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The chirality selectivity in the uptake of platinum (II) complexes with 1,2-cyclohexanediamine isomers as carrier ligand by human erythrocytes

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The uptake kinetics of cisplatin analogs of 1,2-cyclohexanediamine(dach) isomers with various leaving groups, by human erythrocytes in plasma isotonic buffer, were studied. The experimental results showed that the uptake rate constants (*k* values) decrease with the change of leaving group in the sequence: chloride (Cl) > squaric acid (SA) > oxalate (OX) > demethylcantharic acid (DA), with the same dach isomer as carrier group. It is noteworthy that for the platinum (II) complexes with the same leaving group, the *k* values always reduce as: 1*R*, 2*R*-dach > 1*R*, 2*S*-dach > 1*S*, 2*S*-dach. This result reflects the chirality selectivity. No differences in reactivity to protein thiols and effects on membrane permeability were found for the *R*,*R*-, *R*,*S*-, *S*,*S*-isomeric complexes. It is proposed that the chirality selectivity in uptake is due to the recognition of the chirality of the platinum complexes by the erythrocyte membrane. The interactions between the chiral platinum complexes and the head groups of the membrane phospholipid molecules are probably involved.

Keywords: chirality, 1,2-cyclohexanediamine, human erythrocyte, platinum complexes, uptake

Introduction

Among the cisplatin analogs studied, those with 1,2-diaminocyclohexane(dach) as carrier ligand have been noted as second-generation anticancer platinum drugs because of their higher activity andlower toxicity. There are three isomers of dach: *R*,*R*-, *R*,*S*- and *S*,*S*-dach. It was noted that the Pt(II) complexes of these isomers have different antitumor activity, and that those of *R*,*R*-dach are the most active (Speer *et al*. 1978, Kidani & Noji 1991). The difference in activity has been suggested to be the result of these isomers interacting with DNA in different ways (Inagaki & Kidani 1986, Inagaki *et al*. 1990).

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In recent years, the uptake of cisplatin and its analogs has received increased attention, since the activity, toxicology and resistance of these complexes have been shown to be closely related to their uptake and intracellular accumulation. Reile *et al*. proposed that the penetration of the Pt(II) complexes into the tumor cell seemed to be the critical factor causing the different activity of the stereoisomeric diaqua[1,2-bis(4-fluorophenyl) ethylenediamine]platinum(II) sulfate (Reile *et al*. 1992) because they found that the uptake for racemic mixture of higher anticancer activity was faster than the *meso*-isomer of lower anticancer activity. For this reason, we cannot exclude the contribution of the uptake of these isomers to their different bioactivities. In the present work, we attempt to clarify the relation between the uptake and chirality of these complexes.

Materials and methods

$PtCl_2$ (*R*,*R*-, *R*,*S*-, *S*,*S*-dach) and Pt(OX) (*R*,*R*-, *R*,*S*-, *S*,*S*-dach) were generously donated by Professor Kidani (Nagoya City University, Nagoya, Japan); Pt(SA) (*R*,*R*-, *R*,*S*-, *S*,*S*-dach) and Pt(DA) (*R*,*R*-, *R*,*S*-, *S*,*S*-dach) were synthesized in our laboratory (Zou 1995). The chemical structures of these platinum(II) complexes are shown in Figure 1. The *N*-doxyl stearic acid spin label containing ^{14}N nitroxide group at carbon 5 position (5DS), the spin label 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (MSL) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co., USA. Fresh human blood was obtained from Beijing Blood Central Bank. The plasma isotonic buffer (PIB) (pH 7.4) was composed of 140 mmol l^{-1} NaCl, 5 mmol l^{-1} KC1, 0.1 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} Na_2HPO_4 and 5 mmol l^{-1} glucose.

Transport rate of platinum complexes

The red blood cells were suspended in PIB at a final hematocrit of 10% and incubated at 37°C with each of the platinum complexes (initial concentration, C_0 = 16 μg Pt ml^{-1}). The trans-membrane transportation was terminated at various incubation intervals. The cells were centrifuged at 4000 rpm for 10 min. After discarding the supernatant, the cell pellet was washed thoroughly with PIB. The cells were lysed with deionized water and the cytosol was obtained by resedimenting the membrane pellet at 10 000 rpm for 15 min. The uptake rate constants of each platinum complex, and their concentration dependence, were determined by measuring the platinum concentration in the cytosol at various times (C_t, μg ml^{-1}) by AAS (Zhang *et al*. 1993). Each of the experiments was repeated two or three times.

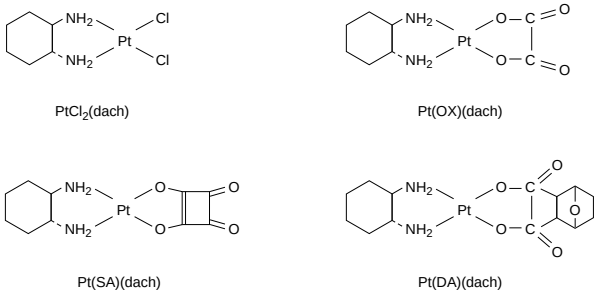

Figure 1. Chemical structures of 1,2-cyclohexanediamine platinum(II)_complexes.

Preparation of human erythrocyte membrane

The plasma membrane was prepared according to Dodge's method (Dodge *et al*. 1963). 50 ml of red blood cells (2×10^8 ml^{-1}) were broken in 2.0 l PBS buffer (5.0 mmol l^{-1}, pH 8.0) at 4°C for 2 h, after which the membrane was washed three times with PBS at 4°C. The separated erythrocyte membrane was collected and stored in PBS buffer (50 mmol l^{-1}, pH 7.4) at –20°C for use. The concentration of membrane proteins was determined by Lowry's method.

Determination of residual sulfhydryl on membrane protein

The membrane was incubated with each of the platinum complexes separately for 5 h at 37°C and centrifuged at 10 000 rpm for 10 min. The membrane was washed with PBS, and then allowed to react with 0.01 mol l^{-1} DTNB for 15 min at room temperature. The mixture was centrifuged and the supernatant was withdrawn. The concentration of residual thiols on membrane protein was determined spectrophotometrically at 412 nm with the molar absorption coefficient A = 13 600 l mol·cm^{-1} (Ellman 1959).

ESR measurement of MSL-spin labeled membrane

The modified Sandberg's method (Sandberg *et al*. 1969) was used to prepare the samples. The membrane suspension (3.0 mg ml^{-1}) was labeled with MSL at a molar ratio of 100:1 by standing in the darkness for 16 h at 4°C. The solutions of the platinum complexes were then added to a final concentration of 0.2 mmol l^{-1} (in the control, PBS was used). After incubation (0.5 h at 37°C), the samples were washed by centrifugation until no ESR signal of free spin label was found in the supernatant. The ESR spectra

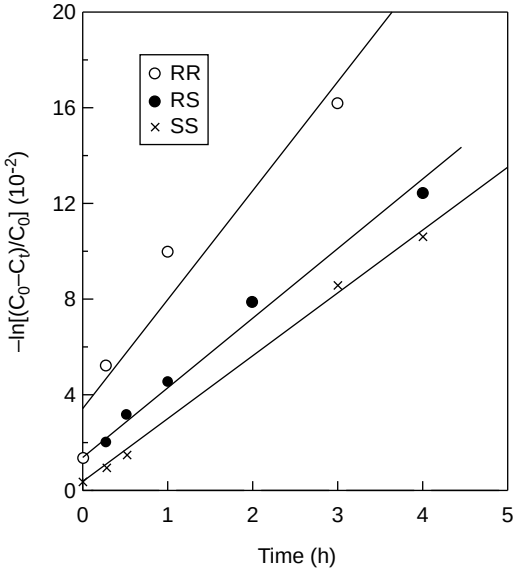

Figure 2. Curves of –ln[C0 – Ct)/C0] vs. incubation time at 37°C for PtCl2(dach) in PIB, pH 7.4.

were recorded using a Bruker ESP-300 instrument operated at scan range 10 mT, radiation power 10 mW, modulation amplitude 0.20 mT and field modulation 100 kHz.

Study of the effect on membrane permeability

The membrane suspensions (2.8 mg ml^{-1}) were incubated with the platinum complexes (a final concentration of 0.2 mmol l^{-1}) at 37°C for 0.5 h; the membrane was then labeled with TEMPO at 25°C for 10 min. The membrane was centrifuged at 3000 rpm for 3 min and the supernatant was discarded. While a freshly prepared ascorbate solution was added with 10-fold excess molar concentration into the membrane suspension at 0°C, the measurement was started immediately. The ESR spectra were recorded every 2 min, with the scan range 20 mT, radiation power 2 mW, modulation amplitude 0.16 mT, and field modulation 100 kHz.

Results

The uptake rate constants of platinum complexes and their concentration dependence

As shown in Figures 2 to 5, the trans-membrane transport of all the platinum complexes studied follows a first-order kinetic process. The first-order uptake constants of these platinum complexes are summarized in Table 1. It is evident that for the same leaving group, the uptake rates of 1*R*, 2*R*-dach complexes are always the highest and 1*S*, 2*S*-dach

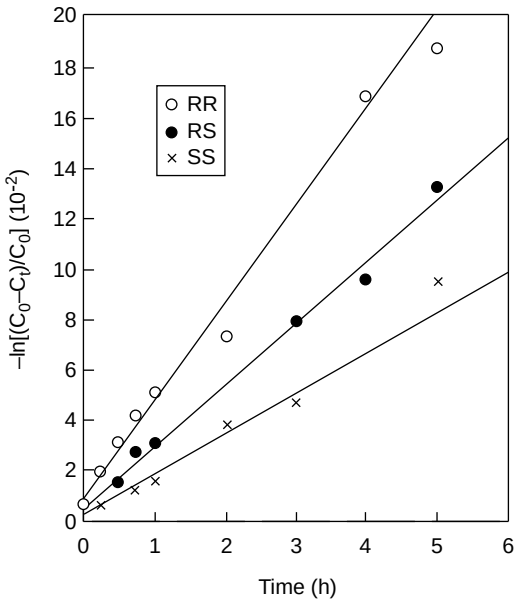

Figure 4. Curves of $-\ln[(C_0 - C_t)/C_0]$ vs. incubation time at 37°C for Pt(SA)(dach) in PIB, pH 7.4.

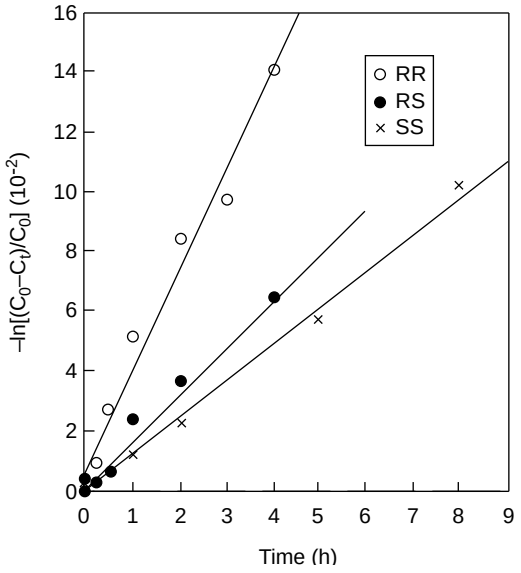

Figure 3. Curves of $-\ln[C_0 - C_t)/C_0]$ vs. incubation time at 37°C for Pt(OX)(dach) in PIB, pH 7.4.

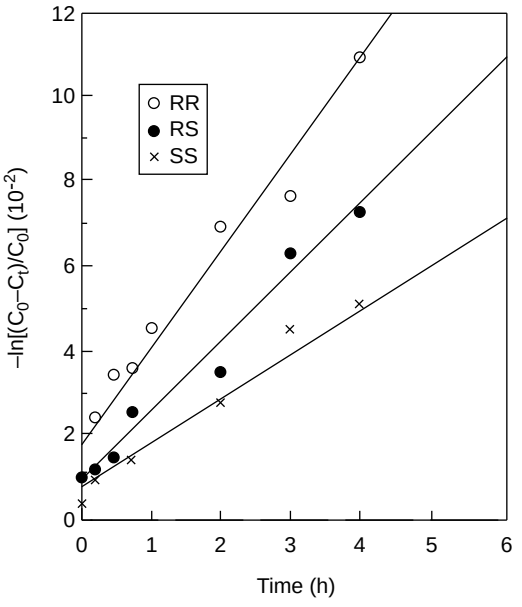

Figure 5. Curves of $-\ln[(C_0 - C_t)/C_0]$ vs. incubation time at 37°C for Pt(DA)(dach) in PIB, pH 7.4.

Table 1. The first-order uptake constants of the platinum complexes (37°C)

Sample	k(h^{-1})	Sample	k(h^{-1})	Sample	k(h^{-1})	Sample	k(h^{-1})
$PtCl_2$(*R*,*R*)	0.0455	Pt(SA)(*R*,*R*)	0.0363	Pt(OX)(*R*,*R*)	0.0330	Pt(DA)(*R*,*R*)	0.0230
$PtCl_2$(*R*,*S*)	0.0292	Pt(SA)(*R*,*S*)	0.0242	Pt(OX)(*R*,*S*)	0.0165	Pt(DA)(*R*,*S*)	0.0164
$PtCl_2$(*S*,*S*)	0.0267	Pt(SA)(*S*,*S*)	0.0176	Pt(OX)(*S*,*S*)	0.0123	Pt(DA)(*S*,*S*)	0.0116

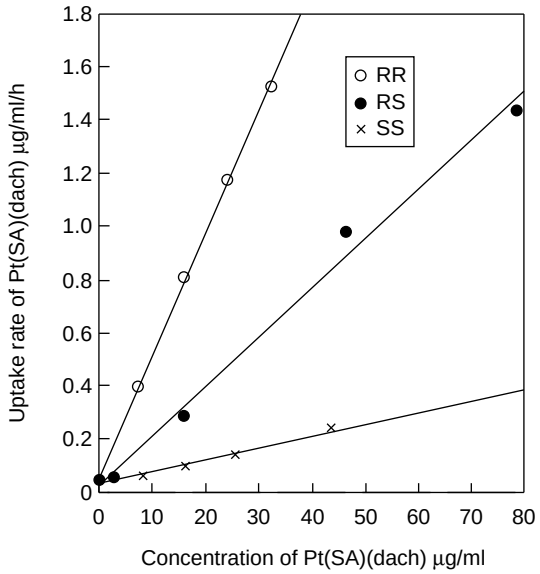

Figure 6. Plot showing dependence of uptake rate on Pt(SA)(dach) concentration.

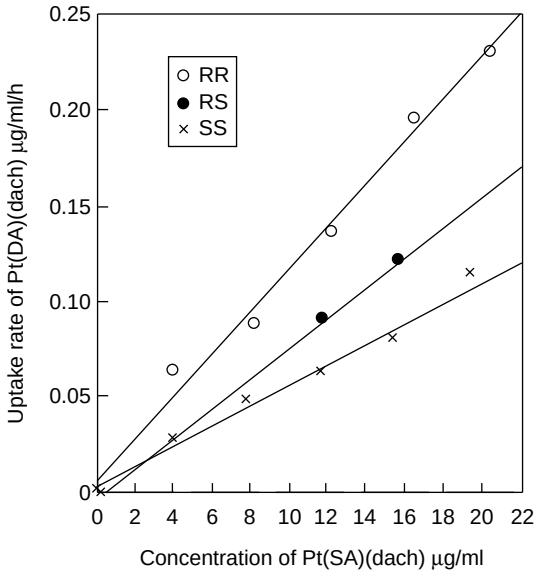

Figure 7. Plot showing dependence of uptake rate on Pt(DA)(dach) concentration.

Table 2. The relative reactivity of Pt(OX)(dach isomers) with thiols of membrane proteins

Sample	Control	Pt(OX)(1*R*,2*R*-dach)	Pt(OX)(1*R*,2*S*-dach)	Pt(OX)(1*S*,2*S*-dach)
Relative reactivity	1.0	0.82	0.86	0.81
Residual thiols (nmol mg^{-1} protein)	62.3 ± 1.7	51.2 ± 2.1	53.3 ± 1.5	50.3 ± 3.7

complexes are always the lowest. For the same isomer of dach as carrier ligand, the k values decrease in the following sequence of leaving groups: Cl > SA >OX > DA. The dependence of uptake rate on the concentration of Pt(SA)(dach isomers) and Pt(DA)(dach isomers) follows a straight line in all cases (Figures 6 and 7).

The reactivity of the oxalatoplatinum(II) complexes with thiols of membrane proteins

The reactivity of the oxalatoplatinum(II) complexes with thiols of membrane proteins was determined by measuring the residual thiols after incubation. The results are tabulated in Table 2.

The effects of the oxalatoplatinum(II) complexes on the conformation of membrane proteins

MSL is bound covalently to the thiols of membrane proteins, and its ESR spectrum is featured by two components, corresponding to different mobility of the spin label, i.e. an extremely immobile and a highly mobile fraction (Lammel & Maier 1980). As shown in Figure 8, P_2 and P_4 are mainly due to very rapidly tumbling spins, whereas P_1 and P_5 are due to very slowly tumbling labels. In the middle of the spectrum (P_3), both components are superimposed. The ratio of peak heights P_1 and P_2, i.e. the ratio of strongly and weakly immobilized thiol groups (S/W), and the correlation time (τ) of the

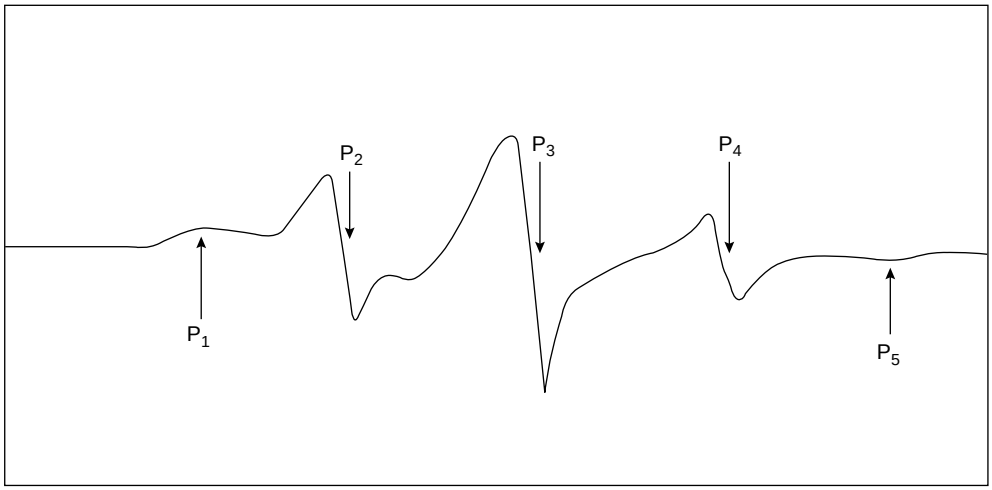

Figure 8. A typical ESR spectrum of MSL-membrane proteins: experimental conditions are given in the text.

rapidly tumbling spin label were used to evaluate the effects of the Pt(II) complexes on the freedom of the labeled thiols. The values of τ (in seconds) were obtained from the following equation (Keith *et al*. 1970):

$$\tau = 6.5 \times 10^{-10}\ \Delta H_0[(h(0)/h(-1))^{1/2} - 1]$$

where ΔH_0 is the peak to peak distance of P_3, and $h(0)$, $h(-1)$ are the peak to peak heights of P_3 and P_4, respectively. The parameters thus obtained for three isomers of oxalato complexes are given in Table 3.

The effects of the oxalatoplatinum(II) complexes on membrane permeability

At 0°C, the TEMPO molecules diffuse outwards through the phospholipid bilayer, but the ascorbate cannot diffuse inwards. The paramagnetic nitroxide groups of those TEMPO in the extracellular space were reduced by ascorbate, and the ESR signal amplitude was reduced.

The time dependence values for the decay of the signal amplitude (H_t/H_0) of TEMPO are shown in Table 4. Here, H_0 is the amplitude of ESR signal recorded at 2.0 min after mixing samples. The values of half-time (Table 4) were derived from the normalized decay curves and were used to estimate the change in permeability (Kornberg & McConnell 1971, Lu *et al*. 1995a).

Discussion

The uptake kinetics of PtX(dach) isomers revealed the chirality selectivity of human erythrocytes. In all

Table 3. The S/W and τ values of MSL spin label

Sample	Control	Pt(OX)(1*R*,2*R*-dach)	Pt(OX)(1*R*,2*S*-dach)	Pt(OX)(1*S*,2*S*-dach)
S/W	0.169 ± 0.001	0.212 ± 0.008	0.139 ± 0.007	0.145 ± 0.005
$\tau(10^{-10}$ s)	5.68 ± 0.05	5.88 ± 0.03	5.31 ± 0.05	5.41 ± 0.03

Table 4. The effects of Pt(OX)(dach isomers) on membrane permeability

Time (min)	2	4	6	8	10	Half-time (min)
Control	1.0	0.85	0.70	0.58	0.48	9.6
Pt(OX)(1*R*,2*R*-dach)	1.0	0.82	0.69	0.57	0.47	9.5
Pt(OX)(1*R*,2*S*-dach)	1.0	0.91	0.75	0.64	0.54	10.8
Pt(OX)(1*S*,2*S*-dach)	1.0	0.85	0.70	0.58	0.48	9.5

cases the *R*,*R* isomers were preferentially transported according to their different leaving groups. Several factors are considered to contribute to this behavior. First of all, in the course of uptake, the binding of platinum complexes to membrane components, such as proteins, is involved and probably causes retardation of uptake, i.e. stronger binding leads to less uptake. The decrease in thiol level of cell membranes is the most straightforward evidence for stable binding of platinum to the membrane proteins. Since no significant disparities were found in the reactivity of protein thiol groups among the three isomeric dach complexes, the origin of the chirality selectivity is not due to the difference in reactivity toward proteins.

The chirality selectivity is also unlikely to be the result of the difference in permeability changes caused by different isomeric complexes, although the uptake of platinum complexes is evidently related to the membrane permeability (Wang *et al*. 1996) and the platinum binding may increase the membrane permeability (Lu *et al*. 1995b). In the present work, all the isomers of Pt(OX)(dach) gave no significantly different effect on permeability.

Since the reactivity to protein thiols was shown to be the same for all the three isomers, but the conformational change of membrane proteins is different, we speculated that the membrane-bound molecules are subject to perturbation to different extents. This is not only due to the platinum binding to the protein, but is also related to the interaction with other target molecules in the membrane, mostly the phospholipid. The interaction might be transmitted to the protein and manifested as an additional factor leading to the difference of conformational change.

To interpret the chirality selectivity, we have to consider the way by which the complexes enter the cell. For cisplatin, either active transportation or passive diffusion has been suggested, although Gately and coworkers have suggested a model for cisplatin uptake in which both mechanisms are present (Gately & Howell 1993). It is reasonable that in Gately's model the contributions of active and passive transportations will be different with various complexes. The uptake of the complexes studied in this work is mainly passive, because the relationship between uptake rate and concentration is linear in all cases (Figures 6 and 7), and no saturation phenomenon was observed. Moreover, the uptake rate constants of those complexes with the same carrier ligand were found to decrease with increasing molecular size.

An inward passive transport process of a solute from extracellular space includes at least two fundamental steps: it starts from the extracellular aqueous phase entering the lipid medium phase, and is followed by diffusion along the acyl chains of lipid molecules (diffusion process). The chiral selectivity is likely due to chirality of the phospholipids comprising the cell membrane. These are all in *R* configuration and are thus organized in a specific way which recognizes the different isomers; the interaction with PtX(1*R*, 2*R*-dach) is much favored, such that the *R*,*R*-complexes concentrate preferentially on the surface of the membrane and their diffusion is promoted by the higher concentration gradients.

In summary, the chirality selectivity in uptake of the PtX(dach) complexes by the erythrocytes is not due to the difference in the reactivity with proteins, nor to the effects on membrane permeability. Since in each case the uptake is by passive diffusion, the reasonable interpretation is that the chirality of the complexes is recognized by the membrane, which is constructed of chiral phospholipid molecules.

Acknowledgement

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References

Dodge JT, Mitchell C, Hanahan DJ. 1963 The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch Biochem Biophys* **100**, 119–130.

Ellman GL. 1959 Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**, 70–77.

Gately DP, Howell SB. 1993 Cellular accumulation of the anticancer agent cisplatin: a review. *Br J Cancer* **67**, 1171–1176.

Inagaki K, Kidani Y. 1986 Difference in binding of (1,2-cyclohexanediamine) platinum(II) isomers with d(GpG). *Inorg Chem* **25**, 1–3.

Inagaki K, Nakahara H, Alink M, Kidani Y. 1990 Characterization of (1,2-cyclohexanediamine) platinum(II) isomers and their d(GpG) adducts by means of ^{1}H NMR spectroscopy. *Inorg Chem* **29**, 4496–4500.

Keith AD, Bulfield G, Snipes W. 1970 Spin-labeled neurospora mitochondria. *Biophys J* **10**, 618–629.

Kidani Y, Noji M. 1991 Developmental approach to prepare new types of antitumor platinum complexes with dual function. In: Howell SB, ed. *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy*. New York: Plenum Press; 127–137.

Kornberg RD, McConnell HM. 1971 Inside–outside transitions of phospholipids in vesicle membranes. *Biochemistry* **10**, 1111–1120.

Lammel B, Maier G. 1980 A spin labeling study of the effects of inorganic ions and pH on the conformation of spectrin. *Biochim Biophys Acta* **622**, 245–258.

Lu JF, An PD, Ma YL, *et al.* 1995a Study of permeability of *cis*-spin labeled platinum complexes through human erythrocyte membrane. *Acta Biophysica Sinica* **11**, 195–200.

Lu JF, Wang K, Sun XZ, *et al*. 1995b Effects of cisplatin and its analogues on the permeability of human erythrocyte membrane. *Metal-Based Drugs* **2**, 73–80.

Reile H, Bernhardt G, Koch M, *et al*. 1992 Chemosensitivity of human MCF-7 breast cancer cells to diastereoisomeric diaqua (1,2-diphenylethylenediamine) platinum (II) sulfates and specific platinum accumulation. *Cancer Chemother Pharmacol* **30**, 113–122.

Sandberg HE, Bryant RG, Piette LH. 1969 Location of sulfhydryl groups in erythrocyte membranes with magnetic resonance spin probes. *Arch Biochem Biophys* **133**, 144–152.

Speer RJ, Hall LM, Stewart DP, *et al*. 1978 Antitumor activity of platinum complexes of 1,2-diaminocyclohexane isomers. *J Clin Hematol Oncol* **8**, 44–50.

Wang K, Lu JF, Li RC. 1996 Events happening when cisplatin molecules encounter the cell. *Coord Chem Rev* **151**, 53–88.

Zhang WM, Li LZ, Li RC, *et al.* 1993 The kinetic studies of across-erythrocyte membrane transport of platinum (II) complexes. *Chemical J Chinese Univ* **14**, 1351–1353.

Zou J. 1995 *Ph.D. thesis*, Nanjing University, China.