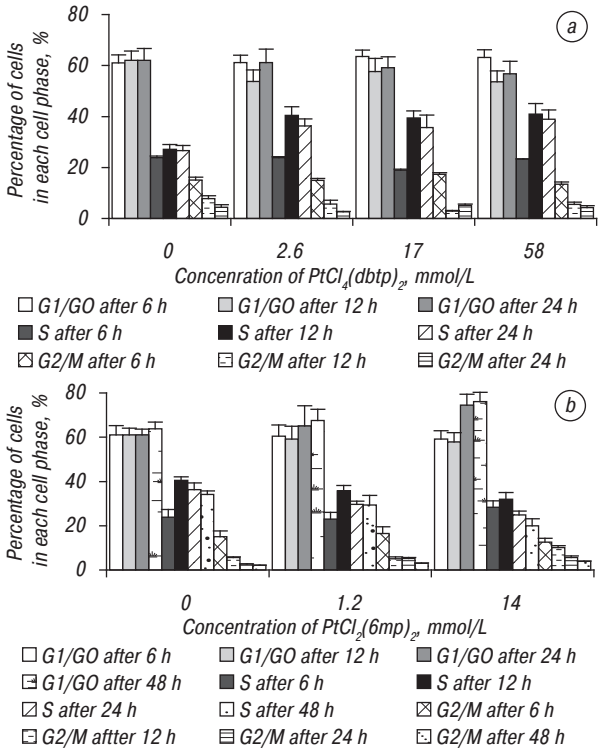

Fig. 4. Cell cycle phases in B16 cells after exposition on $PtCl_4(dbtp)_2$ (*a*) and on $PtCl_2(6mp)_2$ (*b*)

Due to low solubility, only LC_{10} and LC_{50} of $PtCl_2(6mp)_2$ were taken for apoptosis assay. Percentage of B16 apoptotic cells after 6 and 12 h incubations with $PtCl_2(6mp)_2$ is shown on Fig. 3, *b*. High correlation was found between percent of apoptotic cells and $PtCl_2(6mp)_2$ concentrations. Correlation coefficients for 6 and 12 h incubation times were $r = 0.99$ ($p < 0.01$) and $r = 0.73$ ($p < 0.05$), respectively. High negative correlations were estimated for viable cells after 6 h and 12 h, $r = -0.99$ ($p < 0.01$) and $r = -0.96$ ($p < 0.05$).

Influence of $PtCl_4(dbtp)_2$ and $PtCl_2(6mp)_2$ on cell cycle in B16 cells. Cell cycle phases of B16 cells after 6, 12 and 24 h incubation times with $PtCl_4(dbtp)_2$ are presented on Fig. 4, *a*. It was impossible to perform analysis after 48 h, due to small number of living cells. Cell number in G0/G1 correlated with $PtCl_4(dbtp)_2$ concentrations in 12 and 24 h assays, $r = -0.55$ and $r = -0.70$, respectively. Similar correlations were found for S phase ($r = 0.5$; $r = 0.6$). Inhibition of S-phase was observed after incubation with $PtCl_4(dbtp)_2$.

Cell cycle phases of B16 cells after 6, 12, 24 and 48 h incubation times with $PtCl_2(6mp)_2$ are presented on Fig. 4, *b*. An accumulation of cells was observed within the G0/G1 phase after 12, 24 and 48 h of incubation with $PtCl_2(6mp)_2$. Cell number in G0/G1 phase was highly correlated with concentrations in 24 and 48 h assays, $r = 0.98$ ($p < 0.05$) and $r = 0.98$ ($p < 0.05$), respectively. Cell number decreased in B16 cultures after 12, 24 and 48 h exposition to $PtCl_2(6mp)_2$ ($r = -0.87$; $r = -0.86$; $r = -0.97$, $p < 0.05$). The increasing cell number in G2/M after 12, 24 and 48 h incubation was observed. Strong correlations were noticed between cell number in G2/M phases and $PtCl_2(6mp)_2$ concentrations for 12 and 48 h, $r = 0.98$ ($p < 0.02$) and $r = 0.90$ ($p < 0.05$), respectively, while after 24 h only weak correlation was measured ($r = 0.66$).

DISCUSSION

During the last decade the development new platinum drugs could be observed. These drugs which contained amino ligands belong to the third generation of cisplatinum derivates which derived from cisplatinum. The advantages of the platinum(IV) complexes is that they some of them can be used as oral drugs. This is the reason for testing activity of those compounds against cancer [9].

Cisplatinum belongs to alkylating agents, its anticancer action is connected with forming adducts between platinum and DNA strands. Cisplatinum also binds to cytoplasmatic proteins. Adducts are responsible for cell cycle arrest and apoptotic response. Cytotoxic effect is usually connected with these two phenomena [5]. Covalent bindings between platinum complexes and DNA decrease rate of replication, transcription and following translation. Inhibiting the cell cycle is connected with excessive repair processes. N7 positions of guanine and adenine are the main binding places for platinum [10]. DNA can be additionally destroyed in secondary process by free radicals, which are also responsible for apoptosis induction after treatment with platinum compounds [8, 11]. Apoptosis is one of the most important processes responsible for resistance during cancer chemotherapy [12].

$PtCl_4(dbtp)_4$ stopped the B16 growth *in vitro*. The both complexes partially block cell divisions and triggers apoptotic cascade within tested cells.

$PtCl_4(dbtp)_2$ could be dissolved easily in DMSO while $PtCl_2(6mp)_2$ could not. Probably the good solubility of $PtCl_4(dbtp)_2$ was partially responsible for better cytotoxic effect, because higher availability of platinum containing molecules. The solubility was the reason of not achieving LC_{90} for $PtCl_2(6mp)_2$. $PtCl_4(dbtp)_2$ complex

was prepared on the basis of $PtCl_4(dmtp)_2$, a compound which was previously tested against B16 and ClS91 cells. In our previous study toxicity against B16 line was high, but the solubility was low [5]. In current study four CH_3 — ligands to the $PtCl_4(dmtp)_2$ were added and this complex reached better solubility while preserving good cytotoxic properties. LC_{50} for $PtCl_4(dbtp)_2$ and $PtCl_4(dmtp)_2$ were 17.0 and 0.13 μmol/l, respectively.

B16 cells were more resistant for $PtCl_2(6mp)_2$, what can be related to poor solubility of this complex in buffered saline. Intrinsic or acquired resistance to cisplatinum are the main drawbacks in cancer management with cisplatinum regimens [1, 13].

Cytotoxic properties of $PtCl_4(dbtp)_2$ and $PtCl_2(6mp)_2$ are related to heterocyclic ligand (imidazole N-Pt bonded), which is recognized as a donors for hydrogen binding. The general scheme of many anticancer platinum complexes can be presented as cis-$[PtX_2(NHR_2)_2]$, where R is an organic ligand, X — leaving group, i. e. chloride dicarboxylic ions. $PtCl_4(dbtp)_2$ has a chloride ions and pyrimidine, while $PtCl_2(6mp)_2$ has a 6-mercaptopurine instead of pyrimidine.

When analyzing these two complexes it was obvious that $PtCl_4(dbtp)_2$ was more potent to induce apoptosis within melanoma cells than $PtCl_2(6mp)_2$. The both of tested complexes were able to trigger apoptosis with B16 cells even after 6 h. Many of drugs which induce apoptosis are related on p53 protein and block cell cycle between G1 and S phases [14]. It seems that $PtCl_2(6mp)_2$) acts in similar manner. On the other hand, $PtCl_4(dbtp)_2$ caused inhibition in S phase. This observation can be connected with extensive DNA destruction, which was not possible to repair by the cellular system. We suggest that apoptosis was evoked in cells which were stopped in G0/G1 or G2/M check points. Similar observations were made by Bergamo et al., (1999) who tested cisplatinum on Lewis and pulmonary cell cancer lines.

The most of designed cytotoxic drugs were able to interact with DNA strands which leaded to destroy of information saved in nucleic acid and in the consequence cell death [15, 16]. There are many mechanisms responsible for development of resistance during chemotherapy. One of them is lack of apoptotic response following systemic treatment [17, 18, 19].

In conclusion, $PtCl_4(dbtp)_2$ express higher cytotoxic against B16 cells than $PtCl_2(6mp)_2$. Apoptosis was the main mechanism of cell loss in cultures during incubation with the both tested complexes. $PtCl_4(dbtp)_2$ leaded to accumulation in S phase, while $PtCl_2(6mp)_2$ in G1/G0.

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