COMPLEXES OF PALLADIUM(II) WITH 1-PHENYL-1-HYDROXYMETHYLENE BISPHOSPHONIC ACID AND THEIR ANTITUMOR ACTIVITY

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Complex formation of K₂[PdCl₄] with 1-phenyl-1-hydroxymethylene bisphosphonic acid (PhHMBP, H₄L) has been studied by pH potentiometry, electron and NMR spectroscopy. It was found that in aqueous solution with physiological concentration of chlorine anions (0.15 mol/l KCl), anionic complexes of the equimolar compositions [PdLCl₂]³⁻ (lgβ = 24.51 (0.3)) and [PdLCl]⁺ (lgβ = 20.74 (0.02)) are formed. In the first coordination sphere palladium was surrounded by two oxygen atoms of two phosphonic groups of the bidentate coordinated ligand with closure of six-membered [O, O] ring, and two chlorine anions. The formation of palladium(II) equimolar complexes with PhHMBP and bidentate coordination of the ligand to the central metal cation was confirmed by ³¹P NMR spectroscopy. Cytotoxic activity (IC₅₀ based on metal content) of the synthesized Pd(II) complexes with PhHMBP against human MG-63 osteosarcoma and MCF-7 mammary tumor cells was compared with cisplatin on in vitro models. It was established that cytotoxic activity of the Pd complexes was lower than that of cisplatin. The acute toxicity (LD₅₀ based on metal content) of solutions of Pd(II) complexes with PhHMBP was found to be lower compared to cisplatin. It was shown that the use of solutions of palladium(II) complexes with PhHMBP inhibited tumor growth in mice with sarcoma 180.

Key words: palladium(II) complexes, bisphosphonic acid, cytotoxic activity, toxicity.

Cisplatin is still one of the most famous and efficient anticancer drugs used in the clinical practice [1]. Nevertheless, searching for new compounds with antineoplastic action continues to eliminate the serious side effects, to improve the clinical efficacy and to broaden the antitumor spectrum. In particular, palladium(II) bisphosphonates are promising species for the treatment of bone cancer and metastases: complexes include a cytotoxic metal (palladium) and a bisphosphonic acid, which possesses affinity for bone tissue and can ensure targeted delivery of the cytotoxic metal to the lesion site [2-9]. The P-C-P moiety present in bisphosphonic acids provides their active link to the bone matrix, and two side substituents determine their physico-chemical and pharmacologic properties. To study the effect of the structure of complex and chemical nature of the substituents bonded to the carbon atom of bisphosphonate moiety on the biological activity, the formation of palladium(II) complexes with 1-phenyl-1-hydroxymethylene bisphosphonic acid was investigated, which, besides two phosphonic groups, contain a hydroxy group and a phenyl moiety.

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The aim of the work is to determine the composition, structure, stability and biological activity of palladium (II) complexes with PhHMBP.

**Materials and Methods**

The following reagents were used in the work: 1-phenyl-1-hydroxymethylene bisphosphonic acid (PhHMBP, H$_2$L) synthesized by the procedure presented in [10], concentrated solution of pure carbonate-free potassium hydroxide, palladium black. Potassium tetrachloropalladate was prepared according to the standard scheme [11]:

$$\text{Pd}^{\text{HNO}_3+3\text{HCl}} \rightarrow \text{PdCl}_2 2\text{KCl} \rightarrow \text{K}_2\text{PdCl}_4$$

The palladium content of PdCl$_4$ was determined by the reduction of a weighed amount of the compound in a hydrogen stream to metallic palladium [12].

The standard solution of hydrochloric acid was prepared from a titrimetric standard substance. The potassium hydroxide solution was prepared right away before using from a saturated KOH solution. The exact concentration of PhHMBP was determined by titration with KOH.

Weighting was performed on a Vibra HT-120 CE balance. The measurements were made a Metrohm 827 pH lab pH meter. The device was calibrated using standard buffer solutions with pH 1.68, 4.01, 7.00, 9.18, 11.00 (range 50000-11000 cm$^{-1}$). The recording was performed in quartz cuvettes with $l$ = 1 cm.

To prepare solutions for pH potentiometric and spectrophotometric studies, freshly distilled water was used.

The formation constants of complexes and their equilibrium concentrations were calculated using the PSEQUAD program [13] from the data of EASs and pH of equilibrium solutions from the formulas:

$$\beta_{\text{H}_{2}\text{L}} = [\text{H}_2\text{L}] / [\text{L}] [\text{H}]^2 \quad (1);$$

$$\beta = [\text{M}_x\text{L}_y\text{H}_z\text{Cl}_4] / [\text{M}]^x [\text{L}]^y [\text{H}]^z [\text{Cl}]^4 \quad (2).$$

The formation constants of palladium(II) chloroaqua complexes: lg$\beta$[PdCl$_6^{2-}$] = 4.47; lg$\beta$[PdCl$_4^{3-}$] = 7.76; lg$\beta$[PdCl$_4^{2-}$] = 10.17; lg$\beta$[PdCl$_4^{3-}$] = 11.54 (I = 1 mol/l HClO$_4$ + NaClO$_4$, T = 25 °C) [14].

The $^{31}$P NMR spectra with proton decoupling ($^{31}$P-$\{^1$H$\}$) were recorded on a Bruker AVANCE 400 spectrometer without deuterium stabilization of the resonance conditions. The value of $^{31}$P NMR chemical shift was determined relative to 85% H$_3$PO$_4$.

The pH potentiometric and spectrophotometric studies of complex formation in solutions with physiological concentration of chloride ions (0.15 mol/l KCl) were carried out for the systems with a constant $K_{\text{L}}$[PdCl$_4^{2-}$] concentration (C$_{\text{Pd(II)}}$ = 2‧10$^{-3}$ mol/l) and a variable concentration of PhHMBP, neutralized with three KOH equivalents (C$_{\text{KOH}}$ = 4‧10$^{-4}$-4‧10$^{-5}$ mol/l), and for the systems $K_{\text{L}}$[PdCl$_4^{2-}$] - PhHMBP = 1:1 and 1:2 (C$_{\text{Pd(II)}}$ = 2‧10$^{-3}$ mol/l, C$_{\text{KOH}}$ = 2‧10$^{-5}$, 4‧10$^{-4}$ mol/l) depending on pH. Sets of solutions with the molar ratios $K_{\text{L}}$[PdCl$_4^{2-}$]+PhHMBP = 1:1 (C$_{\text{Pd(II)}}$ = 0.05 mol/l) and 1:2 (C$_{\text{Pd(II)}}$ = 0.025 mol/l, C$_{\text{KOH}}$ = 0.05 mol/l) were investigated by $^{31}$P NMR spectroscopy.

All measurements were carried out a day after the preparation of solutions, the period required for the establishment of chemical equilibrium.

To carry out the studies of biological activity, solutions of a Pd(II) complex with PhHMBP (C$_{\text{Pd(II)}}$ = 10.0 mg/ml (0.094 mol/l); C$_{\text{KOH}}$ = 0.094 mol/l, C$_{\text{KCl}}$ = 0.15 mol/l, pH = 4.56) were prepared. To study in vitro cytotoxic activity, we used MG-63 human osteosarcoma and cells of the MCF-7 line (cell line obtained from human breast cancer). The cells were cultured in a DMEM culture medium (Biowest, France) from 10% fetal calf serum (FCS) (HyClone, France) at 37 °C in a humid atmosphere in the presence of 5% CO$_2$. The obtained cell suspensions were placed on a 96-well plate in a concentration of 1.2–1.5‧10$^4$ cells/well (in a volume of 0.1 ml) and incubated for 72 h at 37 °C in the presence of 5% CO$_2$. The solution of palladium complex under investigation was added four or two times (100 μl each time) with two-fold dilution from a concentration of 200 μg/ml in the first wells to a concentration of 1.56 μg/ml. The wells intact cell culture were used as a control. After the addition of a solution of palladium complexes, the test and control cultures in the plates were incubated for 72 h.

The cytotoxicity of palladium(II) complexes with PhHMBP was assessed on the basis of the number of alive cells in the test and control wells (MTT test) [15]. To this end, after the completion of incubation, the supernatant with dead cells and detritus was completely removed, and 50 μl of a crystal violet solution was put in each well to fix and color the cells.
on the substrate. The plate was incubated for 10 min at room temperature. Then the dye was removed, the plate wells were washed with water and dried at room temperature. The optical density, which is proportional to the number of alive cells in the wells, was determined with a Labsystem Multiscan Plus spectrometer at an excitation wavelength of 540 nm.

The in vivo toxicity of Pd(II) complexes with PhHMBP was assessed by determining the half-lethal dose (LD$_{50}$) and studying the percentage of sarcoma 180 (S-180) growth inhibition in while mice in the case of introducing solutions of Pd(II) complexes with PhHMBP. The half-lethal dose of Pd(II) complexes with PhHMBP (LD$_{50}$) was determined according to [16]. The solutions were injected intraperitoneally in while mice once before feeling in different doses. The screening studies of the anticancer activity of Pd(II) complexes with PhHMBP were carried out on while nonlinear mice of the same sex with a body weight of 18–20 g on a sarcoma 180 model [17]. The number of animals in the test group was 7 and in the control group 22. The substance under investigation was injected 24 h after tumor transplantation in a dose of 1/3 LD$_{50}$ daily (7 injections).

The effect parameter was the percentage of tumor growth inhibition by mass. The minimum activity criterion is tumor growth inhibition of over 50%.

Results and Discussion

According to the results of a pH potentiometric titration in the pH range 2.2–11.3, PhHMBP is a tetrabasic acid (H$_4$L). The determined formation constants of PhHMBP forms of different degree of deprotonation are listed in Table 1. They were calculated from the data of two parallel titrations (each titration included more than 50 points) (according to the measurement data in two parallel experiments). The first dissociation constant value for PhHMBP (pK$_1$) is close to 2, which complicates its calculation from the data of pH potentiometric titration. The values of pK$_2$, pK$_3$ and pK$_4$, which correspond to the consecutive dissociation of OH protons from the phosphonic groups, within the experimental error agree with the published data [18]. The geminal hydroxy group remains protonated below the pH of 13.

The diagram of the equilibrium concentration distribution of PhHMBP forms of different degree of protonation as a function of solution pH is shown in Fig. 1. It is seen from the diagram that in the range 6≤pH≤8, [H$_2$L]$^2^-$ and [H$_3$L]$^-$ are predominating forms.

It should be taken into account that depending on the concentration of chlorine anions in aqueous solutions of K$_2$[PdCl$_4$], palladium(II) exists as chloro aqua complexes of the composition [PdCl$_{4-n}$·(H$_2$O)$_n$]$^{2-}$ (n = 0-4). In a 0.15 mol/l KCl solution with a K$_2$[PdCl$_4$] concentration of 2·10$^{-3}$ mol/l, the following Pd(II) complex species are in equilibrium: [PdCl$_4^{2-}$] (1.51·10$^{-3}$ mol/l), [PdCl$_3$(H$_2$O)]$^-$ (4.78·10$^{-4}$ mol/l) and [PdCl$_2$(H$_2$O)$_2$] (1.20·10$^{-5}$ mol/l), with the total absorption at 21650 cm$^{-1}$.

When a K$_2$[PdCl$_4$] solution is titrated in 0.15 mol/l KCl with a solution of PhHMBP neutralized with three KOH equivalents, the maximum in the electronic absorption spectra (EASs) shifts from 21650 cm$^{-1}$ to approx. 24800 cm$^{-1}$ (pH 4.91-5.24) due to the formation of a palladium(II) complex with PhHMBP (Fig. 2).

Similar changes are observed in the EASs of the K$_2$[PdCl$_4$]: PhHMBP = 1 : 1 system within the pH range 2.59-7.04 (Fig. 3).

The formation constants of complexes of the compositions [PdHCl$_2$]$_3^-$ (lgβ = 24.51(0.3)) and

<table>
<thead>
<tr>
<th>lg β[HL]$^3^-$</th>
<th>lg β[H$_2$L]$^2^-$</th>
<th>lg β[H$_3$L]$^-$</th>
<th>pK$_4$</th>
<th>pK$_3$</th>
<th>pK$_2$</th>
<th>Ionic strength</th>
<th>Literature data</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.80</td>
<td>17.47</td>
<td>20.00</td>
<td>10.80</td>
<td>6.67</td>
<td>2.53</td>
<td>0.10 mol/l KNO$_3$ and NaCl, 25 °C</td>
<td>[18]</td>
</tr>
<tr>
<td>10.77(0.02)</td>
<td>17.59(0.02)</td>
<td>20.33(0.02)</td>
<td>10.77</td>
<td>6.82</td>
<td>2.74</td>
<td>0.15 mol/l KCl, 20 °C</td>
<td>Our data</td>
</tr>
</tbody>
</table>
The values of formation constant calculated for complexes of equimolar composition were used to construct a diagram of the equilibrium concentration distribution of palladium complexes as a function of pH (Fig. 4).

It is seen from the diagram that the anionic complex of the equimolar composition \([\text{PdLCl}_2]^{4-}\) is predominant in the solution at the physiological concentration of chlorine anions and pH close to 7.

The composition of the complexes and the mode of the coordination of ligand to the central metal ion, determined from spectrophotometric data, are corroborated by the results of investigation of the system \(K_{\text{2[PdCl_4]}} : \text{PhHMBP} = 1 : 1 \) and \(1 : 2\) by the \(^{31}\text{P}\) NMR method.

The \(^{31}\text{P}\) NMR spectra (Fig. 5, a) of a set of solutions with the ratio \(K_{\text{2[PdCl_4]}} : \text{PhHMBP} = 1 : 1\) were calculated from spectrophotometric and pH potentiometric data.

The coordination mode of ligand to the central metal ion was established on the basis of the dependence of the position of the absorption band maximum on the composition of the first coordination sphere (chromophore) of square-planar palladium complexes [19]. In particular, in the case of bidentate coordination of ligand by two oxygen atoms of phosphonic groups the absorption band with maximum at ~24800 cm\(^{-1}\) indicates the formation of a complex with chromophore of the composition \([\text{Pd; 2Cl; 2OPO}_3]\).
Fig. 3. $EASs$ of the $K_{2}[PdCl_4] : PhHMBP = 1 : 1$ system ($C_{Pd(II)} = C_{H_4L} = 2 \cdot 10^{-3}$ mol/l, pH: 1 – 2.59, 2 – 4.36, 3 – 4.57, 4 – 4.85, 5 – 5.35, 6 – 7.04)

exhibit a redistribution between the integral intensities of the signals of the phosphonic groups of free PhHMBP ($\delta^{31}P$ 15.97-15.51 ppm) and coordinated ligand ($\delta^{31}P$ 38.79-38.36 ppm) by increasing pH from 1.50 to 4.92. The position of the signals of phosphorus nuclei in the range 38.79-38.36 ppm corresponds to the formation of a complex with PhHMBP bidentately coordinated to the central metal ion. At pH 4.92, PhHMBP is almost completely involved into a complex with palladium (II).

The $^{31}$P NMR spectra of solutions of the system with the ratio $K_{2}[PdCl_4] : PhHMBP = 1 : 2$ exhibit signals with similar values of chemical shifts (Fig. 5, b). By increasing pH, the integral intensity of the signals of coordinated phosphonic groups increases and is approximately equal to that of the signal of free acid. This evolution in the spectra of the $K_{2}[PdCl_4] : PhHMBP = 1 : 2$ system indicates the formation of the complexes only of equimolar composition, in which ligand is bidentately coordinated to palladium(II) by two oxygen atoms of two phosphonic groups.

It should be noted that the $^{31}$P NMR spectra of solutions with pH<3 exhibit low-intensity signals in the $\delta^{31}P$ range 24.3–23.5 ppm, which probably correspond to a complex with PhHMBP monodentately coordinated to palladium(II) by phosphonic group oxygen atom. This was not established by electron spectroscopy and pH potentiometry ($C = 2 \cdot 10^{-3}$ mol/l).

The $^{31}$P NMR spectrum of bidentately coordinated PhHMBP consists of three signals with close $\delta$ values. As we have previously shown, the observed nonequivalence of the phosphorus nuclei in the coordinated ligand may be both due to the conformation isomerism of the formed complexes and a result of forming an intraligand hydrogen bond [20].

The in vivo anticancer activity of a palladium(II) complex with PhHMBP was determined on an MG-63 and MCF-7 cell line models ($IC_{50}$ 21

Fig. 4. Distribution diagram of the equilibrium concentration of complexes with different compositions as a function of pH in system $K_{2}[PdCl_4] : PhHMBP = 1 : 1$ ($C_{Pd(II)} = C_{H_4L} = 2 \cdot 10^{-3}$ mol/l)
Fig. 5. $^{31}P$ NMR spectra of the $K_{[PdCl]}$ – PhHMBP systems: $a$ – $C_{Pd(II)} = C_{HMBP} = 0.05$ mol/l, pH: 1 – 1.49, 2 – 2.22, 3 – 3.04, 4 – 3.82, 5 – 4.92, 6 – 5.80; $b$ – $C_{Pd(II)} = 0.025$ mol/l, $C_{HMBP} = 0.05$ mol/l, pH: 1 – 1.51, 2 – 2.48, 3 – 3.56, 4 – 3.97, 5 – 6.32
The acute toxicity of solutions of palladium(II) complexes with PhHMBP (LD$_{50}$ = 41 mg/kg (based on palladium content)) is lower than that for cisplatin, for which LD$_{50}$ is found to be 8.42 mg/kg (based on palladium content).

When determining the antineoplastic action of solutions of palladium(II) complexes with PhHMBP on sarcoma 180 bearing mice, the found percentage of tumor growth inhibition is over 50% (minimum activity criterion) (Table 2).

Depending on pH, the PhHMBP ligand forms in water solutions with the physiological concentration of chlorine anions (0.15 mol/l) the anionic complexes of the equimolar compositions, [PdHLCl$_2$]$^3^-$ and [PdLCl$_2$]$^4^+$, as indicated by pH potentiometry, electron and NMR spectroscopy. The first coordination sphere involves two oxygen atoms of two phosphonic groups of ligand with closure of the six-membered [O, O] ring and two chlorine anions.

The solutions of palladium(II) complexes with PhHMBP demonstrates moderate in vitro cytotoxic activity (IC$_{50}$) against MG-63 and MCF-7 tumor cells that does not exceed that of cisplatin. At the same time, solutions of palladium(II) complexes with PhHMBP exhibit lower toxicity as compared with cisplatin. They also provide inhibition for sarcoma 180 growth in mice.

Table 2. Anticancer activity of solutions of palladium (II) complexes with PhHMBP on a sarcoma 180 model

<table>
<thead>
<tr>
<th>System</th>
<th>Ninth day</th>
<th></th>
<th>Tumor growth inhibition, %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mass</td>
<td>Tumor</td>
<td></td>
</tr>
<tr>
<td>Pd(II) complex</td>
<td>0.395 ± 0.097</td>
<td>59.0</td>
<td></td>
</tr>
<tr>
<td>with PhHMBP</td>
<td>Control</td>
<td>0.964 ± 0.106</td>
<td>–</td>
</tr>
</tbody>
</table>

and 41.7 μg/ml (based on palladium content), respectively, Fig. 6). The found values are lower than those found for cisplatin (2.2 and 1 μg/ml (based on palladium content), respectively).

*Fig. 6. Concentration-dependent effects of Pd (II) complexes with PhHMBP on the percentage of alive cells line MG63 and MCF-7*
КОМПЛЕКСНІ СПОЛУКИ ПАЛЛАДІЮ(II) З 1-ФЕНИЛ-1-ГІДРОКСИМЕТИЛЕН-БІСФОСФОНОВОЮ КИСЛОТОЮ ТА ЇХ ПРОТИПУХЛИННА АКТИВНІСТЬ

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Методами pH-потенциометрии, электронной и ЯМР спектроскопии исследовано комплексообразование K$_2$[PdCl$_4$] с 1-фенил-1-гидроксиметиленбисфосфоновой кислотой (PhHMВР, H$_2$L). Встановлено, що у водному розчині з фізіологічною концентрацією анонімів хлору (0, 15 моль/л KCl) в залежності від pH утворюються анионні комплекси еквімолярного складу [PdHLCl$_2$]$^2^+$ (lgβ = 24,51(0,3)) та [PdLCl$_4$]$^2^-$ (lgβ = 20,74(0,02)). Внутрішня координаційна сфера Pd сформована двома атомами кисню (O, O) циклу та двома анионами хлору. Методом спектроскопії ЯМР 31P підтверджено утворення комплексів Pd(II) еквімолярного складу з бідентатно координованою до центрального іона металу PhHMВР двома атомами кисню фосфонових груп. На моделях in vitro порівняно цитотоксичну активність (IC$_{50}$) за вмістом металу розчинів комплексів Pd(II) з PhHMВР та цисплатину по відношенню до клітин місць розміщення MG-63 та пухлини молочної залози MCF-7 льони. Встановлено, що розчини комплексів Pd(II) з PhHMВР виявляли меншу цитотоксичну активність порівняно з цисплатином. Параметри гострої токсичності (LD$_{50}$) за вмістом металу) розчинів комплексів Pd(II) з PhHMВР вказували на їхню меншу токсичність порівняно із цисплатином. Показано, що застосування розчинів Pd(II) із PhHMВР обумовлювало гальмування пухлинного росту в мишей із саркомою-180.

К ЛЮЧ ОВІ СЛОВА: комплексы палладію(II), бісфосфонові кислоти, цитотоксична активність, токсичність.

КОМПЛЕКСНІ СОЕДИНЕНИЯ ПАЛЛАДИИ(II) С 1-ФЕНИЛ-1-ГИДРОКСИМЕТИЛЕН-БИСФОСФОНОВОЙ КИСЛОТОЙ И ИХ ПРОТИВООПУХОЛЕВАЯ АКТИВНОСТЬ

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Методами pH-потенциометрии, электронной и ЯМР спектроскопии исследовано комплексообразование K$_2$[PdCl$_4$] с 1-фенил-1-гидроксиметиленбисфосфоновой кислотой (PhHMВР, H$_2$L). Установлено, что в водном растворе физиологической концентрации анионов хлора (0, 15 моль/л KCl) в зависимости от pH образуются анионные комплексы эквимолярного состава [PdHLCl$_2$]$^2^+$ (lgβ = 24,51(0,3)) и [PdLCl$_4$]$^2^-$ (lgβ = 20,74(0,02)). Внутренняя координационная сфера Pd сформирована двумя атомами кислорода двух фосфоновых групп. На моделях in vitro сравнили цитотоксическую активность (IC$_{50}$ по содержанию металла) рас-
творов комплексов Pd(II) с PhHMBP и цисплатина по отношению к клеткам остеосаркомы MG-63 и опухоли молочной железы MCF-7 человека. Установлено, что растворы комплексов Pd(II) с PhHMBP проявляли меньшую цитотоксическую активность по сравнению с цисплатином. Параметры острой токсичности (ЛД₀₅₀ по содержанию металла) растворов комплексов Pd(II) с PhHMBP указывали на их меньшую токсичность по сравнению с цисплатином. Показано, что применение растворов комплексов Pd(II) с PhHMBP обусловливало торможение опухолевого роста у мышей с саркомой-180.

Ключевые слова: комплексы палладия(II), бисфосфоновые кислоты, цитотоксическая активность, токсичность.

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