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The interaction of the anti-cancer drug cisplatin with phospholipids is specific for negatively charged phospholipids and takes place at low chloride ion concentration

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Abstract

The interaction of the anti-cancer drug *cis*-diamminedichloroplatinum(II) (cisPt) with model membranes was studied, with emphasis on the cisPt and phospholipid species involved. Binding studies using large unilamellar vesicles have revealed that: (i) Interaction involved negatively charged phospholipids only, and (ii) Interaction with negatively charged phospholipids was observed only in buffers with low Cl^- concentration, indicating that aquated, positively charged cisPt is involved. Binding to all negatively charged phospholipids tested was highest at pH 6.0. At pH 7.4 a high and specific binding was observed with phosphatidic acid and phosphatidylserine. The consequences of cisPt binding on the organization of lipids was investigated with differential scanning calorimetry studies. These studies have indicated a higher ordering of dispersions of negatively charged phospholipids in the presence of divalent cationic cisPt. Summarizing, the interaction of positively charged cisPt species with negatively charged phospholipids is significant and should be considered in in vivo experiments.

Keywords: Cisplatin; *cis*-Diamminedichloroplatinum(II); Phospholipid; Large unilamellar vesicle; Membrane.

1. Introduction

The anti-cancer drug *cis*-diamminedichloroplatinum(II) (cisPt) has an established role in the treatment of testicular, ovarian, bladder, small cell lung as well as head and neck cancer [1]. The reactive species of the drug is not the native cisPt, but aquated cisPt. If *cis*-diamminedichloroplatinum(II) is dissolved in water, the chloride atoms are subsequently replaced by water molecules in a stepwise manner, resulting in mono- and diaquated cisPt [2]. The extent of this aquation is determined by reaction time and the concentration of Cl^- present in the buffer. With a H_2O molecule replacing a Cl^- ion, cisPt becomes a positively charged, weak acid. Depending on the pH of the buffer, aquated cisPt contains a net charge of 0, +1, or +2 [1] (Fig. 1A). Aquation of cisPt in vivo is thought to occur in the cytosol, since the pH and Cl^- concentration are lower than that of the blood plasma. The positively charged aquated cisPt species are very reactive towards several cell

Abbreviations: Buffer A, 10 mM Pipes, 100 mM NaCl, 1 mM EGTA, pH 7.4; Buffer B, 10 mM Pipes, 50 mM Na_2SO_4, 1 mM EGTA, pH 7.4; Buffer C, 10 mM Mes, 100 mM NaCl, 1 mM EGTA, pH 6.0; Buffer D, 10 mM Mes, 50 mM Na_2SO_4, 1 mM EGTA, pH 6.0; cisPt, *cis*-diamminedichloroplatinum(II) and its aquated products; CL, cardiolipin from bovine heart; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DSC, differential scanning calorimetry; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; LUVET, large unilamellar vesicles prepared by extrusion; Mes, 2-morpholinoethanesulfonic acid; NFAAS, non flame atomic absorption spectroscopy; PI, phosphatidylinositol from soybean; Pipes, piperazine-1,4-diethanesulfonic acid; Pt, platinum; SM, sphingomyelin from egg.

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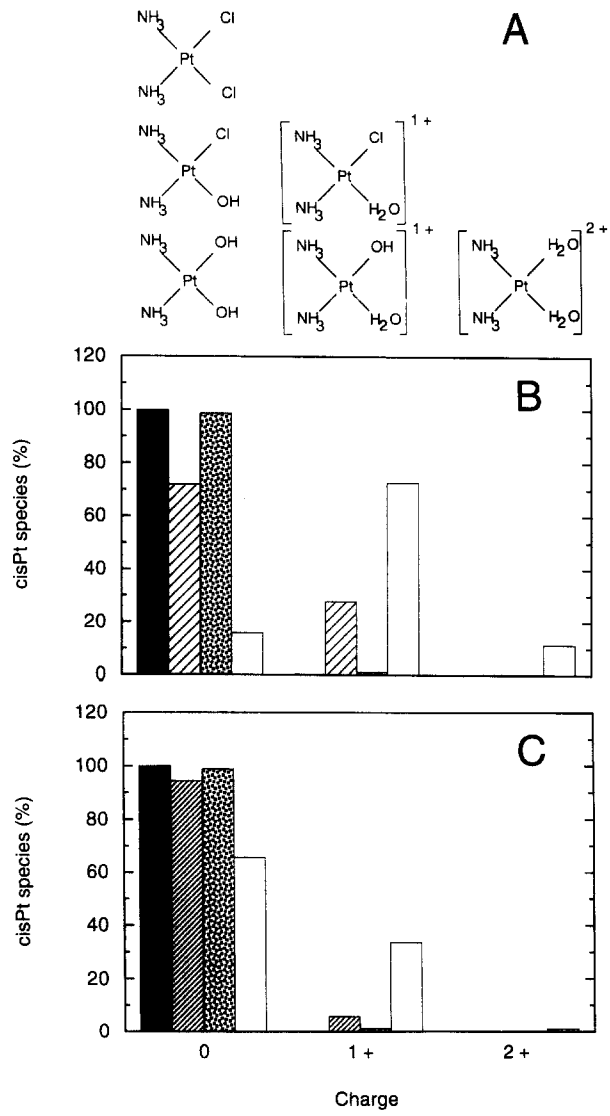

Fig. 1. Equilibrium distribution of the several cisPt species in different buffers. Plotted are uncharged, monovalent and divalent cationic species. (A) Structure of the mononuclear species. Uncharged species are unreactive. Monovalent cations and the divalent cationic species are most reactive. (B) Distribution of 150 μM total cisPt, a concentration representative for the binding experiments. (C) Distribution of 5 mM total cisPt, used in the DSC experiments. Black bars represent distribution in buffer A, arched bars represent distribution in buffer B, dotted bars represent the distribution in buffer C, and white bars represent distribution of cisPt in buffer D. Buffer composition is described in Section 2. Distribution of the species is calculated using the given concentration of Cl^- and total cisPt and an equilibrium constant K of $1.78 \cdot 10^{-3}$ for the first aquation step, $PtCl_2(NH_3)_2 \rightleftharpoons PtCl(H_2O)(NH_3)_2^+ + Cl^-$ and $2.75 \cdot 10^{-4}$ for the second aquation step, $PtCl(H_2O)(NH_3)_2^+ \rightleftharpoons Pt(H_2O)_2(NH_3)_2^{2+} + Cl^-$. Subsequently, for the monoaquated cisplatin the distribution between neutral and 1+ species was calculated using the pH value and a pK_a of 6.41. The distribution between neutral, 1+, and 2+ for the diaquated cisplatin species was calculated using the pH value and pK_a values of 7.21 and 5.37 [13,14].

components. Much attention has been paid to the interaction of cisPt with DNA and -SH groups of proteins and peptides [1,3,4]. However, little is known about cisPt–membrane interactions and virtually nothing has been reported about cisPt–phospholipid interactions, although these interactions may play an important role in the cytotoxic mechanism of cisPt. For other drugs, interactions with membranes or phospholipids have important consequences for their cytotoxicity, side-effects and transport across the plasma membrane. For instance, doxorubicin interaction with phosphoinositides and phosphatidylserine can have an effect on signal transduction [5,6], and its cardiotoxic side-effect is attributed to its interaction with cardiolipin [7]. Aminoglucosides interact with phosphoinositides and this has been associated with nephrotoxicity [8], one of the severe side-effects also observed with cisPt. Furthermore, cisPt-membrane binding studies may help to interpret the results of cisPt influx experiments, of which the mechanism has not yet been resolved [9].

This paper describes the interaction of cisPt with phospholipids. Special attention was paid to the nature of the platinum species involved, i.e. aquated, positively charged, or neutral, by manipulating the buffers in which the experiments were performed, and to the phospholipid species involved.

Part of these results have been presented on the 7th ISPCC, 1995, Amsterdam, The Netherlands (abstract No. 103).

2. Materials and methods

2.1. Materials

cisPt was purchased from Sigma (St. Louis, MO, USA). All lipids, except 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), were obtained from Avanti Polar Lipids (Birmingham, AL, USA). DPPC was prepared in this lab as described [10]. DPPG was prepared from DPPC using phospholipase D mediated base exchange [11].

2.2. Preparation of cis-diamminedichloroplatinum(II) stock solutions and buffers employed

cisPt was dissolved in buffer to a final concentration of 5 mM. The solution was incubated for 1 h at 37–40°C and vortexed occasionally to dissolve the cisPt completely. The buffers in which the stock solution was prepared were of the following composition:

10 mM Pipes, 100 mM NaCl, 1 mM EGTA, pH 7.4 (Buffer A)

10 mM Pipes, 50 mM Na_2SO_4, 1 mM EGTA, pH 7.4 (Buffer B)

10 mM Mes, 100 mM NaCl, 1 mM EGTA, pH 6.0 (Buffer C)

10 mM Mes, 50 mM Na_2SO_4, 1 mM EGTA, pH 6.0 (Buffer D).

Unless stated otherwise these stock solutions were used within 2 h ('freshly prepared'). In order to allow for the

aquation of cisPt to approach equilibrium most experiments involved incubation periods of 3 days. Alternatively, stock solutions of cisPt were allowed to aquate for 3 days in the dark at room temperature ('pre-incubated cisPt').

2.3. Preparation of model membranes

Large unilamellar vesicles (LUVET) employed in the binding experiments were prepared in buffer A, B, C, or D by extrusion through polycarbonate filters with 400-nm pores (Costar-Nuclepore Europe, Badhoevedorp, The Netherlands) as described previously [6]. LUVET were composed of pure phospholipids, except for DOPE. In that case a 1:1 mixture with DOPC was used to prevent the formation of non-bilayer structures.

Multilamellar vesicles (MLV) used for differential scanning calorimetry (DSC) measurements were prepared by hydration of the dried lipid-films in Buffer A, B, C or D in the absence or presence of 5 mM freshly prepared cisPt. The samples were freeze-thawed and vortexed 10 times and transferred to Eppendorf tubes and used for DSC experiments.

2.4. Binding assay

Binding of cisPt to model membranes was assayed as described by [6] with the following modifications: 250 μl of membrane suspensions (20 mM lipid-P_i) were mixed with 750 μl cisPt solution in buffer A, B, C, or D in the range of 25 to 250 μM cisPt (final concentrations). Duplicate samples were incubated in the dark at room temperature for 3 days and freeze-thawed two times immediately after addition of cisPt and two times at the end of the incubation period. Freeze-thaw steps were performed to allow equilibration between the internal and external volume and, since passive diffusion of cisPt across phospholipid bilayers is very slow (Speelmans, G., unpublished data), to allow binding of cisPt to lipids present in the inner leaflet of the bilayer. The model membranes and bound drug were pelleted (45 min at 436 000 × g and 20°C in a Beckman TLA 100.2 rotor). The top 800 μl were collected for determination of free Pt. The amount of lipid-P_i was determined in the supernatant fraction (negligible) and pellet fraction. Blanks without lipid were used to determine the total amount of drug. Corrections were made for binding of cisPt to tubes (which was low). Samples of which the platinum (Pt) concentration had to be determined were stored at −20°C prior to Pt determination.

2.5. Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments were performed with DPPC and DPPG lipids in a Perkin Elmer DSC-2 apparatus calibrated with Indium. Multilamellar vesicles were incubated for 3 days at room temperature in the dark, and centrifuged for 20 min at 14 000 × g at room temperature. The pellet was transferred to a 20 μl sample pan and immediately scanned 5 times. Heating and cooling scans were determined at a rate of 5 K/min in the range from 20 to 55°C against an empty sample pan as reference.

2.6. Platinum determination

Samples containing Pt were thawed and diluted in water immediately before Pt determination such that the concentration was approximately 0.4 μM. The concentration of Pt was determined with a non-flame atomic absorption spectrophotometer, type AAS 4000 Perkin Elmer using a platinum lamp and a deuterium lamp for background correction. For the calibration curve a solution of known concentrations of K_2PtCl_6 was used.

2.7. Other methods

The phospholipid concentration was determined according to Rouser [12].

3. Results

3.1. Conditions

Since it is of primary interest to distinguish between the different cisPt species with respect to their interaction with phospholipids, different buffers were employed to manipulate the distribution of cisPt species. Buffers contained 100 mM Cl^- (Buffer A and C), or no Cl^- (Buffer B and D) and had a pH value of 7.4 (Buffer A and B), or pH 6.0 (Buffer C and D). As Cl^- is slowly released upon aquation of cisPt the initial concentration of cisPt present also influences the extend of aquation in buffer B and D.

In Fig. 1 the distribution of the calculated differently charged cisPt species in buffer A, B, C, and D, is shown based on pK_a values and equation rate constants given in the literature [13,14]. Results are shown for a cisPt concentration of 150 μM (Fig. 1B) which is a concentration representative for the binding experiments, and 5 mM (Fig. 1C) which was employed in the DSC experiments. The concentration of positively charged cisPt is highest in buffers without Cl^- and higher at pH 6.0 than at pH 7.4. Since the aquation of cisPt is a rather slow process, with a half time of several hours [15], stock solutions were either preincubated for 3 days or incubations were performed during 3 days in order to allow for the equilibrium to be approached.

3.2. Binding of cisPt to phospholipid vesicles

Phospholipids were added as large unilamellar vesicles (LUVET) and in order to allow cisPt to react with both

sides of the membrane, freeze-thaw steps were applied. No large differences were observed if Pt binding was calculated from the data of the cisPt concentration in the supernatants, or directly, when determined in the pellet fraction (containing the phospholipids).

In Fig. 2 the results of the binding experiments are shown. The following phospholipid specificity can be observed for binding: Binding is low or negligible to the overall neutral lipids phosphatidylcholine (PC), sphingomyelin (SM) and phosphatidylethanolamine (PE), whereas significant binding is observed to all negatively charged lipids tested; phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI) and cardiolipin (CL). Clear differences in binding can be observed dependent on the buffer employed. Binding of cisPt to negatively charged phospholipids is high in buffers without Cl^- (Fig. 2B,D), whereas hardly any binding is observed in the presence of 100 mM Cl^- (Fig. 2A,C), except for DOPA at pH 7.4 (Fig. 2A). When buffers without Cl^- are compared, it appears that binding is higher at pH 6.0 (Fig. 2D) than at pH 7.4 (Fig. 2B). At pH 6.0 binding to all negatively charged phospholipids is comparably high, but at pH 7.4 a strong binding is observed to PA and PS, whereas binding to CL, PG, and PI is much lower. No apparent binding affinities can be calculated since the binding curves indicate that saturation does not occur at the cisPt concentrations employed. In order to directly compare binding of cisPt to different phospholipids in different buffers, the amount of bound cisPt per phospholipid (mmol/mol lipid-P_i) at a concentration of 100 μM free cisPt is given in Table 1.

Binding of cisPt to phospholipids is highest in buffer with the highest concentration of positively charged cisPt species (Fig. 1), and high for all negatively charged phospholipids tested, but negligible for all zwitterionic phospholipids tested, indicating the involvement of an electrostatic drug–phospholipid interaction.

3.3. Differential scanning calorimetry measurements

With differential scanning calorimetry (DSC) the consequences of the interactions of compounds for the phase

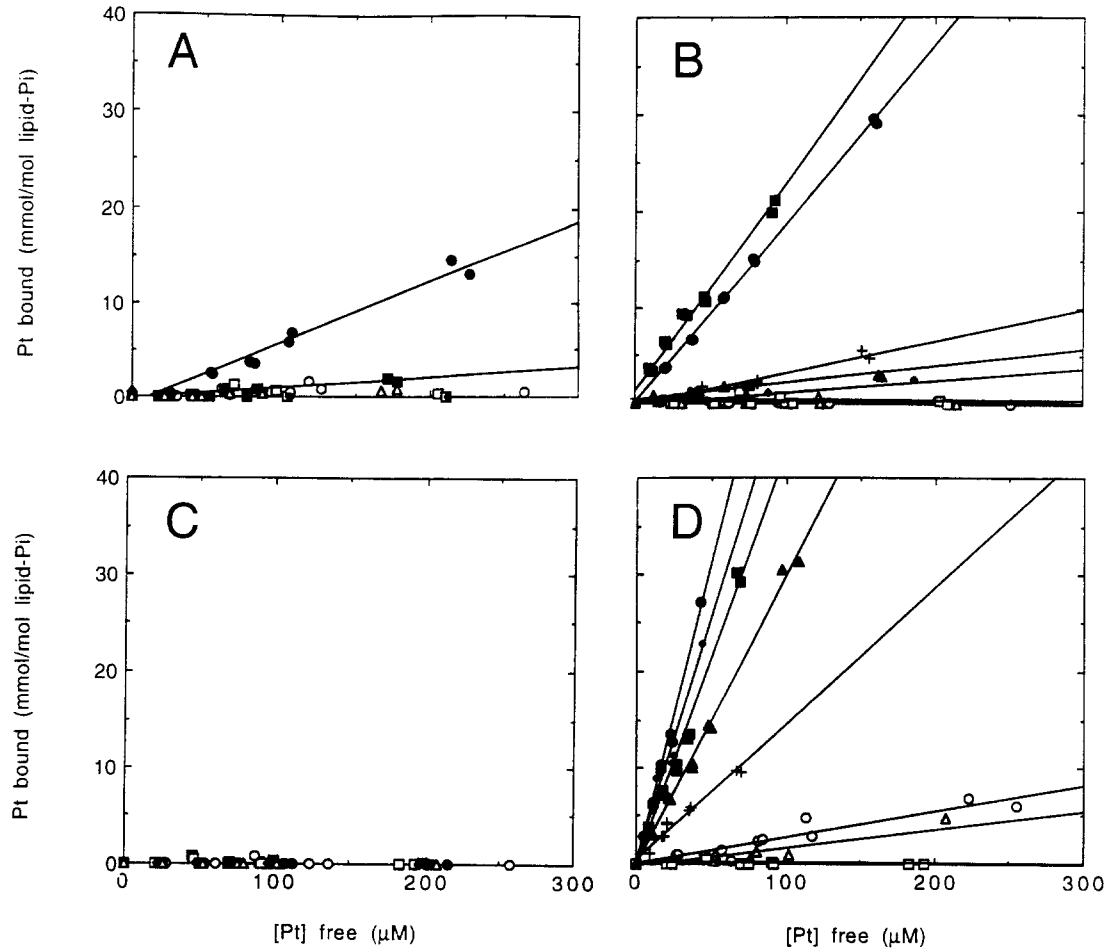

Fig. 2. Binding of cisPt to large unilamellar vesicles composed of various phospholipids in buffers of various composition. Binding experiments were performed as described in Section 2. (A) Buffer with 100 mM Cl^-, pH 7.4. (B) Buffer without Cl^-, pH 7.4. (C) Buffer with 100 mM Cl^-, pH 6.0. (D) Buffer without Cl^-, pH 6.0. LUVET were composed of (○) DOPC, (△) DOPC/DOPE (1:1), (□) SM, (♦) DOPG, (+)CL, (▲) PI, (●) DOPA, and (■) DOPS.

behavior of phospholipids can be studied. The transition temperature and/or enthalpy of the gel to liquid crystalline phase transition of phospholipids can change in the presence of drug molecules and this can give information about the type of interaction. Therefore, it was studied whether the presence of cisPt changed the transition temperature of negatively charged phospholipids (DPPG) in buffer containing a high concentration of Cl^- (Buffer A, C) or no Cl^- (Buffer B, D). The interaction with DPPG was compared to the interaction with the zwitterionic phospholipid DPPC. In Fig. 3A heating scans of DPPG are shown, performed in the absence or presence of 5 mM cisPt after 3 days incubation at room temperature in buffer A or D. When experiments are performed in buffer A (pH 7.4, 100 mM Cl^-) no difference between scans in the absence (scan a) or presence (scan b) of cisPt is observed, with the temperature of the gel to liquid-crystalline phase transition at 41.6°C in both cases. The same phenomenon is observed in buffer C (data not shown). However, in buffer B (pH 7.4, without Cl^-) a small peak broadening and an increase in the transition temperature were observed in the presence of cisPt (data not shown). This effect appears most pronounced in buffer D (pH 6.0, without Cl^-). Scan d shows a large broadening and doubling of the peak. Compared to scan c (performed in the absence of cisPt) the transition temperature is shifted from 41.1 (scan c) to 43.8 and 47.2°C (scan d). This shift to higher transition temperatures is also observed in cooling scans of DPPG with cisPt and was already visible to some extent after 1 day incubation and maximal after 3 days incubation because of the slow aquation of native cisPt (data not shown). In addition, the transition enthalpy (ΔH) of DPPG is increased from 8.7 in the absence of cisPt to 10.1 kcal/mol in the presence of cisPt. In Fig. 3B the results are shown with the zwitterionic phospholipid DPPC. In buffer A (scan a, b) as well as in buffer D (scan c, d) the presence of cisPt (scan b, d) has no effect on the pretransition and main transition temperature of DPPC (of 37.0 and 42.5°C, respectively). This again indicates that the binding of cisPt is specific for negatively charged phospholipids.

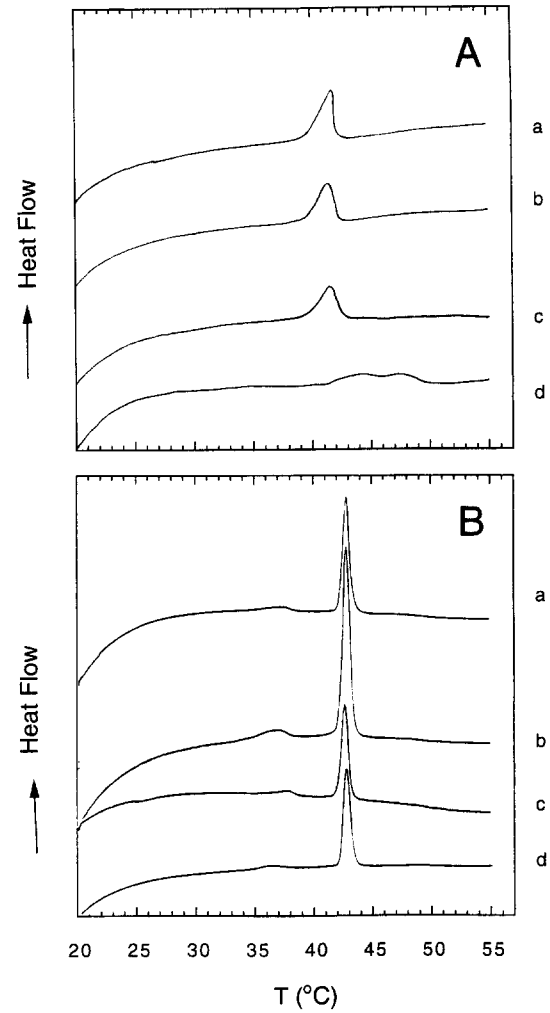

Fig. 3. Differential scanning calorimetry of phospholipids in the presence or absence of cisPt. (A) Heating scans of DPPG in buffer pH 7.4 with 100 mM Cl^- (scan a, b) or in buffer pH 6.0 without Cl^- (scan c, d) in the absence of cisPt (scan a, c) or in the presence of 5 mM cisPt (scan b, d). (B) Heating scans of DPPC in buffer pH 7.4 with 100 mM Cl^- (scan a, b) or in buffer pH 6.0 without Cl^- (scan c, d) in the absence of cisPt (scan a, c) or in the presence of 5 mM cisPt (scan b, d).

Table 1
Amount of mmol cisPt bound per mol phospholipid-P_i at a free cisPt concentration of 100 μM

Phospholipid	Buffer A [a]	Buffer B	Buffer C	Buffer D
DOPC	< 1	< 1	< 1	2.7
DOPE/PC	< 1	< 1	< 1	1.6
SM	n.d. [b]	< 1	n.d.	< 1
DOPA	5.6	18.5	< 1	61.9
DOPG	< 1	1.5	< 1	50.9
CL	n.d.	3.0	n.d.	14.5
DOPS	< 1	22.8	< 1	42.9
PI	n.d.	2.1	n.d.	29.8

[a] Buffer A: 10 mM Pipes, 100 mM NaCl, 1 mM EGTA, pH 7.4; Buffer B: 10 mM Pipes, 50 mM Na_2SO_4, 1 mM EGTA, pH 7.4; Buffer C: 10 mM Mes, 100 mM NaCl, 1 mM EGTA, pH 6.0; Buffer D: 10 mM Mes, 50 mM Na_2SO_4, 1 mM EGTA, pH 6.0.
[b] n.d., not determined.

4. Discussion

The results presented above describe for the first time the interaction of the anti-tumor drug cisPt with phospholipids. Binding studies and DSC experiments did reveal that (i) interaction occurs with all negatively charged phospholipids tested, but with none of the zwitterionic phospholipids tested, and (ii) interaction occurs mainly in buffers without Cl^-, and most strongly at low pH. These are strong indications that electrostatic interactions are involved in cisPt-phospholipid binding. In buffer with 100

mM Cl^-, pH 7.4 some binding to phosphatidic acid (PA) was observed. PA can have two negative charges at high pH and also its small head group, resulting in a high charge density, may account for binding under conditions where the amount of positively charged cisPt is low. In buffer of high pH without Cl^- the two phospholipids with a second ionizable group (PS and PA) show a significant higher binding than the other negatively charged phospholipids. Interestingly, this specific higher binding to PS and PA at higher pH values has also been observed for divalent cations, such as Mg^{2+} [16]. At pH 6.0 in the absence of Cl^- on the other hand, binding to the various negatively charged phospholipids is more comparable, although a decreased binding affinity has been observed: CL < PS, PG, PA. If it is assumed that only mono- and divalent charged cisPt species interact with negatively charged phospholipids, than the amount of cisPt bound to the negatively charged phospholipids in buffer D is in the same order as the amount of positively charged cisPt present in this buffer under equilibrium conditions. The amount of cisPt bound to PA and PS at pH 7.4 in the absence of Cl^- (buffer B) is twice as high as expected from the amount of positively charged cisPt present under equilibrium conditions in buffer B alone. This indicates that upon interaction of cisPt with the phospholipids a shift in equilibrium might take place, upon which more positively charged cisPt is formed, or that other interactions also play a role for these lipids. For PS this indeed seems to be the case (Speelmans et al. 1996, unpublished data).

A broadening, doubling and increase of the transition temperature of negatively charged DPPG was observed in the presence of cisPt at low pH and low Cl^- concentration. This effect was not observed for the zwitterionic phospholipid DPPC. An increased transition temperature indicates a less fluid membrane, which can be explained by neutralization of the negatively charged phospholipids by positively charged cisPt. This rigidifying effect by charge neutralization of negatively charged phospholipids has also been observed by increasing the concentration of H^+, Na^+ or K^+ [17] and especially by increasing the concentration of divalent cations, such as Ca^{2+} and Mg^{2+} [17–19]. The appearance of two transition peaks in the DSC scan is perhaps due to the presence of both mono- and divalent cationic species of cisPt. An increase of the transition temperature of DMPG by 2 K was observed if Ca^{2+} or Mg^{2+} is present in a ratio of 0.06–0.07 mol/mol phospholipid [19]. This is in the same order as the ratio total (charged and uncharged) cisPt/mol phospholipid. A possible explanation is that the hydrolysis equilibria of the cisPt species change upon binding to negatively charged phospholipids and that significantly more positively charged cisPt is formed in the presence of negatively charged phospholipids than in buffers alone.

The comparable interaction of cisPt and divalent cations with membranes is of great interest and may have important physiological implications. Simpkins [20] observed a decrease in the number of Ca^{2+} binding sites on biological membranes, in the presence of cisPt, which may be the result of the same phenomenon as observed by us in model membranes, namely charge–charge interaction between cisPt and phospholipids.

Changes in intracellular cisPt concentrations or distribution, achieved by (i) interaction with amino acids of peptides and proteins and (ii) by active efflux, have considerable implications for antitumor activity [21,22]. If redistribution and competition for binding with DNA might also be achieved by binding to negatively charged phospholipids, this may have important implications for the antitumor activity of cisPt and related derivative compounds. Binding studies at in vivo pH and [Cl^-] and in the presence of proteins and DNA are necessary to ascertain the importance of cisPt–phospholipid interaction. In general the intracellular [Cl^-] is about 4 mM, whereas this is 100 mM in the blood plasma. The internal pH in tumor cells is lower than the pH of 7.4 of the blood plasma [23].

Negatively charged phospholipids play crucial roles in signal transduction, oxidative phosphorylation, and cell proliferation [7,24,25]. Therefore, cisPt–phospholipid interaction may have important consequences for these processes. Especially the strong interaction with PS, located in the inner leaflet of the plasma membrane, is of interest and is currently under investigation.

In summary, we have shown that the interaction of cisPt and its metabolites with phospholipids can be significant and should be considered when studying transport and binding studies of cisPt and other compounds in vivo.

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