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Interaction of the anti-cancer drug cisplatin with phosphatidylserine in intact and semi-intact cells

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Abstract

The anti-cancer drug cisplatin (*cis*-diamminedichloroplatinum(II)) forms a stable coordination complex with phosphatidylserine (PS) in model membrane systems (Speelmans et al., Biochemistry 36 (1997) 10545–10550). Because a similar interaction in vivo would be expected to have important physiological implications we studied cisplatin-PS interaction in human erythrocytes and tumor cell lines. Although cisplatin was efficiently taken up by intact erythrocytes, a cisplatin-PS complex was only detected in cells which had lysed as a result of prolonged storage or hypotonic shock. Despite the use of highly sensitive detection methods, and despite efficient cellular uptake of cisplatin, a complex could also not be detected in four human tumor cell lines, unless cells were permeabilized. In experiments in which cisplatin was incubated with PS-containing liposomes in the presence of an alternative cellular substrate, such as reduced glutathione, the relative affinity of cisplatin for PS was found to be low. Moreover, loading erythrocyte ghosts with physiological concentrations of glutathione strongly reduced cisplatin-PS complexation. Thus, in intact (tumor) cells a complex is not detected, most likely, because of the presence of higher affinity substrates. Though a transient complexation of cisplatin to PS cannot be excluded, our data suggest that cisplatin-PS does not play a direct role in the cellular (cyto)toxicity of cisplatin.

Keywords: Anti-cancer drug; Cancer; Cisplatin; Glutathione; Phosphatidylserine; Phospholipid

1. Introduction

Cisplatin, *cis*-diamminedichloroplatinum(II), is a widely used anti-tumor drug, and very effective in the treatment of testicular, ovarian, bladder, head and neck, esophageal, and small cell lung cancers [1]. The major limitation of cisplatin in the clinic is the development of cellular resistance. Cellular resistance against cisplatin is multifactorial and may involve an increase in thiols such as glutathione, enhanced DNA repair, enhanced tolerance to platinum-DNA adducts, as well as drug accumulation defects [2]. Besides that, cisplatin has a number of serious side effects, in particular nephrotoxicity and peripheral neuropathy [3,4]. Cisplatin enters the cell by a combination of passive diffusion and protein-mediated uptake [5], and then interacts with a variety of intracellular biomolecules [6,7]. In par-

Abbreviations: Cisplatin, *cis*-diamminedichloroplatinum(II); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PS, phosphatidylserine; TLC, thin-layer chromatography

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ticular sulfur- and nitrogen-donor atoms have a high affinity for cisplatin. Interaction with nuclear DNA, especially at the N7 position of guanine bases, appears to be responsible for the anti-tumor activity of cisplatin [8–11]. Apart from DNA and RNA, cisplatin interacts with proteins and peptides. Hydrolysis of cisplatin is the rate-limiting step in the reaction of cisplatin with most biomolecules [6,12]. Hydrolysis involves the replacement of the chloride atoms by water, and results in mono- and diaquated cisplatin. The formation of these highly reactive positively charged species of cisplatin is enhanced at the low-chloride concentrations found intracellularly.

Cisplatin was recently found to interact with negatively charged phospholipids [13,14] forming a coordination complex with phosphatidylserine (PS). Complexation of cisplatin to PS involves loss of both chloride atoms and coordination of platinum to the amine and carboxylate groups of the serine moiety [15]. PS is enriched at the plasma membrane, virtually confined to the inner, cytosolic, leaflet of the membrane where it represents about 20% of the phospholipids. PS plays an important role in cellular processes such as signal transduction [16], cell proliferation and apoptosis [17–19], the blood clotting cascade [20], and the biogenesis of mitochondria [21], and is necessary for the activity of a number of membrane enzymes such as the $(Na^+ + K^+)$-ATPase [22] and protein kinase C [16]. Our initial study describing coordination of cisplatin to PS was performed in vitro using pure lipid model membranes and isolated erythrocyte membranes [15]. A similar interaction in vivo would be expected to have important physiological implications, and might be responsible for some of the side effects of cisplatin. Therefore, we have now extended our study of cisplatin-PS interaction to intact human erythrocytes and well-established human tumor cell lines.

2. Materials and methods

2.1. Materials

Cisplatin, reduced and oxidized glutathione, 5′-GMP, and L-methionine were obtained from Sigma (St. Louis, MO, USA) and digitonin from BDH (Poole, UK). Phospholipids were from Avanti Polar Lipids (Birmingham, AL, USA). Cell culture media and solutions were from Gibco (Glasgow, UK), and culture plastics from Costar (Cambridge, MA, USA). All other chemicals and solvents were of analytical grade.

2.2. Cells

Cells were grown on plastic culture dishes in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin, HepG2 cells in MEM with 10% FCS [23], IGROV-1 [24] in RPMI supplemented with 25 mM HEPES and 10% FCS, Caco-2 (HTB-37, American Type Culture Collection, Rockville, ML, USA) in DMEM with 1% non-essential amino acids and 10% FCS [25], and MCF-7 in DMEM with 10% FCS. All cell lines were routinely checked and found to be free of mycoplasm infections. For experiments, cells were grown on 6 cm dishes and used at 60–90% confluence.

2.3. Cisplatin stocks and incubation buffers

Cisplatin was dissolved in the dark, in buffer or tissue culture medium to a final concentration of 2–8 mM. The solution was incubated for 1 h at 55°C (buffers) or 37°C (culture media) and intermittently vortexed or briefly sonicated (<30 s) using a bath sonicator until properly dissolved. Stock solutions of cisplatin in chloride-free buffers were incubated overnight at 37°C to ensure full equilibration of cisplatin. Cisplatin stocks were prepared in the following buffers and culture media: PIPES-sulfate buffer (10 mM PIPES-NaOH, 100 mM Na_2SO_4, 1 mM EGTA, pH 7.4), phosphate buffer (5 mM phosphate, pH 8.0), HBSS-HEPES buffer (Hanks' balanced salt solution with 10 mM HEPES-NaOH, pH 7.4), HEPES-acetate buffer (20 mM HEPES-KOH, 110 mM KOAc, 2 mM MgOAc, pH 7.2 [26]), complete growth medium (MEM-bicarbonate with 10% FCS), and MEM-HEPES (MEM without bicarbonate, with 10 mM HEPES-NaOH, pH 7.4).

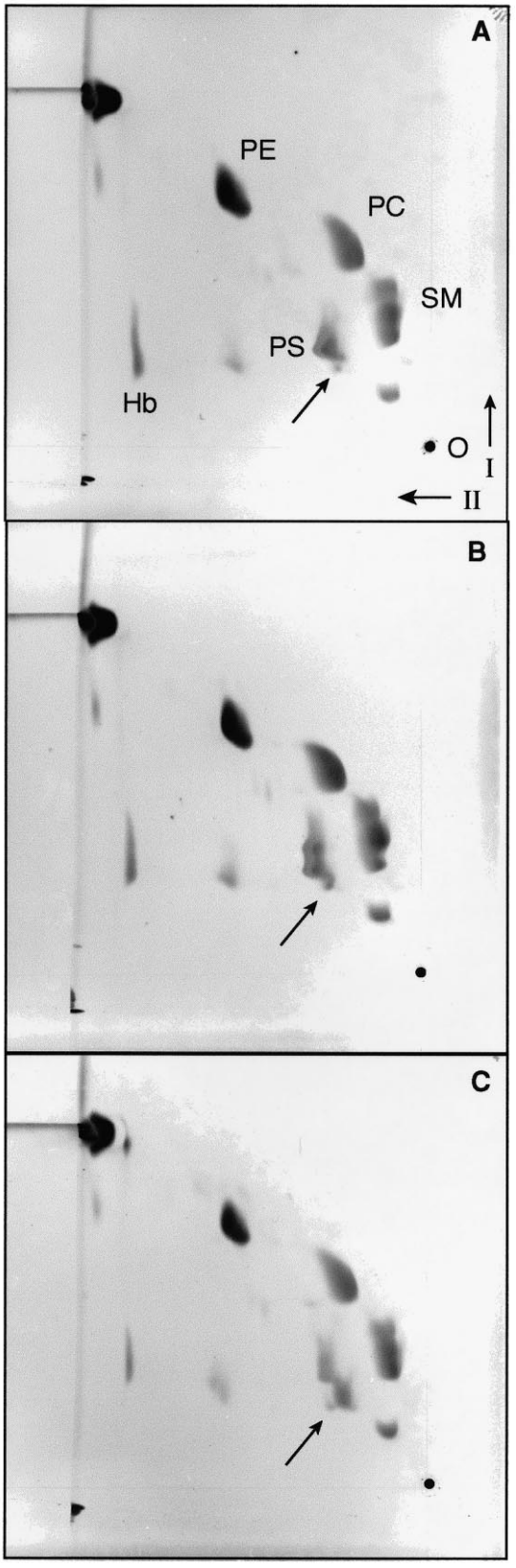
2.4. Interaction of cisplatin with erythrocyte membranes

Isolated human erythrocytes without buffy coat were obtained from the local blood bank in

150 mM NaCl, 50 mM glucose, 30 mM mannitol, and 1.2 mM adenine, and stored at 4°C. Erythrocytes were used either within 1 week after blood donation ('fresh'), or in the second-to-third month after blood donation ('aged'). Before the actual experiment, erythrocytes were washed 3 times in phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM phosphate, pH 7.4) and up to 3 times in the incubation buffer, using centrifugation and resuspension (10 min, $2300 \times g_{max}$), and cell number was determined using a Fuchs-Rosenthal counting chamber. During a typical incubation with cisplatin, the pelleted erythrocytes ('packed cells') were diluted 10 times and aliquots of 1 ml were analyzed. To determine the influence of membrane permeability and of cytosol on cisplatin-PS complexation, packed erythrocytes in PIPES-sulfate buffer were freeze-thawn 10 times, using dry ice in ethanol and a 37°C waterbath, and the released cytosol was removed by repeated centrifugation and resuspension in PIPES-sulfate buffer (10 min, $30\,000 \times g_{max}$ at 4°C). Identical amounts of control and pretreated erythrocytes were diluted 3 times in cisplatin-containing PIPES-sulfate buffer (final concentration of cisplatin of 5 mM) and incubated at 37°C. Cisplatin-PS complexation was also studied in erythrocyte membranes isolated after hypotonic lysis: unsealed ('open') ghost, pink sealed ghost, and white sealed ghost prepared according to Steck and Kant [27], as well as pink resealed ghost prepared according to Wood and Passow [28] (also see [29]). The ghosts were prepared from fresh erythrocytes and used the same day; resealing buffers did not contain Ca^{2+}. Open ghost preparations were freeze-thawn 3 times to maximize membrane permeability and to minimize resealing during incubation with cisplatin. Before use, ghost preparations were washed 3 times in the incubation buffer by centrifugation and resuspension (10 min, $30\,000 \times g_{max}$ at 4°C). The degree of membrane impermeability of ghosts sealed in low ionic strength buffer was determined by assaying the accessibility of intracellular glyceraldehyde-3-phosphate dehydrogenase to externally added substrate [27]. Alternatively, at the end of an incubation with cisplatin the accessibility of cisplatin-PS complex to externally added glutathione was determined. To this end, aliquots were incubated with 100 mM reduced glutathione at 37°C and the fraction of cisplatin-PS complex resisting glutathione treatment was followed in time. To determine the effects of intracellular glutathione on cisplatin-PS complexation, pink ghosts [28] were loaded with 10 mM reduced glutathione in PIPES-sulfate buffer. Extracellular glutathione was removed by repeated centrifugation and resuspension in PIPES-sulfate buffer. As a control, ghosts loaded with glutathione were freeze-thawn 3 times to release most of the intracellular glutathione before incubating with cisplatin.

2.5. Interaction of cisplatin with tumor cells

HepG2, IGROV-1, Caco-2, and MCF-7 cell monolayers on 6 cm culture dishes were washed 3 times with the appropriate incubation medium, and incubated for 1–18 h with 1–5 mM cisplatin in the same medium at 37°C in a waterbath or, if a bicarbonate-containing medium was used, in a CO_2 incubator. The following incubation media were used: HBSS-HEPES, DMEM with 10% FCS (complete growth medium), DMEM-HEPES, and acetate-HEPES; less than 10% of the cells detached during an overnight incubation with cisplatin. After incubation with cisplatin, the cells were rinsed twice with PBS or HBSS-HEPES, scraped, and the lipids extracted and analyzed by two-dimensional TLC using iodine staining (see later). To improve the sensitivity of lipid detection, cells were metabolically labeled with L-[3-^{3}H]- or L-[3-^{14}C]serine (specific activities of 962 GBq/mmol and 2.15 GBq/mmol, respectively; Amersham International, Buckinghamshire, UK) for 20–24 h at 37°C in MEM-bicarbonate, 10% FCS, using 2 ml 75 KBq/ml (^{3}H) or 20 KBq/ml (^{14}C) per dish. Dishes were rinsed 3 times before incubation with cisplatin. Radiolabeled lipids were detected by autoradiography or phosphor-imaging (see later). For experiments on semi-intact cells, cell monolayers were rinsed with acetate-HEPES buffer and cells permeabilized with digitonin, 100 μg/ml (added from a stock of 20 mg/ml DMSO) for 10 min at 20°C. Alternatively, cells were permeabilized by freeze-thawing, floating the culture dish on liquid nitrogen and thawing at 37°C. In both cases, more than 95% of the cells stained blue in a trypan blue exclusion assay (0.2% trypan blue, 2 min at 20°C) indicating that more than 95% of the cells had been permeabilized. Dishes were rinsed 3 times at 37°C before incubation with cisplatin.



2.6. Interaction of cisplatin with phospholipid dispersions

In competition experiments, cisplatin-PS complexation was determined in DOPS/DOPC (1:1, mol/mol) liposomes in the presence of increasing concentrations of alternative substrates for cisplatin. Multilamellar vesicles (lipid concentration 2 mM) were prepared by hydration of a dry lipid film in PIPES-sulfate buffer containing either 1 mM or 5 mM cisplatin, and 0, 1, 5, or 10 mM of reduced or oxidized glutathione, L-methionine, or 5′-GMP. The liposomes were freeze-thawn 10 times and the degree of cisplatin-PS complexation was determined after an overnight incubation at 37°C.

2.7. Lipid analysis

Before lipid extraction, erythrocytes were lysed in distilled water to release the hemoglobin, and the membranes isolated by centrifugation (10 min, $30\,000 \times g_{max}$ at 4°C) and resuspended in saline. Lipids were extracted according to Bligh and Dyer [30]. All aqueous solutions were acidified to 10 mM acetic acid, KCl was added during phase separation (0.44% w/v). Lipids were separated by two-dimensional TLC on HPTLC plates (Si60, Merck, Darmstadt, Germany) using in the first dimension (I in Fig. 1), chloroform/methanol/ammonia, 25%/water (90:54:5.5:5.5, v/v) and in the second dimension (II in Fig. 1), chloroform/methanol/acetic acid/water (90:40:12:2, v/v). Lipids were detected by sulfuric acid or iodine staining, identified by comigration with known standards, and phospholipids quantified by

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Fig. 1. Interaction of cisplatin with PS in the human erythrocyte membrane analyzed by two-dimensional TLC and staining with sulfuric acid. Fresh erythrocytes were incubated with cisplatin (5 mM in PIPES-sulfate buffer, pH 7.4, overnight at 37°C) without pretreatment (A), after being freeze-thawn to induce membrane defects and release cytosol (B), or after being freeze-thawn and depleted of cytosol by repeated centrifugation and resuspension (C). Cisplatin-PS complex (arrow) was only barely detectable in intact erythrocytes (compare A with B,C). The amount of cisplatin-PS complexation was determined by phosphate determination after two-dimensional TLC (see Table 1). I and II, first and second dimension of TLC; O, origin; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Hb, hemoglobin.

phosphate determination [31]. Cisplatin-PS has a lower R_f value than PS [15], and stains in iodine vapor brownish instead of yellow. When liposomes were prepared from a total lipid extract of human erythrocytes or tumor cell lines and treated with cisplatin, the cisplatin-PS complex exactly colocalized with cisplatin-DOPS after two-dimensional TLC (not shown). Tritiated lipid was detected by autoradiography after dipping the TLC plates in 0.4% PPO dissolved in 2-methylnaphthalene supplemented with 10% xylene; preflashed Kodak X-Omat S films were exposed for 2–4 days at −80°C. ^{14}C-Labeled lipids were detected by phosphor image analysis and quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

2.8. Platinum determination

After incubation with cisplatin, erythrocytes and erythrocyte ghost were washed 6 times in PIPES-sulfate buffer using centrifugation (1 min, $1000 \times g_{max}$) and resuspension. The intracellular distribution of cisplatin in erythrocytes was determined by lysing the erythrocytes in 5 mM phosphate buffer (pH 8.0), and pelleting the membranes (10 min, $30\,000 \times g_{max}$ at 4°C). Lysis and centrifugation were repeated, and the Pt content of membrane pellet and the pooled supernatant was determined. Prior to Pt determination, tumor cell monolayers on plastic were washed 6 times in PBS and scraped using a rubber policeman. Samples were digested before Pt analysis: aliquots were transferred to screw-capped Eppendorf cups and dried in a speedvac centrifuge (Swart, Hicksville, NY), after which 200 μl 65% HNO_3 was added and the tightly sealed cups incubated for 2 h at 75°C. The Pt content was determined after dilution in water by flameless atomic absorption spectrometry using a SpectrAA-400 Zeeman spectrometer equipped with an autosampler (Varian, Palo Alto, CA, USA). The method of van Warmerdam et al. [32] was used, with K_2PtCl_6 (Sigma) as a standard. In short, a total volume of 35 μl was introduced into the graphite tube consisting of 5 μl of modifier solution (0.5% Triton X-100 in water, v/v) and 30 μl sample or Pt standard containing 1–8 ng Pt. After drying at 85–120°C for 1 min, and charring at 1400°C for 8 s, Pt absorbance was measured at 2700°C for 3.3 s.

Table 1
Interaction of cisplatin with PS in the human erythrocyte membrane[a]

Sample	Incubation buffer	Cisplatin (mM)	Cisplatin-PS (% of total PS)
Ery, intact	PIPES-sulfate	2	1.1 ± 1.7[b]
Ery, intact	PIPES-sulfate	5	4.7 ± 2.3[b]
Ery, freeze-thawn	PIPES-sulfate	5	10.7 ± 0.4[b]
Ery, freeze-thawn, washed	PIPES-sulfate	5	52.3 ± 0.9[b]
Open ghost	PIPES-sulfate	2	24.8 ± 2.2[c]
Sealed pink ghost[d]	PIPES-sulfate	2	23.6 ± 7.9[b]
Sealed white ghost	PIPES-sulfate	2	21.3 ± 0.7[c]
Open ghost	PIPES-sulfate	5	46.6
Sealed white ghost	PIPES-sulfate	5	54.9 ± 0.6[c]
Open ghost	5 mM phosphate	5	82.4
Sealed pink ghost[e]	5 mM phosphate	5	75.8

[a]Human erythrocyte and erythrocyte membrane preparations were incubated overnight at 37°C with cisplatin, and the amount of cisplatin-PS complexation was determined after two-dimensional TLC as described in Section 2 ([b]average ± S.D. with $n = 3$–6; [c]average and range with $n = 2$). Erythrocytes were used within 1 week after blood donation ('intact'), freeze-thawn to induce membrane defects and release cytosol, or freeze-thawn and cytosol washed away by repeated centrifugation and resuspension. Open erythrocyte ghost and sealed white ghost were prepared according to Steck and Kant [27], and sealed pink ghost according to [d]Wood and Passow [28] or [e]Steck and Kant [27]. Ghosts were resuspended and incubated with cisplatin in PIPES-sulfate buffer or in a low-ionic strength phosphate buffer. The more efficient cisplatin-PS complexation in 5 mM phosphate buffer as compared to that in PIPES-sulfate buffer, is due to the lower ionic strength of the former buffer (see [6]). Over 85% of the ghosts sealed in phosphate buffer were intact at the start of the incubation with cisplatin, while in general about 60% of the pink ghost prepared according to Wood and Passow [28] are properly sealed [44]. For details see text.

3. Results and discussion

3.1. Interaction of cisplatin with intact human erythrocytes

In a previous paper we described that a complex between cisplatin and phosphatidylserine forms in PS-containing model membranes as well as in biomembranes [15]. However, the experiments on biomembranes were performed using open erythrocyte ghost, leaky erythrocyte membranes which almost completely lack any cytosolic contents. Therefore, we extended our original study and compared the interaction of cisplatin with PS in erythrocyte membrane preparations with that in intact erythrocytes. Because it is the positively charged aquated species of cisplatin which reacts with PS [15], experiments were performed in a buffer lacking chloride, i.e. with aquated cisplatin, which is efficiently taken up by intact (tumor) cells [33,34]. Fig. 1 and Table 1 show the results obtained upon incubation of human erythrocytes and various erythrocyte membrane preparations with 2 or 5 mM cisplatin, overnight at 37°C in a neutral pH buffer lacking chloride. A complex between cisplatin and PS was virtually absent if freshly isolated human erythrocytes were used in the experiment, in many cases the amount of complex formed was even too low to be detected by iodine staining indicating that less than 1% of cellular PS had reacted with cisplatin (intact erythrocytes incubated with 2 mM cisplatin, see Table 1). In contrast, if outdated erythrocyte concentrate was obtained from the blood bank and used as starting material, a significant part of cellular PS, about 10%, reacted with cisplatin (data not shown). Upon prolonged storage, erythrocytes release hemoglobin indicative of a deterioration of the barrier properties of the plasma membrane. Therefore, the fact that cisplatin-PS complexation was facilitated in aged erythrocyte preparations might be related to an increased permeability of the erythrocyte membrane. To examine this possibility, membrane damage was induced in freshly isolated erythrocytes by freeze-thawing. Indeed, when freshly isolated packed erythrocytes were freeze-thawn and subsequently incubated with cisplatin, again about 10% of cellular PS was converted into the cisplatin-PS complex (Fig. 1B, Table 1). Prolonged storage and freeze-thawing not only result in an increase in membrane permeability but also in release of cytosolic components. To answer the question which of these events was responsible for the increase in cisplatin-PS complexation, packed erythrocytes were freeze-thawn and the released cytosol was removed. Over 50% of cellular PS complexed to cisplatin (Fig. 1C, Table 1), strongly suggesting that in the intact erythrocyte, cytosol competes with PS for cisplatin.

Almost pure erythrocyte membranes can be obtained after hypotonic lysis using well-established methods [27,28], the so-called open erythrocyte ghosts contain large holes which reseal in a suitable buffer; as long as Ca^{2+} is absent from the resealing buffer, the asymmetric phospholipid distribution is not affected by these procedures [35,36]. The intactness of sealed ghost preparations can be determined by assaying the latency of a membrane-bound intracellular enzyme, glyceraldehyde-3-phosphate dehydrogenase ([27] and references therein). This enzyme latency assay, which can only be applied at low ionic strength, indicated that over 85% of the pink ghost sealed in a low ionic strength buffer was intact at the start of the cisplatin incubation. Attempts to monitor the intactness of sealed ghost during incubation with cisplatin failed; enzyme latency or leakage of residual hemoglobin could not be reliably measured because cisplatin strongly reduced the activity of glyceraldehyde-3-phosphate dehydrogenase and shifted the absorption spectrum of hemoglobin (data not shown).

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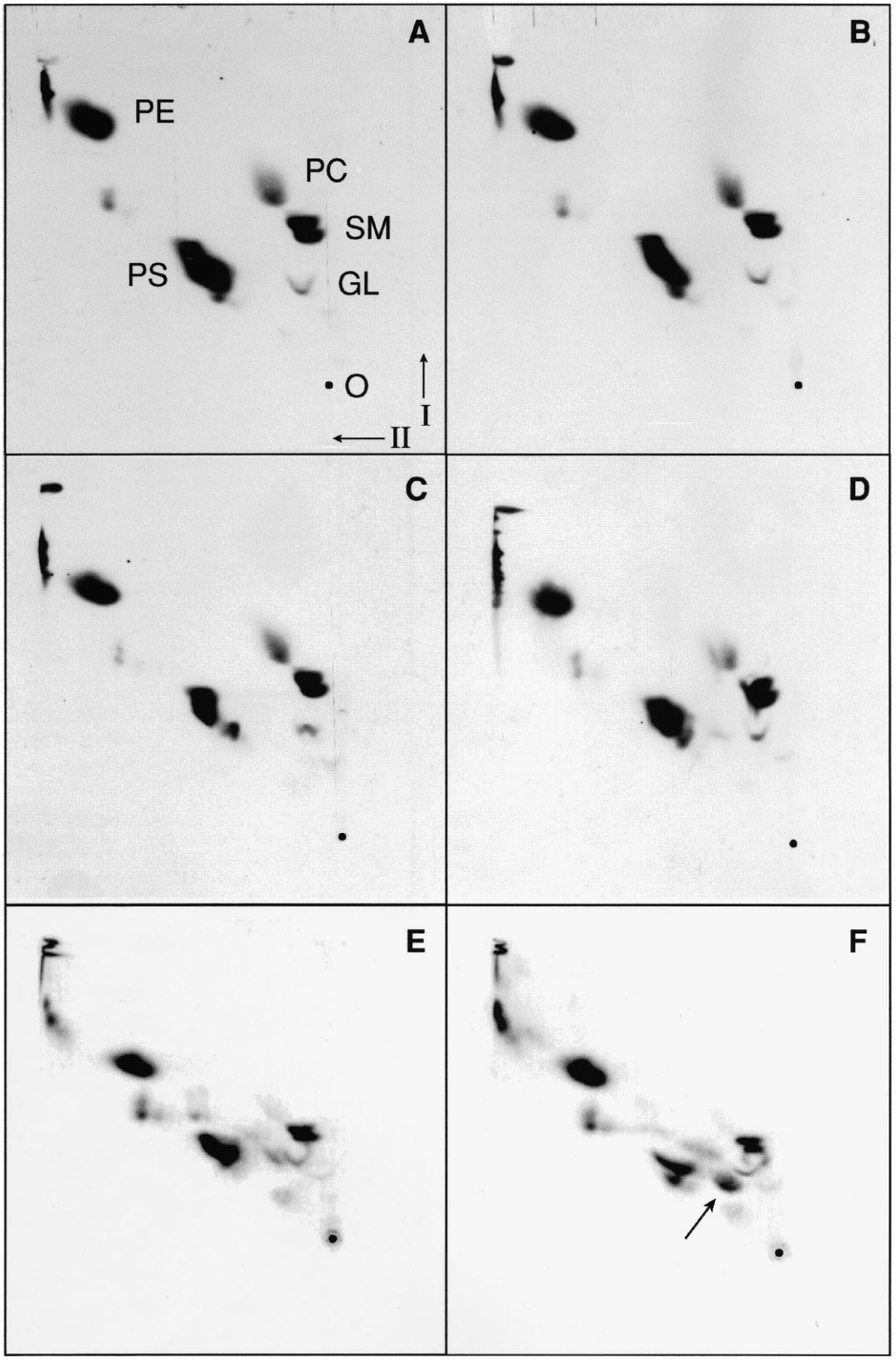
Fig. 2. Interaction of cisplatin with PS in human tumor cell lines. IGROV-1 cell monolayers metabolically labeled overnight with L-3-[3-^{3}H]serine (A–D) or L-3-[3-^{14}C]serine (E,F) were incubated at 37°C with 5 mM cisplatin in HBSS-HEPES (A–D) or acetate-HEPES (E,F). In A–D, the lipid composition was followed in time (0, 1, 3, and 18 h incubation with cisplatin, respectively). In F, cells were permeabilized by freeze-thawing, prior to incubation with cisplatin for 18 h (E, control, intact cells incubated with cisplatin for 18 h). Lipids were separated by two-dimensional TLC and detected by autoradiography (A–D) or phosphor-imaging (E,F) as described in Section 2. An additional spot, the cisplatin-PS complex (arrow in F), was only detected in permeabilized cells; this spot was not observed in control (permeabilized) cells incubated in the absence of cisplatin (not shown). Identical results were obtained in HepG2, Caco-2, and MCF-7 cells (not shown), for details see text. GL, glycolipid (other abbreviations are explained in the legend of Fig. 1).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However, it is reasonable to assume that a significant fraction of the sealed ghosts remained intact. Thus, the fact that in open and sealed ghosts cisplatin reacted with PS to an almost identical extent (Table 1) indicates that membrane passage of cisplatin is not a limiting factor, and strongly suggests that the absence of cisplatin-PS complex in intact erythrocytes is not due to a failure of cisplatin to pass the plasma membrane, but to the presence of cytosol. Cytosolic components apparently prevent a stable association between intracellular cisplatin and the PS present in the inner leaflet of the erythrocyte membrane. This conclusion is supported by measurements of cisplatin uptake and binding. After an overnight incubation with 2 mM cisplatin in PIPES-sulfate buffer, intact erythrocytes, open, and resealed pink ghost [28] contained 11.8, 0.8, and 1.3 nmol cisplatin/nmol PS, respectively (data not shown). Thus, intact erythrocytes contained 9–15 times more cisplatin than open or sealed ghost, yet were virtually devoid of cisplatin-PS complex. Moreover, freeze-thawing of these erythrocytes released $79 \pm 3\%$ ($n = 2$) of the cell-associated cisplatin indicating that in intact erythrocytes cisplatin is predominantly present in the cytosol (data not shown; in agreement with [37]).

3.2. Interaction of cisplatin with human tumor cell lines

Next we examined the interaction of cisplatin with PS in a number of human tumor cell lines, hepatoma HepG2, colon carcinoma Caco-2, breast MCF-7, and ovarian IGROV-1. After two-dimensional TLC and iodine staining, cisplatin-PS complexation was not detected in any of the tumor cell lines (results not shown). To check whether the inability to detect cisplatin-PS was due to the insensitivity of the detection method, cells were metabolically labeled with L-[3-^{3}H]- or L-[3-^{14}C]serine overnight and the resulting radiolabeled lipids were detected after two-dimensional TLC by autoradiography and phosphor-imaging respectively. Phosphatidylserine was efficiently radiolabeled in all four cell lines; Fig. 2A shows a typical result for IGROV-1 cells labeled overnight with [3-^{3}H]serine. After radiolabeling, the cells were incubated with cisplatin and the cisplatin-PS complexation was followed in time at 37°C. Fig. 2B–D show the results obtained for IGROV-1 cells incubated for up to 18 h with 5 mM cisplatin in HBSS-HEPES: cisplatin-PS complexation was not detected. Identical results were obtained using concentrations of cisplatin of 0.1–5 mM, and incubation media such as DMEM with 10% FCS (complete growth medium), DMEM-HEPES, or an acetate-HEPES buffer lacking chloride [26]. Cisplatin-PS complexation was not detected in any of the cell lines as long as cells remained intact; however, cisplatin-PS was detected in cells permeabilized by freeze-thawing or digitonin treatment. A typical example is shown in Fig. 2F, IGROV-1 cells labeled with [^{14}C]serine, permeabilized by freeze-thawing, and incubated overnight with 5 mM cisplatin in acetate-HEPES buffer (cf. Fig. 2E, intact cells treated with cisplatin): 25.9% of PS is complexed to cisplatin. Data obtained on the cellular uptake of cisplatin in intact IGROV-1 cells indicate that during an overnight incubation with 5 mM cisplatin 33.5 ± 0.1 nmol cisplatin is taken up per nmol PS ($n = 2$; data not shown). Thus, in analogy to the results obtained on cisplatin-PS complexation in the erythrocyte membrane, the failure to detect cisplatin-PS complex in intact cells is not due to inefficient uptake of cisplatin but most likely to competing substrates being present in the cytosol.

3.3. Interaction of cisplatin with PS and alternative cellular substrates

The cytosol contains a number of nucleophilic ligands which are potentially competing with PS for cisplatin. The nucleophilicities of sulfur atoms are particularly high, and eukaryotic cells contain relatively high concentrations of thiols, especially glutathione. Reduced glutathione, present in the cytosol at concentrations between 0.5 and 10 mM, exists in equilibrium with an about 500-fold lower concentration of oxidized glutathione [38]. Other potential substrates for cisplatin are methionine-containing proteins and peptides, and especially the guanine bases in RNA and DNA [6,7]. In order to determine the relative affinity of cisplatin for PS, PS-containing liposomes were incubated with cisplatin in the presence of increasing concentrations of the alternative cellular substrates, reduced and oxidized glutathione, L-methionine, and 5′-GMP. The extent to which cisplatin-PS complexation still occurred was determined after an overnight incubation at 37°C (Fig. 3). In

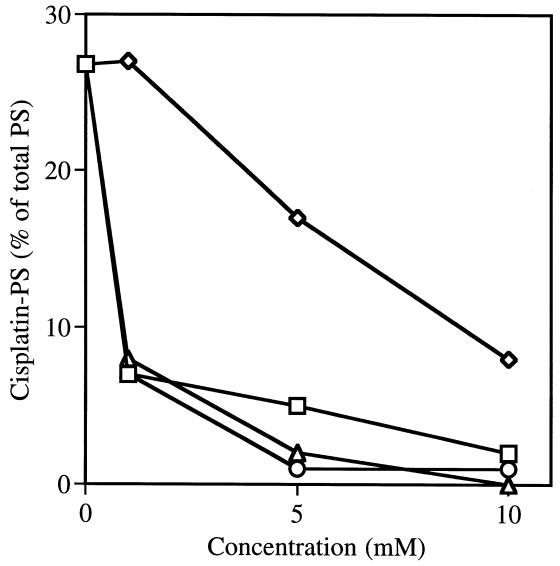

Fig. 3. Cisplatin-PS complexation in the presence of alternative substrates for cisplatin. Multilamellar DOPS/DOPC (1:1, mol/mol, 2 mM lipid) vesicles in PIPES-sulfate buffer were incubated overnight at 37°C with 1 mM cisplatin and up to 10 mM of an alternative substrate (□, reduced glutathione; ◇, oxidized glutathione; ○, L-methionine; △, 5′-GMP), and the degree of cisplatin-PS complexation was determined after two-dimensional TLC (see Section 2). An equimolar concentration of the alternative substrate relative to PS strongly reduced cisplatin-PS complexation; the same result was obtained using 5 mM instead of 1 mM cisplatin (not shown).

most cases, an equimolar concentration of the alternative substrate relative to PS (1 mM) already reduced cisplatin-PS complexation by a factor 3–4; only if oxidized glutathione was used, a molar excess was required to inhibit cisplatin-PS complexation.

Simple calculations show that reduced glutathione may very well prevent cisplatin-PS complexation. Human erythrocytes have an average volume of 90 μm^3 [39], and packed human erythrocytes contain per ml, about 5.10^9 cells, 2100 nmol glutathione [40], and 510 nmol PS (determined by lipid analysis). Thus, the cytosolic glutathione concentration is close to 5 mM and the molar ratio of glutathione to PS is 4. In the case of nucleated cells, accurate morphometric data are only available for a limited number of cell lines, such as dog kidney MDCK-I cells [41]. These cells have a volume of 1500 μm^3 and contain 2.5 nmol PS per 10^6 cells [42], while the glutathione concentration in (human) kidney is about 3 mM (see [43]); from these data a glutathione-to-PS molar ratio of 1.8 can be calculated. These calculations show that in most cells reduced glutathione will be present in 2–4-fold molar excess relative to PS, which, based on the results of the competition experiments (Fig. 3), would be expected to inhibit cisplatin-PS complexation almost completely.

To obtain more direct evidence for the role of glutathione in preventing cisplatin-PS complexation, erythrocyte ghosts were sealed in the presence and absence of glutathione, washed, and incubated with cisplatin (Table 2). When ghosts were sealed in buffer and incubated overnight with 2 mM cisplatin, 31% of cellular PS was converted into cisplatin-PS. Com-

Table 2
Influence of glutathione on the interaction of cisplatin with PS in human erythrocyte membranes[a]

Pink ghost loaded with	Cisplatin (mM)	Cisplatin-PS (% of total PS)
Buffer	2	31.0
Glutathione	2	17.3
Buffer (freeze-thawn)	2	32.9
Glutathione (freeze-thawn)	2	38.6
Buffer[b]	2	
Post-incubation with 100 mM glutathione at 37°C (min)		
0		31.0
30		12.7
120		15.8

[a]Ghosts were incubated overnight at 37°C with cisplatin in PIPES-sulfate buffer and the amount of cisplatin-PS complex was determined. Pink ghosts [28] were sealed in PIPES-sulfate buffer, or in PIPES-sulfate buffer containing 10 mM reduced glutathione; extracellular glutathione was removed by repeated centrifugation and resuspension. Ghosts were freeze-thawn to induce membrane permeabilization and, in the case of ghost loaded with glutathione, release intracellular glutathione.

[b]The intactness of the ghosts (loaded with buffer) after an overnight incubation with 2 mM cisplatin was determined by assaying for the accessibility of the cisplatin-PS complex to externally added glutathione (100 mM, 37°C). The results of a typical experiment are shown. For details, see text.

plexation was strongly reduced if ghosts were loaded with 10 mM glutathione, only 17% complex was formed. To determine whether cisplatin-PS complexation was inhibited by intracellular glutathione or by glutathione possibly released from the ghosts, part of the samples were freeze-thawn prior to incubation with cisplatin. Freeze-thawing resulted in a complete loss of the inhibitory effect of glutathione on cisplatin-PS complexation, indicating that in our experimental setup released glutathione was diluted to such an extent that it did not interfere with cisplatin-PS complexation. The small increase in complexation observed in freeze-thawn versus intact ghosts is likely due to an increase in membrane permeability induced by freeze-thawing. Together these data clearly show that glutathione, at physiological concentrations, strongly inhibits cisplatin-PS complexation. Based on the competition experiments (Fig. 3), 10 mM glutathione would be expected to reduce cisplatin-PS complexation by at least 90%. The reason why loading ghosts with 10 mM glutathione reduced complexation by only 43%, is the rather low efficiency of ghost resealing, and the fact that glutathione will only inhibit complexation in sealed ghosts. In general about 60% of the ghosts reseal properly during ghost preparation [44], and part of these sealed ghosts may become leaky during overnight incubation with cisplatin. Because the assays normally used to monitor membrane permeability of erythrocyte ghost failed in the presence of cisplatin (see Section 3.1), a novel method was developed to determine the fraction of intact ghosts at the end of the cisplatin incubation. The method makes use of a post-incubation with a high concentration of glutathione and is based on the fact that glutathione is capable of reversing cisplatin-PS complexation but does not pass intact membranes. Thus, if glutathione is added to ghosts treated with cisplatin, only the cisplatin-PS complex present in leaky ghosts will be lost; an incubation with 100 mM glutathione for 1 h at 37°C is sufficient to remove all accessible cisplatin-PS [15]. When sealed ghosts were treated with 2 mM cisplatin overnight, and subsequently incubated with 100 mM glutathione, cisplatin-PS complexation was reduced by 50–60%, indicating that 40–50% of cisplatin-PS was protected inside properly sealed ghosts. Thus, only 40–50% of cisplatin-PS complexation can be prevented by loading ghosts with glutathione, and the observed reduction in complexation by 43% shows that 10 mM glutathione is capable of nearly completely preventing cisplatin-PS complexation in intact erythrocyte ghosts.

3.4. Role of cisplatin-PS complexation in cisplatin-induced (cyto)toxicity

Cisplatin is generally used in the clinic at a dose of 100 mg/m^2 given intravenously, and the peak concentration in the blood measured directly after i.v. injection is about 30–35 μM [45]. Although cisplatin efficiently forms a stable coordination complex with phosphatidylserine in vitro, both in pure lipid model systems and purified biomembrane systems, we were unable to detect the cisplatin-PS complex in intact erythrocytes or tumor cells, even at cisplatin concentrations much higher than the clinically achievable concentration. Therefore we consider it unlikely that cisplatin-PS complexation plays a direct role in the cellular (cyto)toxicity of cisplatin.

In view of the molar excess of glutathione relative to PS, and the efficiency with which glutathione reduces cisplatin-PS complexation, glutathione is probably one of the key factors responsible for the absence of cisplatin-PS complex in intact cells treated with cisplatin. Glutathione is both capable of preventing cisplatin-PS complexation by competing with PS for cisplatin binding, as well as capable of reversing cisplatin-PS complexation [15]. Thus, either cisplatin-PS complexation does not occur because cisplatin reacts preferentially with glutathione, or cisplatin-PS complexation does occur but is followed by a rapid transfer of cisplatin to other cellular substrates, such as glutathione. The latter possibility is not excluded because while entering the cell, cisplatin passes the PS present in the cytosolic leaflet of the plasma membrane before contacting alternative substrates in the cytosol.

An intriguing question remains as to how cisplatin reaches its ultimate target, nuclear DNA, despite the presence of scavengers for cisplatin, such as glutathione, being present in the cytosol. Although for some of the cytosolic nucleophiles binding to cisplatin appears to be reversible allowing transfer of the platinum compound to DNA, in vitro experiments also indicated that such a transfer is extremely unlikely in the case of glutathione [46–48].

The positively charged aquated species of cisplatin are most likely responsible for DNA adduct formation and the induction of apoptosis. The fact that the cytosolic face of the plasma membrane and many intracellular membranes contain high concentrations of negatively charged phospholipids, in particular phosphatidylserine, may facilitate aquation and thus activate cisplatin. Our future research will focus on the potential role of membrane lipids, in the cellular uptake of cisplatin, and in the intracellular activation of this highly potent anti-tumor drug.

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