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PAPER

Inhibitory effect of platinum and ruthenium bipyridyl complexes on porcine pancreatic phospholipase A_2

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Pancreatic phospholipase A_2 (PLA_2) plays an important role in cellular homeostasis as well as in the process of carcinogenesis. Effects of metallo-drugs used as chemotherapeutics on the activity of this enzyme are unknown. In this work, the interaction between porcine pancreatic PLA_2 and two selected transition metal complexes—tetrachloro(bipyridine) platinum(IV) ([$PtCl_4$(*bipy*)]) and dichloro (bipyridine) ruthenium(III)chloride ([$RuCl_2$(*bipy*)$_2$]Cl)—was studied. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and fluorescence spectroscopy have been used to analyse the enzyme activity in the absence and presence of metal complexes and to verify potential binding of these drugs to the enzyme. The tested metal complexes decreased the activity of phospholipase A_2 in an uncompetitive inhibition mode. A binding of the ruthenium complex near the active site of the enzyme could be evidenced and possible modes of interaction are discussed.

Introduction

Phospholipases A_2 are esterolytic or more specifically lipolytic enzymes[1] that hydrolyze membrane phospholipids producing free fatty acids (FFA) and lysophospholipids (LPLs), thus providing precursors for the biosynthesis of proinflammatory lipid mediators.[1–3] Pancreatic PLA_2 belongs to the type I PLA_2 enzymes that have been shown to elicit receptor-mediated cellular responses such as stimulation of prostaglandin production, secretion of steroid hormones,[4–6] cell proliferation,[7–9] and vascular smooth muscle contraction.[10] In the formation of arachidonic acid and derived biomediators, this enzyme displays a critical role in cellular homeostasis due to catalysis of a rate-limiting step. Accordingly, it has been shown that pancreatic PLA_2 plays a role in carcinogenesis, immune suppression, stimulation of cell proliferation, invasion and metastasis.[11–13] Several studies have shown that human pancreatic PLA_2 stimulates the growth of a human pancreatic cancer cell line *via* binding to a specific receptor.[9,14]

It also stimulates extracellular matrix invasion by normal and cancer cells.[15] In addition, this enzyme is a target protein for triggered liposomal drug delivery to tumor tissues.[16] Anti-tumor therapy by transition metal complexes such as cis-platin, carbo- and oxaliplatin has been known over decades, but there are no data dealing with the interaction of these metallo-drugs with enzymes involved in phospholipid metabolism. Although metal complexes are efficient anti-proliferative agents in solid tumors, there are numerous side effects of these drugs such as nephrotoxicity and tumor cell resistance that must be overcome by synthesis of new more effective complexes and new approaches to the drug administration. Among other metal complexes, Ru(III) complexes are of particular interest, as they exhibit less toxic effects than platinum complexes.[17] They usually interact with nucleic acids, but there are some data implying also their interaction with cellular proteins.[18–21] Several Ru(II) drugs have reached the level of clinical investigations (NAMI-A and KP1019).[22,23] In particular platinum(IV) and ruthenium(III) complexes containing aromatic ligands, specially with bipyridine ligands, appear to be suitable candidates for studying the basic features of interaction with biomolecules.[24]

In this study, we investigated the interaction of Pt(IV)- and Ru(III)-bipyridine complexes with porcine pancreatic PLA_2. This protein is a useful model for secretory PLA_2 enzymes that display an important role in host-defense as well as clearance of necrotic tissues.[25,26] MALDI-TOF MS has been already shown to be a powerful technique for the analysis of bipyridine metal complexes,[27] and therefore it is used here as a method of first choice. Tested metal complexes affect the enzymatic

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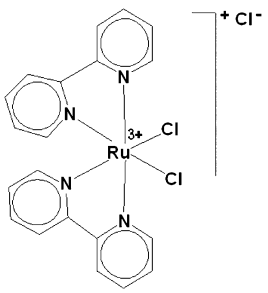
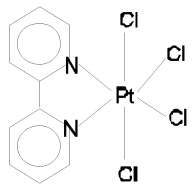
activity, which might cause significant changes in the subsequent processes of membrane remodeling and production of important regulatory molecules in host defense against tumor cells. Thus, detailed knowledge of interaction between phospholipase A_2 and metallo-drugs can be later exploited in further development of therapeutic strategies.

Materials and methods

Chemicals

Platinum and ruthenium complexes ([$PtCl_4$(*bipy*)] tetrachloro-(bipyridine) platinum(IV) and [$RuCl_2$(*bipy*)$_2$]Cl dichloro-(bipyridine) ruthenium(III)chloride) were synthesized as described in the literature.[28,29] Chemical analysis, UV-VIS and nuclear magnetic resonance (^{1}H-NMR) spectral data of these complexes were in good agreement with those obtained in previous preparations. Structural formulae and relative masses of complexes used are given in Table 1. Porcine pancreatic phospholipase A_2 was purchased from Fluka (Neu-Ulm, Germany) showing an activity of 163 U mg^{-1} protein according to the manufacturer (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^{-1} chloroform solutions and phosphatidylcholine extracted from hen's egg yolk.[30] The applied detergent used to increase the surface area and decrease the substrate's surface concentration, deoxycholic acid (DOC), was purchased from Sigma (Taufkirchen, Germany). Matrices for MALDI-TOF MS, 2,5-dihydroxybenzoic acid (DHB) and sinapic acid (SA), as well as solvents (chloroform and methanol) and trifluoroacetic acid (TFA) were purchased from Fluka and Sigma (Taufkirchen, Germany) and were used without further purification.

Table 1 Structural and empirical formula with molecular masses of metal complexes. Molecular mass is given for an isotope mixture considering the natural abundance of all isotopes

Structural formula	Empirical formula	Molecular mass/ g mol^{-1}
	[$RuCl_2$(*bipy*)$_2$]Cl	519.8
	[$PtCl_4$(*bipy*)]	493.1

Preparation of samples for MALDI-TOF MS analysis

For investigation of kinetics of PLA_2 by MALDI-TOF MS, lipid and lysolipid samples were prepared as described in the literature.[27] Phospholipids isolated from hen's egg yolk were resuspended in Tris buffer (50 mM TRIS, 100 mM NaCl, 5 mM $CaCl_2$, 5.4 mM deoxycholate, pH 8.5). Concentrations of starting solutions were 10 mg ml^{-1} for phospholipids, 0.2 mg ml^{-1} for the internal standard LPC 14 : 0, 0.2 mg ml^{-1} for PLA_2 and 1 mM for platinum and ruthenium complexes. Incubation of phospholipids with the enzyme was performed for different substrate concentrations, metal complex concentrations and time intervals. Incubated samples contained: phospholipids (concentrations: 0.312, 0.625, 1.250, 1.875, 3.125, 6.250 mg ml^{-1}) and porcine pancreatic PLA_2 (0.0125 mg ml^{-1}). After 30 s, 1, 5, 10, or 30 min incubation, internal standard LPC 14 : 0 (0.05 mg ml^{-1}) was added to all samples and the reaction was stopped by addition of the chloroform/methanol (1 : 1, v : v) mixture. After vortexing and centrifugation, the chloroform layer containing phospholipids and lysophospholipids was evaporated to dryness. A residual film was resuspended in the matrix DHB (0.5 M in methanol with 0.1% TFA). The procedure of sample preparation[31] requests premixing of the sample and the matrix before application on the sample plate, in favor of better reproducibility. The sample was applied as 1.5 μl droplet on the sample plate and dried immediately under a warm stream of air.

For monitoring the changes in mass spectra in the m/z range 10 000–15 000, fresh solution of PLA_2 c = 0.02 mg ml^{-1} in 50 mM Tris-HCl buffer (37 °C and pH = 8.5) was prepared and applied on the sample plate premixed with sinapic acid, as control. Metal complexes were dissolved in the same buffer to different final concentrations and incubated with the enzyme for three hours and then applied on the sample plate with sinapic acid.

Quantification of product LPC 16 : 0 using internal standard

Concentration of internal standard LPC 14 : 0 (peaks at m/z = 468.3 and m/z = 490.3) added in all samples was kept constant, 0.2 mg ml^{-1}. The intensity of the peak corresponding to the product of digestion, LPC 16 : 0 (m/z = 496.3 and m/z = 518.3, the proton and the sodium adduct, respectively), decreases in comparison to peaks arising from internal standard. The mean signal-to-noise ratio of peaks corresponding to adducts of LPC 16 : 0 is used to calculate their concentration, following the equation: c(product) = (S/N of product peak)(c of int. std.)/(S/N of internal standard). In all cases we considered mass peaks corresponding to sodium adducts of product LPC 16 : 0 and internal standard LPC 14 : 0. Only signal to noise ratios (S/N) from the same mass spectra were compared for different compounds (product and internal standard) in order to calculate concentrations. The main values of concentration of the product were calculated from min. five different sample spots and under the same experimental conditions of acquisition.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry DE Pro Workstation (PerSeptive Biosystems, Framingham, MA, USA) and an Autoflex II (Bruker Daltonics, Leipzig, Germany) MALDI-TOF mass spectrometer, both equipped with a pulsed nitrogen laser (337 nm). For the m/z ranges 10 000–15 000, each mass spectrum was obtained operating in the positive linear mode (voltage 20 kV) with a low mass gate of 10 000 and by averaging 150 laser shots at the 27% laser power. For the lower m/z ranges, no low mass gate was set and all mass spectra were obtained using the reflector mode. The spectra recorded using Autoflex II were calibrated externally using a standard calibrant mixture (Protein Calibration Standard I; Bruker Daltonics), while the data files were transferred to Flex Analysis version 2.4 (Bruker Daltonics) for automated peak extraction. Mass spectra recorded on a Voyager spectrometer were calibrated by setting the peak of the protonated DHB matrix to its appropriate value (155.034 Da).

Fluorescence spectroscopy

Fluorescence measurements were performed on a Jobin Yvon Spex FluoroMax 2 spectrofluorimeter (Edison, NJ, USA) at 37 °C. The excitation wavelength was 280 nm and fluorescence emission spectra were recorded from 300 to 500 nm using a wavelength increment of 1 nm. The initial volume of PLA_2 (10 μM in PBS) was 1200 μl and changes in fluorescence spectra were monitored after adding 0, 5, 10, 20, 30, 40, 50 μl of each metal complex (1 mM in PBS).

Results

Determination of the PLA_2 activity

Kinetic analysis of PLA_2 is rather complicated by the presence of a lipid/water interface and the dependence of the enzyme activity on the aggregation state, structure and surface concentration of the phospholipid substrate.[32] There are numerous methods established for monitoring the PLA_2 activity, but they usually require radioactive or fluorescence labeling of the substrate, which implies the use of a non-native substrate for the enzyme. In addition, these methods are time-consuming for experimental preparation, while colorimetric methods, which might be applied for this purpose, suffer from low sensitivity.[33,34] Previously, MALDI-TOF MS was shown to be a suitable method for sensitive detection of the PLA_2 activity.[31] We selected this approach for monitoring concentration changes of LPLs, as products of the PC digestion with PLA_2 with and without metal complexes. Along with high sensitivity and possibility to acquire high number of information from a single spectrum, the main advantage of MALDI-TOF MS is that no labeling of the substrate is required.[35]

Briefly, this approach for the determination of the PLA_2 activity is based on the quantification of the product (lysolipid) in the MALDI-TOF mass spectra depending on the time of incubation with the enzyme.[31,36,37] The data obtained showed that all PL and LPL classes can be easily detected and even measured simultaneously, using an appropriate internal standard.[35,38] The internal standard used in this work was LPC 14 : 0, as it gives the best results, yielding peaks easily distinguished from sample peaks (Fig. 1).

The positive ion MALDI-TOF mass spectra of phospholipids extracted from hen's egg yolk incubated with porcine pancreatic PLA_2 for different times (1, 2, 5, 15 and 30 min) as well as unperturbated phospholipid spectra are presented in Fig. 1. Peaks of interest in this study have values m/z = 760.6 and m/z = 782.6 for the proton and sodium adduct of PC 16 : 0; 18 : 1, respectively. Other intense mass peaks were found at m/z = 758.6 and 780.6 corresponding to the proton and sodium adduct of PC 16 : 0; 18 : 2. Upon incubation of phospholipids with phospholipase A_2 these mass peaks decreased continuously as a function of time. Two peaks, present only in trace amounts in the initial spectrum, increased concomitantly

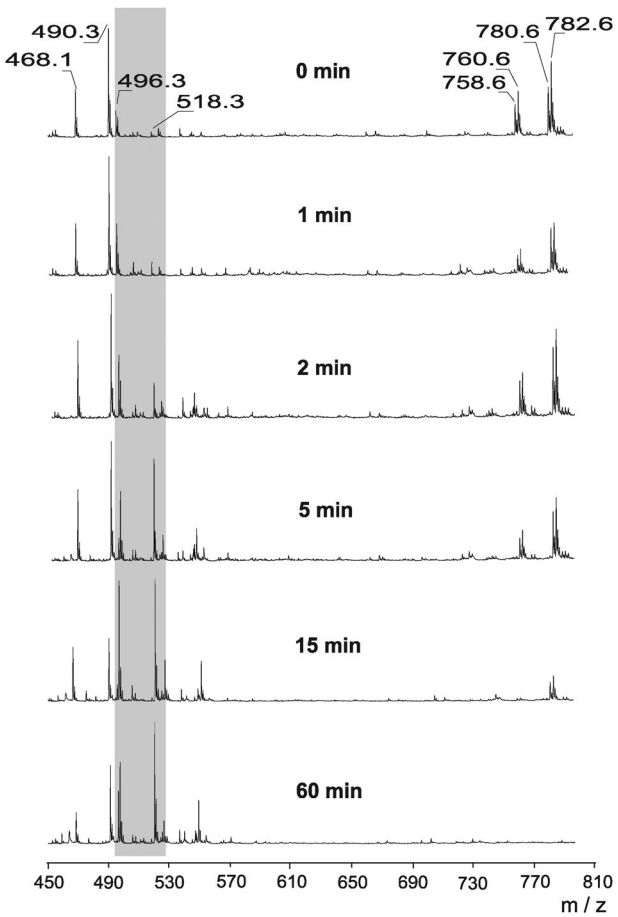


Fig. 1 Positive ion MALDI TOF mass spectra of phospholipids isolated from hen's egg yolk digested by phospholipase A_2 for 0, 1, 2, 5, 15 and 60 min. Labeled peaks belong to the following ion species: m/z = 760.6 and m/z = 782.6 for proton and sodium adducts of PC 16 : 0, 18 : 1, respectively; m/z = 496.3 and m/z = 518.3 for proton and sodium adducts of LPC 16 : 0, respectively. Proton and sodium adducts of internal standard used, LPC 14 : 0, yield peaks at m/z = 468.3 and m/z = 490.3, respectively. Peaks with m/z values 758.6 and 780.6 correspond to the proton and sodium adducts of PC 16 : 0, 18 : 2. Concentrations of PLA_2 and internal standard were 0.0125 mg ml^{-1} and 1.07 × 10^{-4} M, respectively, while the initial concentration of the substrate was 4.11 × 10^{-3} M. DHB was added using a sample : matrix ratio 1 : 1. For detailed experimental conditions, see the section Preparation of samples for MALDI-TOF MS in Materials and methods. A representative data set from three independent experiments is shown.

at m/z = 496.3 and m/z = 518.3 corresponding to the proton and sodium adducts of LPC 16:0, respectively. A given amount of the lysophospholipid LPC 14:0 (peaks at m/z = 468.3 for the proton adduct and at m/z = 490.3 for the sodium adduct) has been added to all samples as internal standard, and the concentration of the product (LPC 16:0) was determined as previously described.[31]

After obtaining the concentration of enzymatically generated LPC 16:0 (*i.e.* the concentration of the product of the reaction) the more precise analysis was performed, Lineweaver–Burk diagram constructed and K_m and V_{max} values for this enzymatic reaction determined. The K_m value was (8.63 ± 3.62) × 10^{-3} M, while the V_{max} value corresponded to (5.33 ± 1.28) × 10^{-5} M min^{-1}, and the effect of selected compounds on these values will be investigated.

Effect of transition metal complexes on the PLA_2 activity

In the next set of experiments the activity of PLA_2 was investigated in the presence of the metal complexes $[RuCl_2(bipy)_2]Cl$ dichloro(bipyridine) ruthenium(III)chloride and $[PtCl_4(bipy)]$ tetrachloro(bipyridine) platinum(IV). Both complexes yielded additional peaks in the mass range used to detect phosphatidylcholines and lysoproducts (Fig. 2). The ruthenium complex is characterised by a couple of peaks from 565 to 573 with a peak at 569.3 having the highest intensity (Fig. 2A). This distribution is caused by the broad natural abundance of different ruthenium isotopes ranging from ^{96}Ru to ^{104}Ru. The original $[RuCl_2(bipy)_2]Cl$ (mass 519.8) is apparently converted in the aqueous environment into a new species (mass 569.3), replacing all chloride anions with one more *bipy* ligand.[27] The presence of this peak groups does not disturb the analysis of lysophospholipids. In contrast, the peak group for the corresponding platinum complex is found from 494 to 498 in relation to the broad distribution of natural platinum isotopes (Fig. 2B). The LPC peak at 496.3 was completely covered by the platinum metal complex. Thus, only the sodium adduct of LPC 16:0 at 518.3 can be used for quantitative assessment of the PLA_2 activity.

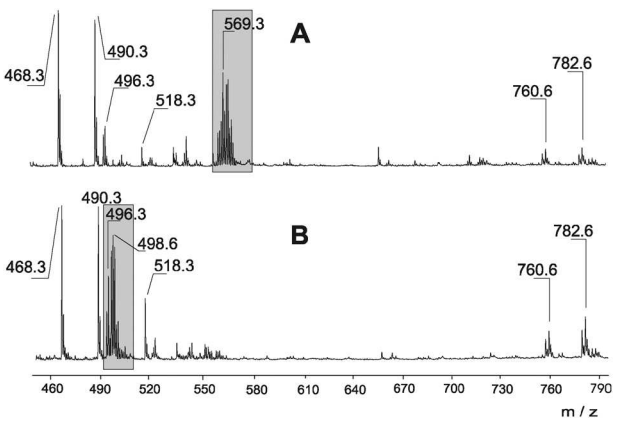


Fig. 2 Positive MALDI-TOF mass spectra of hen's egg yolk phospholipids incubated with pancreatic phospholipase A_2 and $[RuCl_2(bipy)_2]Cl$ (A) or $[PtCl_4(bipy)]$ (B). Peaks of interest are labeled: 486.3 and 490.3 for proton and sodium adducts of LPC 14:0 used as internal standard; 496.3 and 518.3 for proton and sodium adducts of LPC 16:0; 760.6 and 782.6 for proton and sodium adducts of PC 16:0, 18:1, while the shaded groups of peaks with m/z values 569.3 and 498.6 originate from ions of ruthenium and platinum complexes, respectively. The peak group around 569.3 was assigned to $[RuCl_2(bipy)_2]Cl$ which had lost one chloride anion, followed by elimination of two neutral HCl molecules and addition of one more bipyridine ligand. The peak group around m/z = 498.6 corresponded to the sodium adduct of $[PtCl_4(bipy)]$ after replacement of one neutral HCl molecule by H_2O.

Both metal complexes affected considerably the activity of porcine pancreatic phospholipase A_2. Metal complexes were incubated with the phospholipid suspension and then the enzyme was added. The final concentrations of metal complexes were 0.625 × 10^{-5} M and 0.625 × 10^{-7} M. Both, the K_m and V_{max} values of the enzyme were significantly lower in the presence of platinum and ruthenium complexes in comparison to the values obtained without metal complexes (Fig. 3).

The addition of $[RuCl_2(bipy)_2]Cl$ at a molar concentration of 0.625 × 10^{-7} M lowered the K_m value of phospholipase A_2 to 50% of the starting value and when the concentration of the complex was 100-fold higher, the K_m value was lowered to about 10% of the initial value (Fig. 3A). When $[PtCl_4(bipy)]$ was added at the same concentrations K_m was lowered to about 60% or 30% of the initial K_m value (Fig. 3C). Similar changes were found for the V_{max} values by both metal complexes (Fig. 3B and D). Lineweaver–Burk plots were constructed (Fig. 4A and B) with the slopes (K_m/V_{max} values) in the range of standard deviations and these indicate that the platinum and ruthenium complexes exhibit mainly an uncompetitive mode of inhibition of porcine pancreatic PLA_2.

Interaction of metal complexes with the enzyme

Next, we wanted to test whether the interaction of transition metal complexes with PLA_2 yielded a stable complex between

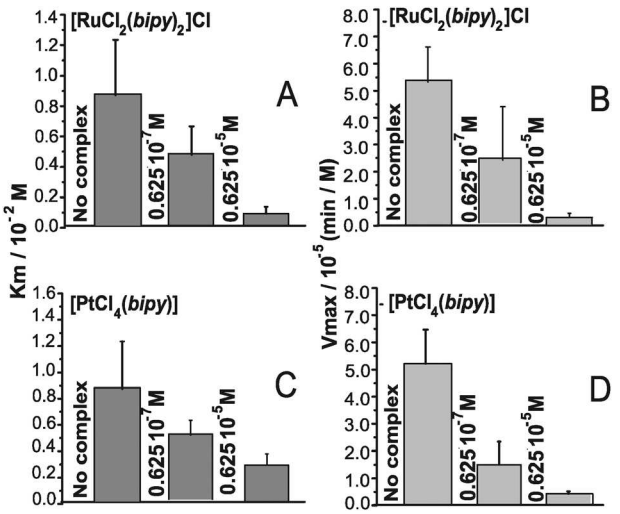


Fig. 3 K_m (A and C), V_{max} (B and D) values obtained for phospholipase A_2 in the presence of different concentrations of $[RuCl_2(bipy)_2]Cl$ (A–B) and $[PtCl_4(bipy)]$ (C–D). Experimental conditions are described in the section: Preparations of samples for MALDI-TOF MS in Materials and methods, in detail. Lipid samples were incubated with the metal complexes at the indicated concentrations and the enzyme is added. Production of lysolipid LPC 16:0 is measured after 0.5, 1, 2, 5, 15, 30 min. Means and standard deviations from three independent experiments are given.

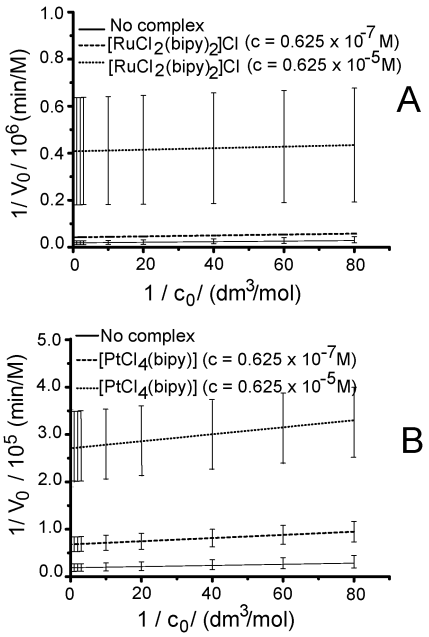


Fig. 4 Lineweaver–Burk plots for the formation of LPC 16 : 0 are given with different concentrations of $[RuCl_2(bipy)_2]Cl$ (A) and $[PtCl_4(bipy)]$ (B). The experimental conditions are same as for Fig. 3. Means and standard deviations from three independent experiments are given.

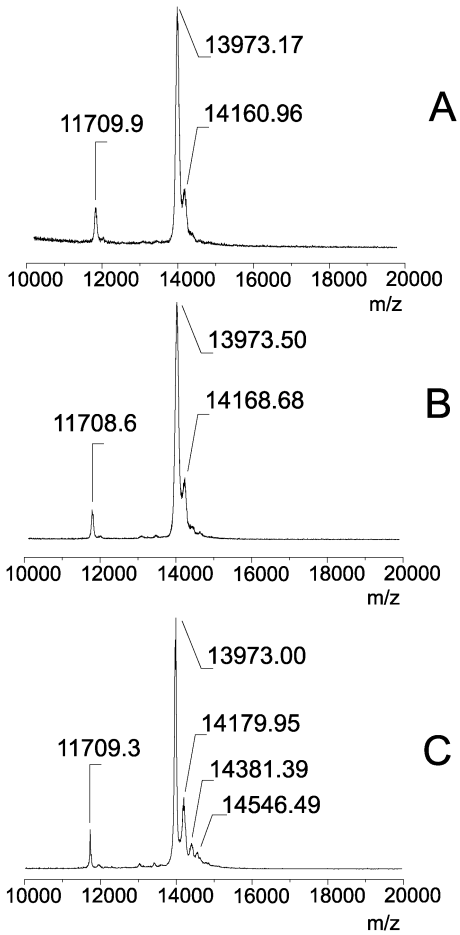


Fig. 5 MALDI-TOF mass spectra of porcine pancreatic PLA_2 (0.125 mg ml^{-1}) incubated with $[RuCl_2(bipy)_2]Cl$. Molar concentrations of the complex used were 10^{-5} M (A), 10^{-4} M (B), or 10^{-3} M (C) and time of incubation for each was three hours.

both partners. Porcine pancreatic phospholipase A_2 was incubated for 3 hours with each metal complex using different concentration ratios. In all samples, the concentration of PLA_2 was kept constant (0.125 mg ml^{-1}), while the final concentrations of metal complexes were 10^{-5}, 10^{-4} and 10^{-3} M. After incubation, all samples as well as the untreated PLA_2 were applied on the sample plate together with sinapic acid as matrix and MALDI-TOF mass spectra were recorded.

In Fig. 5, the MALDI-TOF mass spectra of the enzyme incubated with $[RuCl_2(bipy)_2]Cl$ are shown. Low concentrations of the ruthenium complex (10^{-5} M, Fig. 5A or 10^{-4} M, Fig. 5B) did not change the appearance of characteristic peaks at m/z = 13973.2 and m/z = 14160.9 for PLA_2. The first peak arises from the enzyme bearing a single positive charge, whereas the later signal corresponds to an isoform of the enzyme with higher mass present in the preparation. The peak at lower m/z ratio around 11 709 has not been assigned so far, but it might correspond to a fragment of PLA_2, possibly obtained during the process of enzyme preparation.

However, additional peaks in the mass spectra were detectable at a higher concentration of the metal complex (10^{-3} M, Fig. 5C). There are two new low intense, but rather broad peaks centred at m/z = 14 381 and at m/z = 14 546. Considering the fact that $[RuCl_2(bipy)_2]Cl$ rearranges in aqueous solution, as it has been described above, these two new peaks can be regarded as association complexes of these two later ruthenium complexes with PLA_2. To determine the exact mass differences is challenging because of the broad mass distribution of ruthenium isotopes, but the mass differences between the new peak groups and the unperturbed enzyme correspond well to the masses of both complexes.

Further evidence for potential interaction of the ruthenium complex with PLA_2 was obtained from fluorescence measurements. The fluorescence of PLA_2 when excited at 280 nm is mainly due to the presence of a single tryptophan residue (Trp3) which is located on the surface of the enzyme molecule and exposed to the environment.[39] All fluorescent spectra of PLA_2 solution were recorded in the presence of increasing concentrations of metal complexes.

The resulting fluorescent spectra of PLA_2 obtained before and after the addition of increasing amounts of $[RuCl_2(bipy)_2]Cl$ are presented in Fig. 6A. The ruthenium complex caused a concentration-dependent quenching of the tryptophan fluorescence of PLA_2.

It is well known that fluorescence quenching originates from collision between the fluorophore and the quencher (dynamic quenching) and/or due to complex formation between the fluorophore and the quencher (static quenching). To determine the mechanism of interaction between PLA_2 and $[RuCl_2(bipy)_2]Cl$, fluorescence intensity data were analyzed by the Stern–Volmer equation:

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

where F and F_0 represent the fluorescence intensity with and without the metal complex. K_{sv} is the Stern–Volmer quenching constant, and [Q] is the concentration of the metal complex in

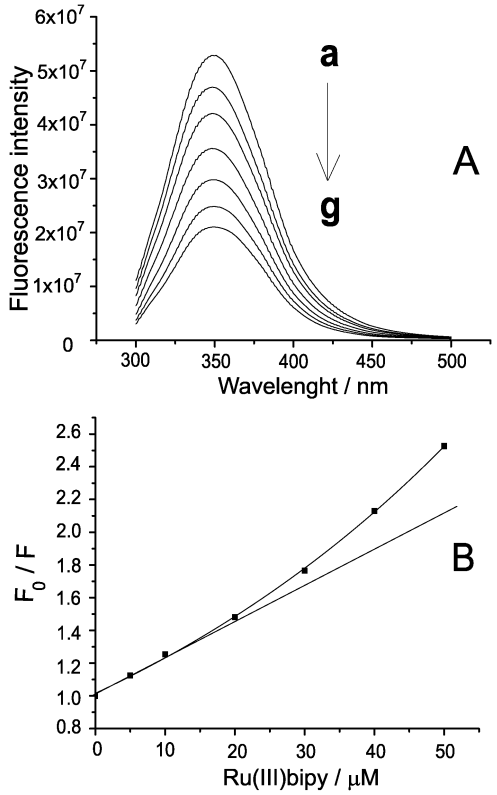


Fig. 6 (A) Fluorescence spectra of phospholipase A_2 at different concentrations of Ru(III)bipy at 25 °C. Concentration of PLA_2 was 10 μM and the complex concentrations were: (a) 0; (b) 5; (c) 10; (d) 20; (e) 30; (f) 40 and (g) 50 μM. (B) Stern–Volmer plot of the interaction of Ru(III)*bipy* with PLA_2.

the sample. The corresponding Stern–Volmer plot for the tryptophan fluorescence of PLA_2 by $[RuCl_2(bipy)_2]Cl$ is given in Fig. 6B. At low metal complex concentrations, data can be fitted to a straight line, but there is a clear deviation at higher concentrations. Thus, the ruthenium metal complex causes both a dynamic and static quenching. In other words, the complex formation seen in mass spectrometry can be verified by fluorescence quenching data.

In contrast, no signs for a complex formation with porcine pancreatic PLA_2 could be observed for the $[PtCl_4(bipy)]$ complex. There were no additional peaks in mass spectra and no quenching of tryptophan fluorescence (data not shown).

Discussion

Both metal platinum and ruthenium metal complexes bearing bipyridine ligands $[RuCl_2(bipy)_2]Cl$ and $[PtCl_4(bipy)]$ inhibited strongly the activity of porcine pancreatic phospholipase A_2. Data analysis revealed an uncompetitive inhibition mode of this enzyme for both metal complexes, as both the V_{max} and K_m values decreased significantly. That means, these metal complexes prevent the product formation by interaction with the enzyme–substrate complex. These data are in accordance with observations of other authors that other types of secreted phospholipase A_2 are inhibited in the same way with a nonapeptide or lysophosphatidylcholines.[40]

We have selected MALDI-TOF MS for the study of the PLA_2 activity because this method is sufficiently sensitive and does not require any additional derivatization of the samples for the analysis. In addition, it allows analysis of both the substrate and the product of the enzyme-catalyzed reaction in a single spectrum. Moreover, alterations in the phospholipid substrate and lysoproducts derived from PLA_2 activity can simultaneously be visualised in the same mass spectrum. Even quantitative information can be obtained by MALDI-TOF MS with the addition of the internal standard, as demonstrated in previous publications.[31,41–44]

The only problem, related to the analysis of MALDI-TOF mass spectra of the products of enzymatic activity of PLA_2, was that signals of the $[PtCl_4(bipy)]$ complex are located in the mass region where the proton adduct of lysophosphatidylcholine 16 : 0 (m/z = 496.34) was found. We have overcome this by using the sodium adduct of lysophosphatidylcholine 16 : 0 (m/z = 518.32) for the assessment of enzyme activity.

In order to get more information about the interaction of both metal complexes with porcine pancreatic phospholipase A_2, we applied further mass spectrometric approaches and fluorescence quenching to analyse a ligand binding. The ruthenium complex ($[RuCl_2(bipy)_2]Cl$) yielded two new, but low intense, mass peaks in the mass spectrum for PLA_2 (Fig. 5C), indicating that this complex has partly been bound to the enzyme. An exact evaluation of these new peaks was not possible because of their low intensity and the fact that ruthenium exhibits a broad distribution of isotopes. The original $[RuCl_2(bipy)_2]Cl$ is apparently converted in the aqueous environment into two different species, one losing all chloride anions, and another replacing them with one more *bipy* ligand. Thus, the two new mass peaks can be regarded as association complexes of these two later ruthenium complexes with PLA_2. In the case of $PtCl_4(bipy)$ no additional peaks in the mass spectrum were observed (data not shown).

A potential association of $[RuCl_2(bipy)_2]Cl$, but not for the $[PtCl_4(bipy)]$ complex, with phospholipase A_2 was also verified by fluorescence quenching analysis of the tryptophan fluorescence. Porcine and bovine pancreatic phospholipase A_2 have only one tryptophan residue at position 3 in their polypeptide chain. In contrast, human pancreatic phospholipase A_2 is free of tryptophan. As evidenced by the 3D-structure, the tryptophan residue is located at the surface near the substrate binding channel and the active catalytic centre of PLA_2.[45] The reason for different results for the association of $[RuCl_2(bipy)_2]Cl$ and $[PtCl_4(bipy)]$ complexes with PLA_2 remains unknown. We can only assume that $[RuCl_2(bipy)_2]Cl$ forms a more stable bond with the enzyme than the corresponding Pt-complex. The inhibition of enzyme activity by $[PtCl_4(bipy)]$ revealed that at least a transient interaction of this complex with PLA_2 should occur. As stated above, this is mostly an interaction with the enzyme–substrate complex as given by the uncompetitive inhibition mode.

Interestingly, our unpublished observations demonstrated that the addition of these transition metal complexes to the large unilamellar vesicles formed from PC, which contained carboxyfluorescein, increased significantly the leakage of this dye. This effect might be caused by changes in the phospholipid organization after addition of transition metal complexes.

In agreement to this, there are data in the literature proving the binding of metal complexes to phospholipids, mostly to negatively-charged species.[46,47] These data also endorse a potential interaction of transition metal complexes with phospholipids.[46–48]

Pancreatic secretory PLA_2 is distributed throughout the gastrointestinal tract and is particularly abundant in pancreas and duodenum.[49] This enzyme is involved in control of secretin release, which is important for water homeostasis and overall metabolism. The association of pancreatic PLA_2 with cancer progression has not been demonstrated so far and the effect of transition metal complexes on the activity of this enzyme has not been studied. In this work, this enzyme serves only as a model system for the study of the effect of these chemotherapeutic drugs on the PLA_2 activity. One can assume that transition metal complexes will exhibit similar effects on other types of secretory PLA_2 enzymes.

Whereas the expression of cytosolic and calcium-independent PLA_2 enzymes has been investigated for several carcinomas,[50–52] the expression of secretory enzymes with tumor progression has poorly been addressed. It has been recently shown that the secretory PLA_2 type IIA protein content is decreased in colon carcinoma cells, whereas this enzyme could be detected at higher concentrations in apoptotic, necrotic cells and in the invasive front of colorectal carcinoma.[53] However, it remains unknown whether this activity can be correlated with its increased expression. One can assume that PLA_2 enzymes are involved in the process of clearance of apoptotic and necrotic tumor cells, whereas their increased content in the invasive front might promote penetration of tumor cells into the healthy tissue. We can only speculate that potential inhibitory effect of metallo-drugs on the secretory PLA_2 might slow down the process of protrusion of tumor cells by decreasing the concentration of mediators of cellular signaling, such as metabolites of arachidonic acid and lysophospholipids. Additional effects of metallo-drugs on the death of tumor cells remain to be further studied.

Prostate cancer cells also secrete higher levels of $sPLA_2$ due to increased activation of intracellular signaling pathways, which are, in turn, also activated by products of $sPLA_2$ activity.[54] In a similar manner to colon carcinoma cells, we can assume that inhibitors of $sPLA_2$, such as transition metal complexes, might contribute to the processes of inhibition of prostate tumor growth and differentiation, *i.e.* that they will have rather beneficial effects. In addition, inhibition of secretory PLA_2 with transition metal complexes might affect the process of host defence under chemotherapy. Since secretory PLA_2 plays a significant role in host defence (for instance the enzyme secreted from stimulated neutrophils), inhibition of this enzyme might imply decreased ability of the host to cope with invading microorganisms. Furthermore, efficiency of the clearance of necrotic tissues, including necrotic tumor cells, might be significantly suppressed under chemotherapy. This all might contribute to a lower efficiency of the anti-tumor therapy with metallo-drugs.

Finally, results presented in this work emphasize the necessity to better clarify the effect of individual metallo-drugs on various types of secretory PLA_2 enzymes, because they possess different amino acids in their active centre and may have different roles in carcinogenesis and inflammation.

Conclusions

In conclusion, both tested complexes $[RuCl_2(bipy)_2]Cl$ and $[PtCl_4(bipy)]$, which share the same ligands, affected the production of lysolipids in a concentration dependent manner as uncompetitive inhibitors. The type of interaction between the Ru(III) complex and the enzyme is a combination of pure collision and complex formation indicating apparently only one class of binding sites on the enzyme for $[RuCl_2(bipy)_2]Cl$. The nature of interaction of the platinum complex, as well as the mechanism of inhibition of the enzyme activity and its recovery, however, remain to be further studied. Further physiological consequences of the inhibition of the PLA_2 activity by transition metal complexes have to be investigated in more detail.

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