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RESEARCH ARTICLE

Inhibitory effect of cisplatin and [Pt(dach)Cl2] on the activity of phospholipase A2

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Abstract

This work has been focused on testing the influence of two selected Pt(II) complexes cisplatin, $Pt(NH_3)_2Cl_2$, and $[Pt(dach)Cl_2]$ on the activity of porcine pancreatic phospholipase A_2 (PLA_2). It has been assumed that this enzyme plays a role in carcinogenesis and that it could be a target in the tumour therapy. The results of this study show that both Pt(II) complexes inhibit the activity of the enzyme, though they bind to it in a different manner. While cisplatin interacts with the enzyme in an acompetitive manner, the stable interaction of $[Pt(dach)Cl_2]$ with PLA_2 could not be detected under our experimental conditions.

Keywords: MALDI TOF MS, metallo-drugs, phosphatidylcholine

Introduction

Although transition metal complexes, such as cisplatin, carboplatin, and oxaliplatin, have been applied as anti-tumour agents over decades, there is a lack of data regarding the interaction of these metallo-drugs with the enzymes involved in phospholipid metabolism. These enzymes, such as phospholipase A, C, or D, are an important part of intracellular signalling pathways, which control the cell growth and differentiation[1,2].

Phospholipases A_2 are esterolytic enzymes which hydrolyze membrane phospholipids producing free fatty acids (FFAs) and lysophospholipids (LPLs), thereby providing precursors for the biosynthesis of proinflammatory lipid mediators. Pancreatic phospholipase A_2 (PLA_2) belongs to the type I PLA_2 enzymes, which have been shown to elicit receptor-mediated cellular responses, such as the stimulation of prostaglandin production, the secretion of steroid hormones[3,4], or cell proliferation[5,6]. This enzyme plays a critical role in cellular homeostasis due to the catalysis of a rate-limiting step in the formation of arachidonic acid and derived biomediators. Accordingly, it has been shown that pancreatic PLA_2 plays a role in carcinogenesis, immune suppression, and the stimulation of cell proliferation, as well as invasion and metastasis[7–9].

The main aim of this study has been to test whether cisplatin (routinely used in the therapy of solid tumours, such as ovarian cancers[10,11]) and dichloro(1,2-diaminocyclohexane)platinum(II) ($[Pt(dach)Cl_2]$), which is one of metabolites of other routinely applied metallo-drug, oxaliplatin[12,13], inhibit the activity of pancreatic PLA_2. We have also studied potential interaction between the complexes and the enzyme. Porcine pancreatic PLA_2 was used as the model system and the results obtained for the two enzyme assays demonstrate a clear inhibitory effect, indicating differences in the mode of interaction between the complexes and PLA_2.

Materials and methods

Chemicals

$[PtCl_2(dach)]$, dichloro(1,2-diaminocyclohexane) platinum(II), was synthesized as described in the literature[14,15]. The chemical analysis, UV-VIS, and nuclear magnetic resonance (^{1}H-NMR) spectral data of this complex were in good agreement with those obtained in previous preparations. Cisplatin, $[PtCl_2(NH_3)_2]$, and

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cis-diamminedichloroplatinum(II) were purchased from Sigma (*Taufkirchen*, Germany). Porcine pancreatic PLA_2 was purchased from Fluka (Neu-Ulm, Germany) showing an activity of 163 U/mg protein; 1 U corresponds to the amount of the enzyme that hydrolyzes 1 μmol of 3-*sn*-phosphatidylcholine in 1 min at 37°C and pH 8.0). The enzyme was used without further purification. In the experiments, we used lipids and lysolipids commercially available from Sigma (*Taufkirchen*, Germany) and Avanti Polar Lipids (Alabaster, MA, USA) as 10 mg/mL chloroform solutions and phosphatidylcholine (PC) extracted from hen's egg yolk[16]. The detergent applied with the aim of increasing the surface area and decreasing the substrate's surface concentration, deoxycholic acid, was purchased from Sigma (Taufkirchen, Germany). Matrices for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), 2,5-dihydroxybenzoic acid (DHB), and sinapic acid (SA), as well as solvents (chloroform and methanol) and trifluoroacetic acid were purchased from Fluka and Sigma (Taufkirchen, Germany) and were used without further purification. Eggs for the isolation of lecithin were purchased at the local supermarket.

Methods

Extraction of egg-yolk PC

The method for the isolation of PC from egg yolk was based on the total lipid extraction by the $CHCl_3$:CH_3OH, 2:1 (v:v) mixture, the removal of water-soluble compounds using a 1% NaCl solution and the precipitation of phospholipids in cold acetone. A detailed description of the procedure can be found elsewhere in literature[16]. The concentration of phospholipids was checked by modified Stewart's colorimetric assay[17] with the standard curve recorded with palmitoyl-oleoyl-phosphatidylcholine (PC 16:0, 18:1).

Preparation of samples for MALDI-TOF MS analysis

For the investigation of the kinetics of PLA_2 by MALDI-TOF MS, egg-yolk PC (1 mg/mL) was re-suspended in a Tris buffer (50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, 5.4 mM deoxycholate, pH 8.5) and incubated with pancreatic PLA_2 (0.1 mg/mL[18]). Separately, the enzyme was incubated with Pt(II) complexes (5×10^{-4} mg/mL) for 2.5 h and, afterwards, the suspension of phospholipids was added. The reaction was terminated after the indicated periods of time by the addition of chloroform/methanol (1/1, v/v) mixture, followed by vigorous vortexing. The chloroform layer was separated by centrifugation and taken for further analysis by MALDI-TOF MS.

The procedure of sample preparation demands the premixing of the sample and the matrix before application on the sample plate, in order to ensure better reproducibility. The sample was applied as 1.5 μL droplet on the sample plate and dried immediately under a warm stream of air.

The application procedure for the analysis of transition metal complexes by MALDI slightly differs from that described for lipids: in this case, the matrix/analyte mixture was left at room temperature to co-crystallize. This has been shown to yield the best results in terms of homogeneity and the reproducibility of the spectra[19].

For the analysis of the binding properties of platinum complexes with PLA_2 by MALDI-TOF MS, the enzyme (0.1 mg/mL) dissolved in a phosphate-buffered saline (PBS) buffer with 5 mM $CaCl_2$ (pH 7.2) was incubated with the complexes (5×10^{-4} mg/mL) in the presence of PC (16:0, 18:1) (1 mg/mL) for 3.5 h. Both complexes and PC were prepared in the same buffer as the enzyme. Subsequently, a small volume of the sample (1 μL) and then the same volume of matrix solution were applied onto the MALDI target plate. This mixture was left at room temperature to co-crystallize.

MALDI-TOF MS

The MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry DE Pro Workstation (PerSeptive Biosystems, Framingham, MA, USA) or Autoflex (Bruker Daltonics) MALDI-TOF mass spectrometers equipped with a pulsed nitrogen laser (337 nm). All spectra were acquired with delayed extraction conditions and under the reflector mode. For the lower *m/z* ranges, no low mass gate was set and all mass spectra of phospholipids and complexes were obtained using the reflector mode. The mass spectra were calibrated by setting the peak of the protonated DHB matrix to its appropriate value (155.034 Da). The MALDI-TOF MS analysis of the enzyme was conducted under delayed extraction conditions and in the linear mode. All spectra were acquired with a 10 mg/mL concentration of SA in an acetonitrile/water solution (volume ratios 1:1).

UV/Vis spectrophotometric titration

UV/Vis spectrophotometric titration was applied in order to detect the interaction between the complexes and the enzyme. Briefly, a series of solutions of PLA_2 with Pt(II) complexes with varying concentrations of complexes was made in PBS (pH 7.4). The concentration of PLA_2 was kept constant at 25 μM and different amounts of platinum complexes (in the concentration range from 2.5 to 250 μM were added). The concentration ratios ($cPLA_2$:cPt(II)) were in the range from 0.1 up to 10. After 1 h incubation at 37°C, all spectra were recorded in the wavelength range 240–400 nm.

Colorimetric assay for enzyme activity

The colorimetric assay according to the description of the manufacturer (Sigma-Aldrich[20]) was applied for monitoring the PLA_2 activity. The precise description of the reaction mechanism could not be found. We assume that the procedure was based on the measurement of the change in the concentration of Fe^{2+} ion (obtained by the reduction in the presence of hydroxylamine) bound to PC. As the concentration of PC decreases upon the action of PLA_2, the measured absorbance value A_{570} also decreases. The scope of the plot of A_{570} in relation with time of incubation

represents the reaction rate and can lead to a conclusion about the effect of the complexes on the PLA_2 activity.

Results

In order to investigate the influence of the selected transition metal complexes on the activity of PLA_2, two methods were applied: colorimetric assay and MALDI-TOF MS. In the first part of our study, the MALDI-TOF mass spectra of Pt(II) complexes and isolated phospholipids were analyzed, whereas in the second part, MALDI-TOF MS was applied for monitoring the activity of the enzyme and the assessment of the effect of the complexes on the production of lyso-phosphatidylcholine (LPC) in the PLA_2-catalyzed reaction. These results were also confirmed by colorimetric assay.

MALDI-TOF MS of Pt(II) complexes

The signals arising from the Pt(II) complexes tested for their influence on the activity of PLA_2 are rather complex because Pt consists of several natural isotopes; the one with the mass of 194.96 is the most abundant. The combination with other elements present in the complex makes its mass spectra even more complicated. Since a more detailed description and explanation of the MALDI-TOF mass spectra of transition metal complexes is given elsewhere in literature[21,22], we will make only a few remarks on that subject.

The spectrum of [Pt(dach)Cl_2] (Figure 1A) consists of two groups of peaks, one of them is generated by the addition of sodium ion to the molecule $m/z = 403.5$, whereas the peak at $m/z = 419.5$ arises from the ion formed by the addition of potassium to the molecule. These peaks are described in detail in our previous study[21,23]. Other two peaks present in the spectra of [Pt(dach)Cl_2] cannot be identified with certainty, but they are most probably generated by the addition of a matrix molecule.

In contrast to [Pt(dach)Cl_2], the MALDI-TOF mass spectrum of cisplatin was rather difficult to acquire, because this Pt complex expresses rather high reactivity[24]. The positive ion MALDI-TOF mass spectra of cisplatin consist of three groups of peaks, one of which cannot be identified with certainty, i.e. the peak at $m/z = 313.3$ could not be explained based on possible fragmentation reactions of this molecule. The presence of platinum in the ion group from which these peaks were generated can be assumed based on the characteristic peak pattern. Peaks around $m/z = 323.4$ and $m/z = 339.4$ are generated by the addition of one sodium ion or one potassium ion to the complex, respectively. It should be pointed out that the pattern of the group of peaks which arise from sodium adduct corresponds almost completely to the theoretical presentation of mass spectra obtained with the Selket program (data not shown).

MALDI-TOF MS of egg-yolk PC

PC—a substrate for PLA_2—was isolated from egg yolk according to the previously described procedure. The organic extract was analyzed by MALDI-TOF MS and the corresponding positive ion mass spectrum is given in Figure 2A. The spectra were acquired with the DHB matrix and the signals obtained from it are indicated by asterisks. Other peaks are labelled according to their m/z ratio and their identity is also indicated in the spectrum trace.

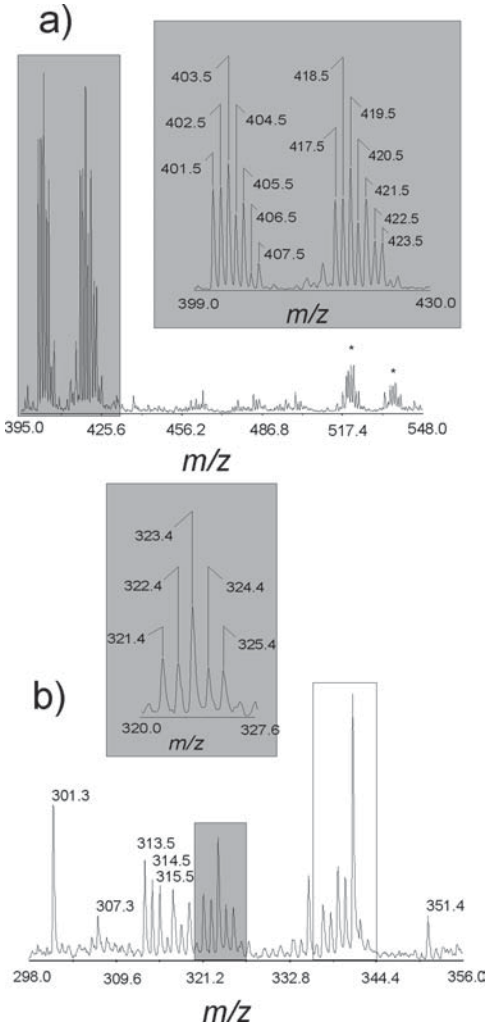

Figure 1. The positive ion MALDI-TOF mass spectra of [Pt(dach)Cl_2] (a) and cisplatin (b). Inserts in both spectra are group of signals arising from the Pt complexes and are given to emphasize their complexity. The signals arising from the complexes are emphasized with rectangles in both spectra. MALDI-TOF mass spectra of Pt(II) complexes are acquired with 2,5-DHB as matrix, under delayed extraction conditions and in the reflector mode.

The MALDI-TOF mass spectra have confirmed that the major species in the isolate is indeed PC; mostly PC containing palmitic acid residue esterified on the *sn*-1 position was identified. The following peaks are present: (i) PC(16:0,18:1) (The fatty acid composition of phospholipids is usually given in such a way that the first number represents the number of C-atoms and the second one the number of double bonds in the fatty acyl chain. The fatty acid on the sn-position of the glycerol backbone of the corresponding phospholipid is listed first.) The corresponding peaks are at $m/z = 760.1$, 761.4 and 783.3,

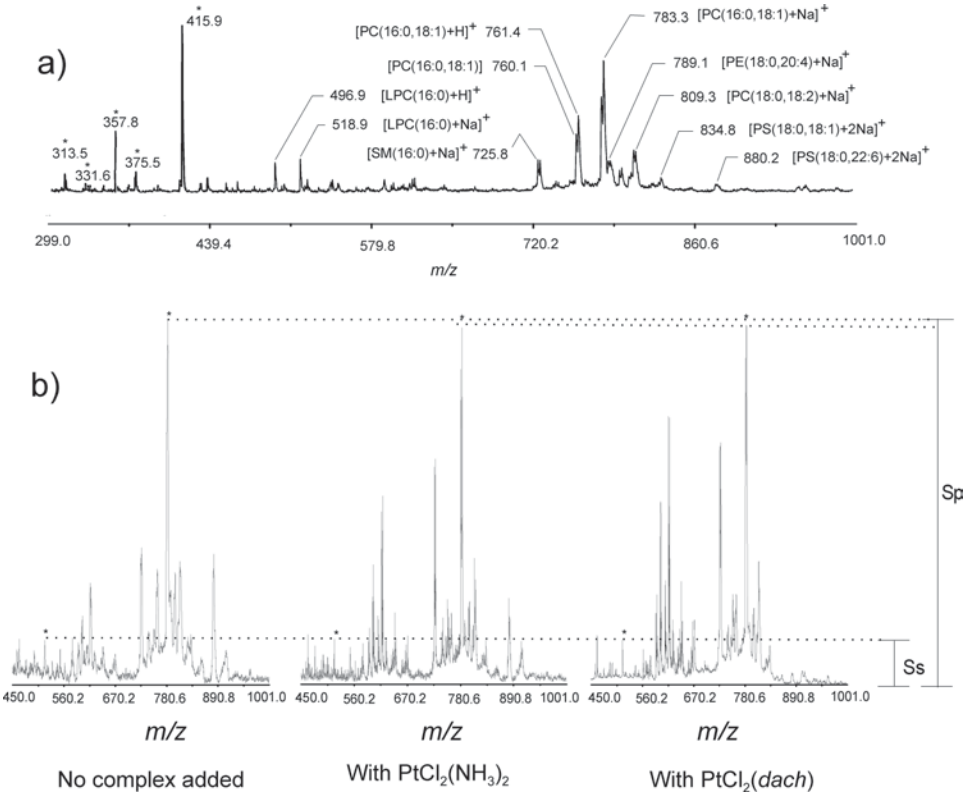

Figure 2. (A) The positive ion MALDI-TOF mass spectrum of egg-yolk lecithin, (B) the mass region of phosphatidylcholine without the Pt(II) complexes (left), with cisplatin (middle), and with [Pt(dach)Cl_2] (right). The signals are indicated by their *m/z* ratio together with their identity. Peaks arising from the matrix 2,5-DHB are labelled with an asterisk. The lines indicating the intensity of the signal arising from PC (S_s) and from the product LPC (S_p) are given in the spectra. All MALDI-TOF mass spectra were acquired with DHB matrix, under delayed extraction conditions and in the reflector mode.

molecular ion, the proton, and sodium adducts, respectively and (ii) PC (18:0, 18:2). Peak at *m/z* = 809.3, the sodium adduct. Besides PC, there are also small amounts of phosphatidylethanolamine (PE), phosphatidylserine, and sphyngomyelin (SM) detectable in the spectra. In addition, certain amount of LPC could be detected, but its intensity significantly increased after the incubation with PLA_2, as it will be shown later. The presence of other phospholipid species in the isolate does not disturb further experiments, as pancreatic PLA_2 uses preferentially PC as the substrate[25]. Therefore, the increase in the content of lyso-phospholipids will be used as the measure of the PLA_2 activity, as it has already been shown in our previous work[18,26].

Since it is demonstrated that Pt complexes might also have esterase activity[27], we have performed control measurements, i.e. the phospholipids were incubated with complexes only and the corresponding mass spectra were acquired and presented in Figure 2B. Only the mass region of phospholipids is presented in order to demonstrate that the ratio between the intensity of signals arising from the sodium adduct of PC (signal of the substrate, S_s) and that arising from the sodium adduct of LPC (signal of the product, S_p) does not change significantly in the presence of the complex. The left trace represents the MALDI-TOF mass spectrum of phospholipids incubated without complexes, the middle trace is acquired from phospholipids incubated with cisplatin, and the spectrum to the right represents the phospholipids incubated with [Pt(dach)Cl_2]. The group of peaks in the mass region, between *m/z* = 560 and 670 (not labelled in the spectra), most probably represents the PEs and SMs and/or certain fragmentation products generated upon laser irradiation[28].

PLA_2-catalyzed production of LPC

Positive ion MALDI-TOF mass spectra of the reaction mixture obtained after the indicated periods of incubation of PC with PLA_2 are given in Figure 3. The peaks are labelled according to their *m/z* ratios and the identity of relevant signals is given in the figure. It is obvious that the hydrolysis of PC catalyzed by PLA_2 takes place very quickly after the addition of the enzyme: already after 2 min incubation, the signals arising from PC are difficult to detect in the spectra. After 40 min, no signals arising from PC could be detected. Since this species is easily detectable in the MALDI-TOF mass spectra[29] due to its preformed positive charge, it is possible to affirm that the entire amount of the substrate has been converted into LPC (16:0). The ratio between the sodium adducts of the reaction product (LPC) and the substrate (PC) was taken as the measure for monitoring the consumption of PC

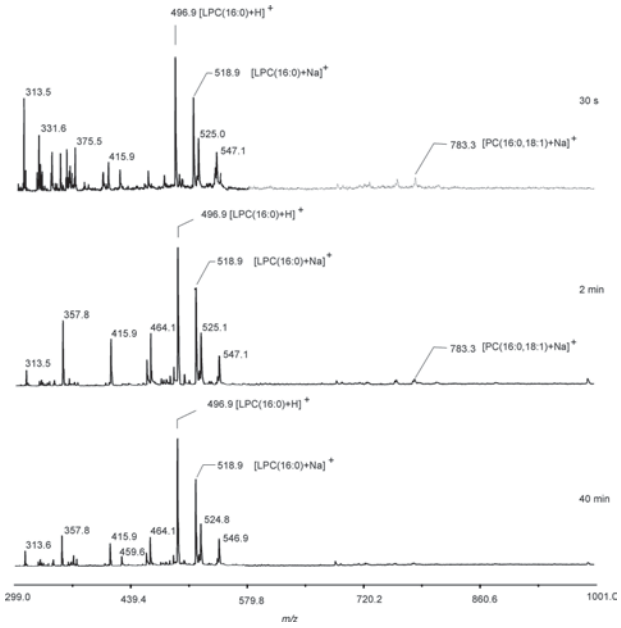

Figure 3. MALDI-TOF mass spectra of egg-yolk PC incubated for indicated periods of times with PLA_2. Signals of interest are indicated according to their *m/z* ratios and their identity is given in the spectra traces. All other experimental conditions are like for the spectra presented in Figure 2.

and the assessment of the influence of the complexes on the enzymatic activity of PLA_2.

In Figure 4A, the ratio between the intensity of the LPC and PC peaks (S_p/S_s ratio) calculated from the MALDI-TOF mass spectra acquired after different incubation periods of egg-yolk PC with PLA_2 with or without Pt(II) complexes is given as a function of the incubation time. This ratio can be used as the measure for the amount of LPC produced; it can also be used to estimate the influence of Pt(II) complexes on the activity of PLA_2, as demonstrated in our previous work. Obviously, compared to the results obtained in the presence of either cisplatin or [Pt(dach)Cl_2], the greatest amount of LPC has been produced in the absence of the complexes. The amount of LPC under all tested conditions increases during the first 200 s after the initiation of the reaction, to subsequently achieve the plateau level. Further incubation of egg-yolk PC with PLA_2 does not result in significant increase in the amount of LPC.

The data obtained by colorimetric assay for the first 3 min of the reaction (with or without Pt(II) complexes), which approximately represents the linear part of the S_p/S_s values calculated from the MALDI-TOF mass spectra (Figure 4A), are presented in Figure 4B. In a similar manner as in the MALDI-TOF mass spectra, the highest reaction rate is observed for the sample in which egg-yolk PC was incubated with PLA_2 without Pt(II) complexes, whereas significant decrease is observed in the presence of both Pt(II) complexes tested in this study. Insignificant differences are observed in the influence of cisplatin compared to [Pt(dach)Cl_2].

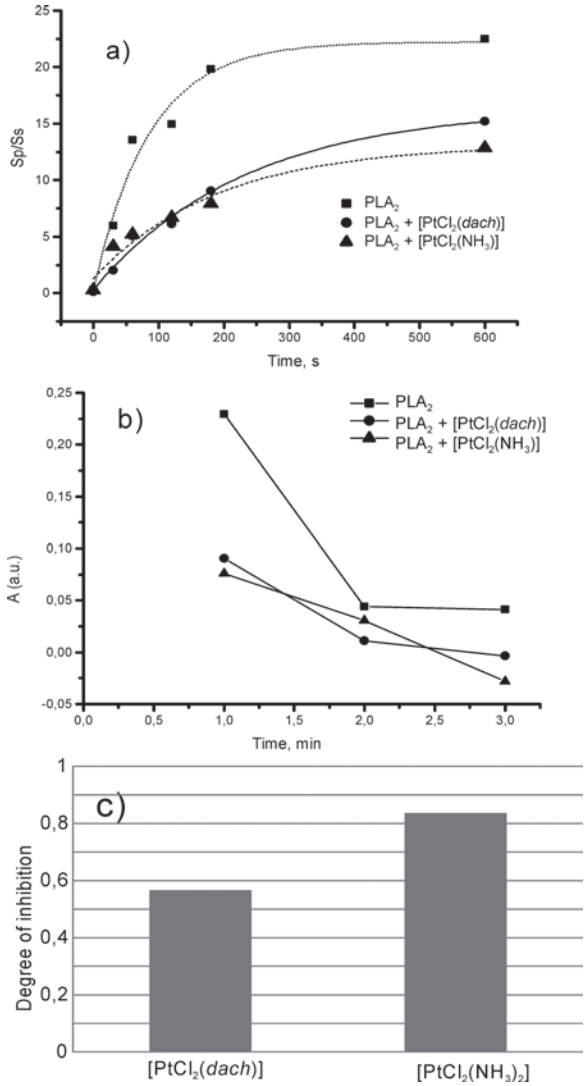

Figure 4. (A) The ratio between the LPC signal intensity and PC signal intensity (S_p/S_s) calculated from the MALDI-TOF mass spectra of egg-yolk lecithin incubated with PLA_2, which is previously incubated with Pt(II) complexes in the relationship with the time of incubation. (B) Absorbance of the reaction mixture monitored for 3 min of incubation of egg-yolk PC (1 mg/mL) with 0.1 mg/mL PLA_2 with or without Pt(II) complexes (5×10^{-4} mg/mL) in dependence of the time of incubation. (C) Degree of inhibition of the PLA_2 activity obtained in the presence of Pt(II) complexes. Each value in both graphs represents the mean value from at least three independent measurements.

The degree of the enzyme inhibition in the presence of two complexes could be calculated from MALDI-TOF mass spectra (Figure 4A) from the first linear part of the graph, according to the equation:

$$i = \frac{v_0 - v}{v_0}$$

where v_0 is the rate of a reaction in the absence of inhibitor (control reaction) and v the rate of a reaction in the presence of inhibitor. The *i* value in the absence of complexes is zero and the values obtained for the enzyme in

the presence of cisplatin and [Pt(dach)Cl_2] were 0.86 and 0.59, respectively (Figure 4C). Obviously, much stronger inhibition of the enzyme activity (86%) was determined in the presence of cisplatin, compared to other complex tested.

Binding of Pt(II) complexes to PLA_2

In the next stage of our experiments, we tried to detect binding of the tested complexes to the enzyme in order to explain the inhibitory effect of the complexes on the enzyme activity. For this purpose, PLA_2 was incubated with the complexes for three and a half hours and the MALDI-TOF mass spectra of a protein were acquired. We expected a mass shift towards higher *m/z* values in the MALDI-TOF mass spectra of PLA_2 after the incubation with both complexes. PLA_2 yields two peaks at $m/z = 13801$ and at a higher mass $m/z = 13991$ (Figure 5A). It is, however, usual that the fraction of PLA_2 isolated from porcine pancreas contains isoform with a higher mass. This spectra pattern does not change with the addition of PC (16:0, 18:1), i.e. the enzyme substrate (trace 5B). In the spectra of the enzyme incubated with cisplatin (Figure 5C), and cisplatin and phosphatidylcholine (Figure 5D), new signal appears at $m/z = 14{,}220$, which corresponds to the addition of $[Pt(NH_3)_2]^{2+}$ to the higher mass isoform of the enzyme. The bond between the enzyme and [Pt(dach)Cl_2] could not be detected by MALDI-TOF MS (data not shown).

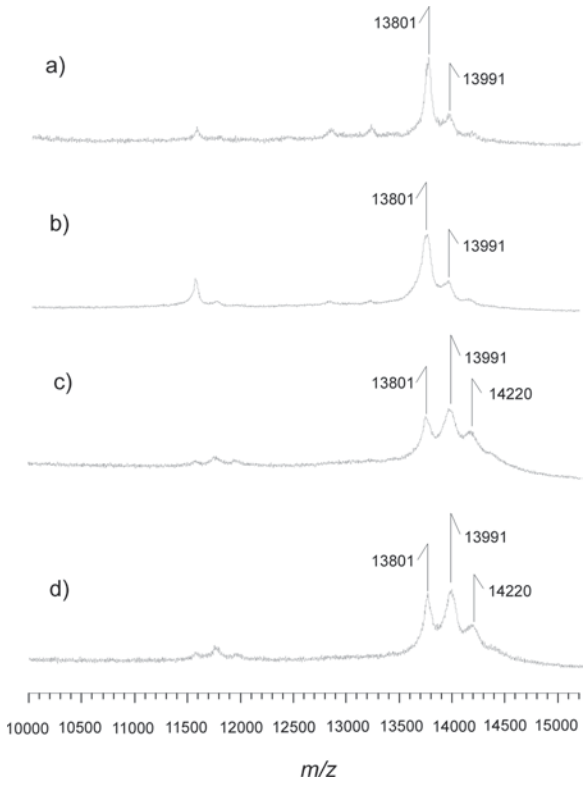

Figure 5. MALDI-TOF mass spectra of PLA_2 (A), PLA_2 incubated with PC (16:0, 18:1) (B), PLA_2 incubated with cisplatin (C), and PLA_2 incubated with the substrate, PC (16:0, 18:1), and with cisplatin (D). Enzyme (0.1 mg/mL) was incubated with the complex (5×10^{-4} mg/mL) and with PC (16:0, 18:1) (1 mg/mL) as described above, for other experiments. The MALDI-TOF mass spectra were acquired with sinapic acid as matrix under delayed extraction conditions and in linear mode. Presented spectra are representative of four measurements and they are average of 100 individual laser shots.

In addition to MALDI-TOF mass spectrometric detection of the interaction between the complexes and the enzyme, we have also tried to detect the interaction of complexes with PLA_2 by spectrophotometric titration, i.e. recording the UV spectrum of the enzyme upon addition of increasing amounts of a complex, i.e. with different *R* values (*R* value represents the ratio between the molar concentration of PLA_2 and the added complex). The coordination of a platinum drug to a protein causes changes in the protein conformation. They are detected as a change in the aromatic chromophores in the UV spectra of the protein. The resulting UV spectra are given in Figure 6: in (A) the spectra recorded upon the addition of increasing concentrations of cisplatin, in (B) the spectra recorded upon the addition of increasing concentrations of [Pt(dach)Cl_2]. The graph given in (C) shows the dependence of A_{280} from the *R* values.

In the case of cisplatin, the hyperchrome effect occurs with a decrease in the *R* value, i.e. an increase in the concentration of cisplatin (Figure 6A). This behaviour was also detected in the studies dealing with the interactions of cisplatin with serum proteins[30]. A different situation has been observed in the case of [Pt(dach)Cl_2] (Figure 6B). In this case, the absorbance at 280 nm increases as the *R* values increase up to $R = 1$, i.e. the equal concentrations of the complex and the enzyme. For the *R* values >1, the effect could be described as hypochromic (Figure 6C).

Discussion

Interaction of Pt(II) complexes with pancreatic PLA_2

The interaction of potential metallo-drugs with enzymes is of great importance having in mind that enzymes are target molecules for the therapy of various diseases. In this study, we have demonstrated that cisplatin and [Pt(dach)Cl_2] inhibit the activity of pancreatic PLA_2. Furthermore, the data obtained in our study imply different mode of the interaction between the selected Pt(II) complexes and the enzyme: [Pt(dach)Cl_2] is less reactive than cisplatin and lower degree of inhibition of the enzyme activity in the presence of [Pt(dach)Cl_2] was obtained from the data presented in this work. The differences in reactivity between these two platinum complexes can be explained by the differences in their structure. Compared to cisplatin, [Pt(dach)Cl_2] is a more sterically crowded complex. Namely, the substitution reactions of square-planar complexes, such as cisplatin and [Pt(dach)Cl_2], proceed through associative pathways (A) involving the transition state with the coordination number 5. Both of these complexes have two labile leaving chlorido ligands in the *cis*-configuration but there is a crucial difference in non-labile ligands. Cisplatin has two non-labile ammine

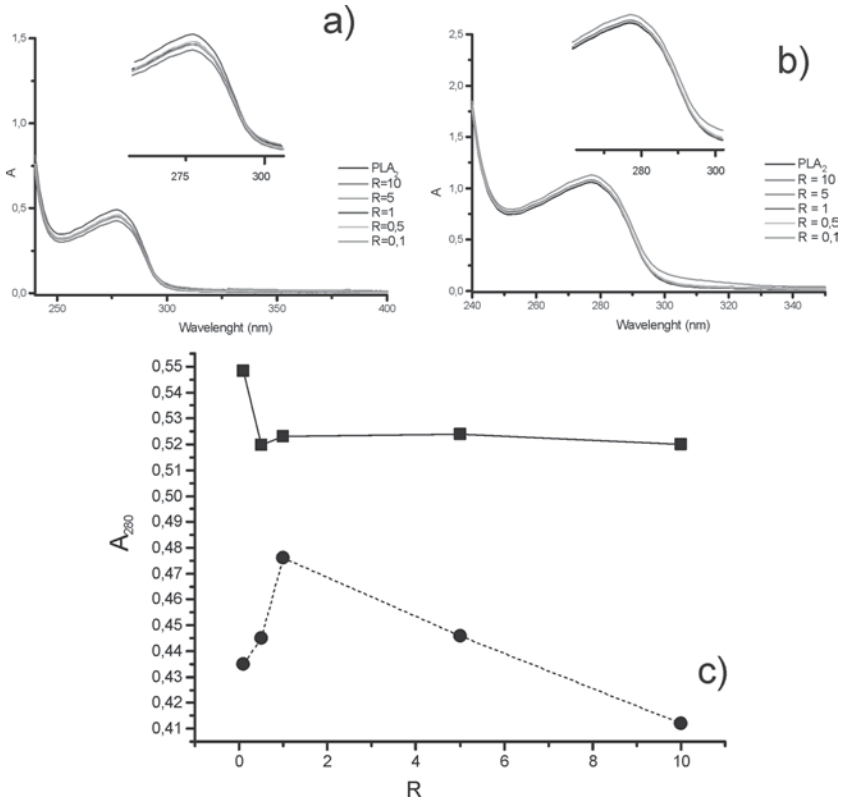

Figure 6. Spectrophotometric titration of the PLA_2 with cisplatin (A) and [Pt(dach)Cl_2] (B). In (C), the dependence of the A_{280} from the R value (the ratio between molar concentration of PLA_2 and the added complex) is given.

ligands, while [Pt(dach)Cl_2] has one bidentate (dach) ligand which is much bulkier. That might indicate that the transition state has higher energy, while the rate of substitution reactions in the [Pt(dach)Cl_2] complex is reduced. However, a more precise analysis of the mechanism will be presented in our future studies.

The interaction of both tested complexes with PLA_2 leads to the inhibition of the enzyme activity as it has been demonstrated. It has already been shown that some Pt(II) complexes exhibit esterase activity[27]. Accordingly, there is a possibility that the LPC detected in the spectra of PC incubated with the enzyme in the presence of Pt(II) complexes is not a result of the PLA_2-catalyzed hydrolysis, but of the esterolytic activity of tested complexes. We have excluded this possibility by simple control experiment (Figure 2B); therefore, we can state that all LPC which appear in the spectra are the result of PLA_2-catalyzed hydrolysis of PC, and that the effect of tested complexes is the effect on the enzyme and not on the substrate.

Additionally, it seems that the inhibition modes of secretory PLA_2 of various transition metal complexes are different. We have recently observed that the Pt and Ru complexes with bipyridine exhibit the acompetitive type of inhibition, and their interaction with PLA_2 could be detected only in the presence of a substrate[31]. This type of inhibitory action implies that the binding of the complex increases after the interaction of the enzyme with the substrate, i.e. PC. This interaction changes the enzyme conformation, making the interaction site on the enzyme more accessible for the complex. Pancreatic PLA_2 as a secretory type of enzyme contains a number of cysteine residues, which are potential binding sites for the interaction with Pt(II) complexes[24,32–36], but the exact site of the interaction with complexes has not been known. On the other hand, the binding of cisplatin to PLA_2 could be detected even without the presence of substrate (Figure 5C), whereas the interaction between the other complex tested in our study, [Pt(dach)Cl_2], with this enzyme is different. The binding of [Pt(dach)Cl_2] to the enzyme could not be detected by MALDI-TOF MS (data not shown), and it can be assumed that the potential transient bond is not sufficiently stable under laser irradiation to be detected by this method. This fact and the data obtained by spectrophotometric titration (Figure 6) indicate a different mode of interaction compared to that in case of cisplatin.

Methodological approach

Readers may express their doubts regarding the experimental techniques used in this study because they are not routinely established neither for the characterization of transition metal complexes nor for the monitoring the enzyme activity of phospholipases. Therefore, one of our tasks was to test the applicability of MALDI-TOF MS for these purposes. A few remarks on the methodology have to be made at this point. First of all, MALDI-TOF MS enables fast and sensitive analyses of transition metal complexes, though some clusters, as

observed in Figure 1A, are detectable in the spectra acquired with organic matrices. This methodological drawback has already been studied[37]. Although the matrix that we have chosen, 2,5-DHB, may not be perfect for the MALDI-TOF mass spectrometric analysis of transition metal complexes, it enables easy identification and analysis of phospholipids[28,29], which will be used in further work as a substrate for PLA_2. However, a group of peaks which arise from the transition metal complexes—in this case from Pt(II) complexes—can be easily distinguished from other peaks in the MALDI-TOF mass spectra. It should be pointed out that the spectra of cisplatin were rather difficult to acquire, most probably due to the high reactivity of this platinum complex. The pattern of the signal corresponding to the sodium adduct of cisplatin matches the theoretical presentation (data not shown); the somewhat more intense first peak in the group (at $m/z = 321.4$) is the only difference. Since the theoretical presentation does not include the matrix molecule (which itself gives several signals in the mass spectra), the higher intensity of this peak might be the result of the superposition with the matrix signals. The other group of peaks, most probably arising from potassium adduct of cisplatin, do not completely match with the theoretical representation, implying some overlapping signals.

In addition to the characterization of the transition metal complexes, MALDI-TOF MS is not routinely used as the method for the determination of enzymatic activity. It is, therefore, necessary to test its capacity for being applied in the assessment of the enzyme activity of pancreatic PLA_2. The MALDI-TOF MS approach offers numerous advantages over other methods[25], and it was selected because of its simplicity (the substrate is in its native state and no derivatization is required), reproducibility, and sensitivity. The high sensitivity of this approach indicates that even rather low concentrations of lyso-phospholipids (those in the femto-molar range), which are the product of the PLA_2 activity, can be detected[38].

However, the S_p/S_s values, which were calculated from the MALDI-TOF mass spectra, should be used with some caution for determination of certain enzyme kinetic parameters[25], as we have done in our work (determination of the degree of inhibition). These values are calculated from the first linear part of the curves only to numerically illustrate the differences in the efficiency of inhibition (which is obvious).

To check results obtained with MALDI, we have also monitored the reaction colorimetrically. The time-dependent decrease in the values of the absorbance represents the reaction rate. The results obtained by this method were in agreement with the MALDI data (Figure 4). Accordingly, MALDI-TOF MS can be used as a reliable method for monitoring the enzyme activity.

In the light of the aim of our study, it is important that the S_p/S_s ratio (the LPC signal intensity over the PC signal intensity) calculated from the MALDI-TOF mass spectra does not change in the presence of the Pt(II) complex, which indicates that all LPC arises from the PLA_2-catalyzed reaction, as discussed above. This ratio has previously been used as the measure for the amount of the LPC produced by the catalytic activity of PLA_2[25]. Therefore, we have confirmed its applicability in this case, as well.

Potential physiological relevance

Results obtained in numerous studies imply that the aberrant expression of secretory type of PLA_2 is common in tumours derived from many different tissues, but the significance of these findings in the tumour development and progression could not be well established[39].

Work of Avoranta and co-workers[9] shows that sPLA type IIA is more expressed in the peritumoral mucosa in patients with colorectal carcinoma than in tumour tissue. Moreover, majority of vital tumour cells fail to express this enzyme, whereas PLA_2 was accumulated in apoptotic and necrotic tumour cells. According to the hypothesis of the same authors[9], PLA_2, which is overexpressed in apoptotic and necrotic cells, might assist the host in the clearance of apoptotic cells and its inhibition can slow down this process.

On the other hand, secretory PLA_2 was found to be upregulated in several types of malignancies, such as breast cancer[40], prostate cancer[41], or gastric cancers[42]. The level of its expression increases with increasing tumour grade and it was demonstrated that enzyme can be a marker for metastasis of some cancer types[43].

In general, the nature and concentration of lipid mediators produced by the catalytic activity of PLA_2, or derived from them, can determine whether the signals for the tumour development will be proliferative, apoptotic, or survival[39]. Some authors suggest that lost asymmetry in the tumour cells membrane results in the increase in the activity of PLA_2IIA[44], and that products of its activity can stimulate important apoptotic pathways via the activation of sphingomyelinases and subsequent release of ceramide[45]. From all these reasons, secretory types of PLA_2 were considered as potential target for anti-tumour therapy[46].

At this stage, we cannot state that the inhibition of $sPLA_2$ with metallo-drugs (Pt, Ru, or Au) will lead to inhibition of the tumour growth; we can only indicate that the degree of enzyme inhibition depends on the nature of the ligand. Platinum complexes are, however, highly reactive species and might react with a number of proteins, although nucleic acids were considered as their primary targets. Therefore, findings presented in this work might be expanded on other enzymes involved in regulation of tumour growth and differentiation. More work should be conducted in order to connect any physicochemical properties of a drug with its physiological effect.

Conclusion

In conclusion, cisplatin and $[Pt(dach)Cl_2]$ interact with hog pancreatic PLA_2, also implying the interaction with

the human enzyme, and this interaction inhibits the PLA_2 activity. Having this in mind, the question arises whether this type enzyme (secretory PLA_2s) could be used as a target for anti-tumour therapy with metallo-drugs. Namely, it has been demonstrated that in the invasive form of the cancer tissue, the concentration of secretory pancreatic PLA_2 is increased[9]. Accordingly, it remains to investigate in detail the impact of the inhibition of the activity of PLA_2 on the tumour progression. The results presented in this study suggest the existence of different modes of binding of the Pt complexes to the enzyme, which may be considered as an advantage that can be further exploited in the development of therapeutical agents.

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Declaration of Interest

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