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The events that occur when cisplatin encounters cells[1]

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Abstract

The chemical studies on the cellular response to platinum anticancer complexes are reviewed by treating the cell as a complex reacting system comprising several critical targets. In this multiple-target system, the contributions of non-DNA targets cannot be underestimated. The biological events observed in the response should be interpreted as the step-by-step results of a process in which the concert of related chemical reactions generates a sequence of chemical events. According to the multiple-target model the molecules in the plasma membrane, phospholipids and proteins, are the frontier targets. It is shown, on the basis of thermodynamic considerations, that the membrane proteins are attacked preferentially, while platinum binding to the phospholipids is relatively weak and reversible. Nevertheless, the weak and transient interaction with the phospholipids causes alterations in conformation which are transmitted and amplified further, resulting in changes in the structure and function of membranes, such as the permeability. The chemical events that occur in the course of platinum–phospholipid interactions include platinum binding, the subsequent conformational changes of the phospholipids and a change in the structure of the bilayer. In addition, the action of platinum complexes on the cytoskeleton is summarized in terms of a perturbation of the process of polymerization of skeletal protein. The sequence of chemical events, platinum binding to the monomeric protein and the induced conformational change and perturbations to the self-association of the monomer, is shown to be important in the cell damaging process. Platinum binding to actin was shown to be fatal to the cell by inducing cross-linking and aggregation and depolymerization and disorganization of the microfilaments. Various platinum complexes have been compared and several trends in their structure–activity are discussed. The other relevant events, apoptosis and drug resistance are also emphasized with the involvement of targets other than DNA.

Keywords: Cisplatin; Cellular response; Anticancer complexes

Abbreviations

CHIP	*cis*-dichloro-*trans*-dihydroxybis(isopropylamine)platinum(IV)
CDCDP	diammine-1,1-cyclobutanedicarboxylatoplatinum(II)
dach	diaminocyclohexane
DADP	diamminediaquoplatinum(II) cation
DBDP	diamminedibromoplatinum(II)
DCDP	diamminedichloroplatinum(II)
DIDP	diamminediiodoplatinum(II)
DPPC	dipalmitoylphosphatidylcholine

FMA	fluorescein mercuric acetate
MDA	malondialdehyde
MDP	dichlorodimethyldopamidoplatinum
MSL	3-maleimidoproxyl spin label
*n*DS	*n*-doxylstearic acid methyl ester
NPM	*N*-(1-pyrenyl)maleimide
PC	phosphatidylcholine
TEMPO	2,2,6,6-tetramethylpiperidine-*N*-oxyl

1. Introduction

1.1. Survey of the current guidelines for exploring new anticancer platinum complexes

Cis-diamminedichloroplatinum(II) (cisplatin, DCDP) is currently used as the first choice drug for the treatment of head–neck cancers, cancers of the genito-urinary tract, lung cancer and metastatic breast cancer, but the problems of toxicity and drug resistance dampen the clinical effect. For this reason, in the past three decades, thousands of platinum complexes have been screened in the search for anticancer active complexes with lower toxicity and drug resistance, but only a few have passed clinical trials [1].

On reviewing these research works critically, it is notable that the foundation for the interpretation of pharmacological and toxicological effects and the guidelines for the designing of new complexes are focused mainly on the interaction with DNA. Actually, the bioactivity and toxicity are the manifestation of all the events that occur in the drug–cell interaction, in which the involvement of non-DNA targets is also important. There is indeed plenty of convincing evidence that indicates that DNA is the critical target and cisplatin binding causes inhibition of DNA synthesis [2], but the conclusion is by no means exclusive. Based on an understanding of the mechanisms of toxicity of "soft" metal ions, proteins, peptides and phospholipids are all important targets.

The structure–anticancer activity relationship was derived mainly from the results of screening of various cisplatin analogues in the early 1970s. These empirical principles are consistent with the structural requirements for specific DNA binding [3]. Thus they are universally recognized and utilized. Nevertheless, there are some things that are inexplicable. Researchers have focused their studies on the mechanism of cisplatin–DNA reactions in order to gain an insight into the structure–activity relationships. The generally accepted mechanism, including the hydrolysis of cisplatin inside the cell and the subsequent binding to DNA, is also in keeping with the basic consideration of DNA as target and basis for the structure–activity relationship. For this reason, most of the studies concentrated on the alteration of leaving groups in order to modulate the ligand exchange rate and the appropriate concentration of the aquo species.

The interpretation of cisplatin's biological effects founded on these postulates disregards that

1. there are cellular targets other than DNA,
2. the relationships between structure and other biological activities should be considered, and
3. the cell response to cisplatin is the overall results of a series of chemical events.

1.2. Non-DNA targets

Although the mainstream of investigation has focused on DNA, a few arguments have been raised justifying a need for studies of non-DNA targets.

1.2.1. Biomedical evidence for non-DNA targets

Several biomedical studies have already suggested the role of non-DNA targets in the anticancer action and toxicity of cisplatin.

(1) It has been reported that cisplatin induces haemolysis [4].
(2) It is well known that some platinum complexes induce allergic reactions [5].
(3) Cisplatin and its aquo analogues tend to bind with plasma proteins [6].
(4) Cisplatin mediates the oxidative stress and leads to cell damage [7].
(5) Cisplatin activates macrophages [8].
(6) The nephrotoxicity of cisplatin is by nature a heavy metal toxicity, which is characterized by the inhibition of critical enzymes, binding to thiol compounds and damage to cell membrane, etc.

Perhaps we may visualize that, before binding to DNA, the platinum complexes have already taken part in a series of chemical reactions. As the result of these reactions, the platinum complexes are distributed to various biomolecules in various sites. About a decade ago, Slater suggested that attention should be paid to the effects on membrane and membrane-bound enzymes [9]. In the past ten years, we have accumulated much data relevant to the discussion of target molecules other than DNA.

1.2.2. Chemical basis for the presence of non-DNA targets

The chemistry of Pt(II) complexes comprises three main aspects, the coordination of Pt(II), the redox chemistry of Pt(II) and electrostatic interactions of charged Pt(II) complexes with charged species. All three are relevant to the reactions with biomolecules.

1.2.2.1. Preferential binding to S-donor groups is the main reaction with proteins. General speaking, platinum(II) as a "soft" Lewis acid has a high affinity for sulphur, and its affinity for N donors is significantly higher than for O donors. We would thus expect that Pt(II) will bind to S and N donors of peptides and proteins [10], as well as DNA. Even in the earlier literature on Pt coordination chemistry, a lot of experimental results revealed the existence of highly stable Pt—S covalent bonds and the coordination of CH_3-S- and C=S groups. On reaction with proteins, the diammineplatinum(II) binds as a bidentate ligand, with one of the leaving groups replaced by a sulphur donor and the other by the neighbouring

N or O donor. For some proteins, the prior hydrolysis of cisplatin is not necessary for binding to the S donor [11], but not for all proteins [12].

The reaction between aquo platinum species with thioether sulphur is more rapid than that with thiol groups, because the ligand exchange reaction is slower than the direct coordination with the thioether sulphur. Thus platinum complexes bind readily with methionine residues of proteins. However, the covalent Pt—S bond is much more stable and reactions with thiols tend to be irreversible; thus the damage is rather permanent. On the contrary, the Pt in Pt—S(met) can be mobilized with other nucleophiles, such as diethyldithiocarbamate and thiourea [13]. In some cases the ammonia ligand *trans* to the coordinated S is activated and expelled from the coordination sphere owing to the *trans* effect. Consequently, the proteins and peptides (including enzymes) are expected to be the most sensitive targets. Since in most cases the SH→SS bonding plays an important role in determining the protein conformation, it is also expected that platinum binding will cause conformation changes and finally the alteration of the structure and biological function.

1.2.2.2. Reacting as cations. It has been reported that, among the hydrolysis products of cisplatin, the predominant species that reacts with the cell components is *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ [14]. In common with most cationic species, they are expected to "bind" *via* charge–charge interactions with the head groups of phospholipids, as well as the other negatively charged sites on the cell surface. The absence of the aquo species in extracellular space would perhaps be an argument against this proposal. However, even in the presence of a relatively high concentration of Cl^- a fraction of cisplatin, although very small, undergoes hydrolysis and, in addition, the cell surface promotes the hydrolysis. Platination on phosphate head groups may induce a change in conformation and bilayer structure is then affected. In addition, the positively charged Pt(II) cations, being Lewis acids, may accelerate the hydrolysis of the neighbouring peptide linkage after binding to protein molecules.

1.2.2.3. Redox chemistry and free radical formation. The redox chemistry of platinum compounds has been well studied, but it is still very ambiguous for reactions occurring in the physiological state, in which the platinum is bound to peptides, phospholipids, nucleic acids and small molecules. In relation to its biological effect, researchers have been interested in whether Pt(II) to Pt(III) transformation is possible and whether, through this single electron transfer route, active oxygen radicals are generated resulting in oxidative damages to lipids and proteins. On the contrary, active oxy radicals might be sustained at a high level by decreased antioxidative ability, such as by depletion of glutathione (GSH) levels. Soft acids, such as Cd(II) and Pt(II), may elevate the peroxide concentration by binding to GSH and depressing the GSH:GSSG ratio.

1.3. The cell response to platinum complexes is the overall manifestation of a complex system [15]

From the time cisplatin approaches the cell surface until it interacts with DNA in chromosomes, Pt(II) encounters a number of biomolecules. As discussed above, it

interacts with these molecules and the cell is affected as a multicomponent system. For simplification, researchers have concentrated their studies on a single target molecule and have neglected the other parts of the cell. The contemporary concept of a living cell requires that the cell is treated as a complex system, which is characterized by the following features.

(1) There is a variety of potential targets, confined in various compartments such as the biomembranes, cellular vesicles, nucleus, etc.

(2) Most of the biopolymers are assembled in a specific way and some are packed in the biomembranes. The assembled molecules are kept in a specific spatial orientation, which is essential for their activities. The polymerization state is sensitive to exogenous substances.

(3) Because of the compartmentalization of intracellular reactants, the reactions occur in specific sites.

(4) The mass transfer of both the reactants and the products is vectorial, depending on the site of generation, the barriers and carriers, the channels and any other pathways.

(5) Since living biosystems are open systems, they are never at equilibrium but are maintained in a non-equilibrated steady state. In such open systems, the kinetics of the reactions are far more important than the thermodynamics aspects.

(6) In the course of drug–cell interactions, the reactions are interlinked kinetically and thermodynamically. The concert of the relevant reactions results in a sequence of chemical events, which is the foundation of the sequence of the biological events.

(7) The whole system is under delicate control which operates by keeping everything and every event in spatial or temporal order. Any perturbation, even simple and transient, might be amplified and propagated to the whole system and cause its destruction.

Based on this model we should review our explanation of the molecular mechanism of the activity, toxicity and side effects of a drug. Several critical clues have been explained on the basis of actions on DNA, but we should extend our view to the whole cell [16]. For instance, the current understanding of cell death comprises two pathways. The necrotic death is characterized by cell lysis and starts from membrane damage with the elevation of intracellular Ca^{2+} ion as an important clue. The programmed death (apoptosis), on the contrary, is characterized by the fragmentation of DNA, condensation of chromatin and loss of nuclei [17], with the local elevation of intracellular Zn^{2+} [18].

2. Events on or in the plasma membrane [19]

It is reasonable to assume that the plasma membrane is the frontier against the exogenous substance, while membrane-bound molecules, lipids and proteins will be the accessible target molecules. Metal binding is doubtless the first set of events. As shown in Table 1, the possible binding sites on, and in, the plasma membrane are different in their nature and affinity of binding.

The subsequent events include the changes in conformation and membrane struc-

Table 1
Potential metal-binding sites on the plasma membrane

Targets	Binding sites	Nature of binding
Phospholipids	$-NMe_3^+$, $-PO_4^{2-}$, $-COOH$, $-NH_3^+$	Electrostatic and coordination
Peptides	$-SH$	Covalent and coordination
	$-SCH_3$, $-COOH$, $-NH_2$, $-CONH_2$	
Oligosaccharides	$-OH$	Coordination
Cholesterol	$-OH$	Coordination

tures. An additional event is the metal ion induced oxidative damage, including lipid peroxidation, the oxidative cross-linking (SH to –SS–) and oxidative degradation of biopolymers. This is caused either by metal ion mediated generation of reactive oxygen species or indirectly by binding glutathione to reduce glutathione peroxidase activity and the antioxidant activity. In most cases, the events that occur in the membrane will be propagated to other targets in other compartments by signal transducing systems, transmembrane transport systems, etc.

2.1. *Binding of cisplatin with membrane-bound molecules*

The membrane proteins and membrane phospholipids compete for binding to the platinum complexes. It has been shown that the positively charged aquo species are reactive in their binding to phospholipids and proteins, but the unhydrolysed cisplatin can also bind to proteins slowly *via* a ligand exchange mechanism.

The covalent Pt–S bond is much stronger for a thiolate rather than a thioether sulphur and the binding is irreversible. Binding to the head groups of phospholipids involves charge–charge interactions, which are weak and reversible. The overall binding constants of Pt–protein range between 10^4 and 10^6, while those of Pt–liposome are only 10^2. By means of a fluorescence quenching method, we have determined the binding capacity and binding constants of $[Pt(NH_3)_2]^{2+}$ to human erythrocyte membranes after a 17 h incubation with *cis*-DADP. For comparison, the binding constants to membrane proteins were also determined. The experimental data were processed by the Scatchard method. The Scatchard diagrams thus obtained are fitted to a biopolymer having two categories of binding sites. The numbers n_1 and n_2 of binding sites and their corresponding apparent binding constants K_1 and K_2 were determined and are listed in Table 2. The results indicate that the bindings to whole membrane and to membrane proteins are comparable, but much higher than the binding to DPPC liposomes. Thus, based on thermodynamic considerations, the platinum complexes bind to membrane proteins preferentially [20].

By the same method the apparent binding constants of $[Pt(NH_3)_2]^{2+}$ to rat Erhlich ascites cancer cells were determined to be as follows: $K_1 = 1.35 \times 10^5$ l mol^{-1} ($n_1 = 6.80 \times 10^{-4}$ mol (g protein)$^{-1}$) and $K_2 = 2.50 \times 10^3$ l mol^{-1} ($n_2 = 1.92 \times 10^{-3}$ mol (g protein)$^{-1}$) [21].

Table 2
Platinum binding to cell membranes

Reaction systems	n_1[a]	K_1 (l mol^{-1})	n_2[a]	K_2 (l mol^{-1})
Erythrocyte membrane	4.00×10^{-3}	6.30×10^{4}	9.11×10^{-5}	3.17×10^{4}
Erythrocyte membrane proteins				
10 min reaction time	2.03×10^{-3}	1.10×10^{5}	5.53×10^{-4}	5.48×10^{4}
17 h reaction time	1.38×10^{-3}	2.56×10^{5}	4.91×10^{-4}	4.52×10^{4}
PC liposome	0.138	6.32×10^{2}	0.0296	1.37×10^{2}

[a] Expressed as moles of Pt per gram of protein for membrane and moles of Pt per mole of PC for liposome.

2.2. *Phospholipids as target molecules*

The phospholipids are likely to be unimportant targets, but the transient, weak interaction with phospholipids can be amplified and prolonged and ultimately interfere with cell function for the following reasons [22].

(1) In the membrane, the phospholipids are assembled into an organized supramolecular system via weak interactions between neighbouring molecules.

(2) The phospholipid molecules in the membrane are in continuous motion, including lateral diffusion, flip–flop and wobbling.

(3) The fatty acid chains in phospholipids are also in a dynamic state, as characterized by the fluidity. Different portions of the chain are subject to different degrees of motion, giving different fluidity.

(4) The conformation of phospholipid is important in the molecular assembly but is very sensitive to the binding of ions or small molecules on the head groups.

(5) The conformation change will induce the consequent alteration of assembly, leading to changes in the dynamics of molecules and, finally, the alteration of membrane structure and functions, such as permeability.

(6) A living cell is in a non-equilibrium state; therefore, even though the metal binding and subsequent conformational changes are transient and recoverable, the cells might be damaged just at this moment.

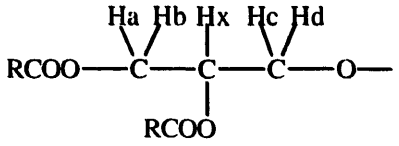
(7) Polyvalent metals may cross-link the neighbouring molecules, lipid to lipid or lipid to protein. The phospholipids are thus immobilized.

(8) Several metal ions may induce oxidative damage to lipids or proteins.

Consequently, it is reasonable that a simple metal binding may become a trigger inducing the changes in membrane structure and functions. The effects depend on the hardness of metal ions, the size and morphology of complexes, and the charge and lipophilicity of species. A recent study on Hg^{2+}–cell interactions gives strong support to these ideas [23].

2.2.1. *The interaction between cis-diaquodiammineplatinum(II) and phospholipids*

By means of ^1H nuclear magnetic resonance (NMR) studies, the interaction between DADP with dipalmitoylphosphatidylcholine (DPPC) in $CDCl_3$ solution



J (Hz)	Jax	Jbx	Jcx	Jdx
without Pt	3.4	4.4	3.9	4.5
with Pt	5.6	4.4	5.2	4.3

Scheme 1.

and in liposome form was investigated [24]. The results revealed the binding mode and the subsequent conformation change. The shift and broadening of the $-NMe_3^+$ resonance from 3.416 to 3.279 ppm, and the change in the $-CH_2CO-$ peak from a quartet to a triplet show that the platinum complex binds to the phospholipid head groups. The binding mode is similar to that in lanthanide–phospholipid complexes, in which the cations interact with the head groups of the phospholipid molecules [25]. In the ^{13}C spectra of DADP and DPPC in $CDCl_3$, the peaks of $-CH_2O-$choline and $-CH_2O-$glycerol shifted in opposite directions after reaction with *cis*-DADP. Since coupling constants after platinum binding are characterized by $J_{ax} > J_{bx}$ and $J_{cx} > J_{dx}$ (Scheme 1), a gauche to trans transformation in the glycerol moiety was postulated [24].

Similar conclusions can be drawn for the reaction with phospholipids in liposomes. When phospholipids assemble into liposomes, the lipid molecules line up in an orderly fashion, with the head groups exposed to the aqueous phase and the aliphatic chains hidden in the hydrophobic zone. Both electrostatic and hydrophobic interactions play important roles in determining the integrity of the membrane. The platinum complexes may induce changes in conformation and membrane structure. In addition to the binding to the head groups, a fraction of complexes will pass through the membrane and reach the liposome core.

The ^1H NMR relaxation curves of DPPC liposomes were determined at 25–65 °C to follow the course of interaction, including platinum binding and conformation changes. The spin lattice relaxation time T_1 values of the head group $-NMe_3^+$, the $-(CH_2)_n-$ and the tail $-CH-$ given in Table 3 reflect that the reaction with *cis*-DADP caused a gradual response [24].

Table 3
The influence of *cis*-DADP on the spin lattice relaxation time T_1 of DPPC liposomes[a]

	T_1 (s)		
	$-NMe_3^+$	$-(CH_2)_n-$	$-CH$
DPPC	0.780±0.011	0.810±0.008	0.940±0.044
DPPC+*cis*-DADP			
1.5 h	0.353±0.007	0.719±0.007	0.941±0.037
60 h	0.616±0.083	0.651±0.012	0.851±0.038

[a] DPPC liposomes (10 mg (ml $D_2O)^{-1}$) were incubated with DADP (prepared from 0.5 mg DCDP) at room temperature for 1.5 h and 60 h. ^1H NMR spectra were recorded at 338 K.

The results indicate that platinum complexes bind to head groups rapidly and as a result induce a conformation change, which is transmitted inward slowly. The effect on the head group is recoverable, but the effect in the chain is sustained for rather a long time.

2.2.2. The interaction between cisplatin and plasma membrane

The interactions between platinum complexes and phospholipids in a real cell membrane are complicated not only by the competition from proteins but also by the reinforced membrane structure with the involvement of proteins. The problem to address is, under such conditions, whether platinum still binds to the head groups of phospholipids and induces alterations in conformation and membrane structure.

IR studies [26] on the reaction products of human erythrocyte membrane and cisplatin showed that after a 2.5 h reaction time at 4 °C the vibration at 1062.5 cm^{-1} shifted and split into a doublet at 1059 cm^{-1} and 1036 cm^{-1} (Fig. 1) and an additional weak absorption appeared at 953 cm^{-1}. The later was assigned to Pt—O—P bonding. All the changes disappeared after the incubation time was extended to 12 h. The results indicate that the platinum still binds to the phosphate group initially, but the effect is recovered slowly.

It is noteworthy that *cis*-DBDP, *cis*-DIDP and *trans*-DCDP gave similar effects, which were not recovered after a 12 h reaction time, and the 953 cm^{-1} peaks were very strong. These results show that the binding of cisplatin is much less pronounced

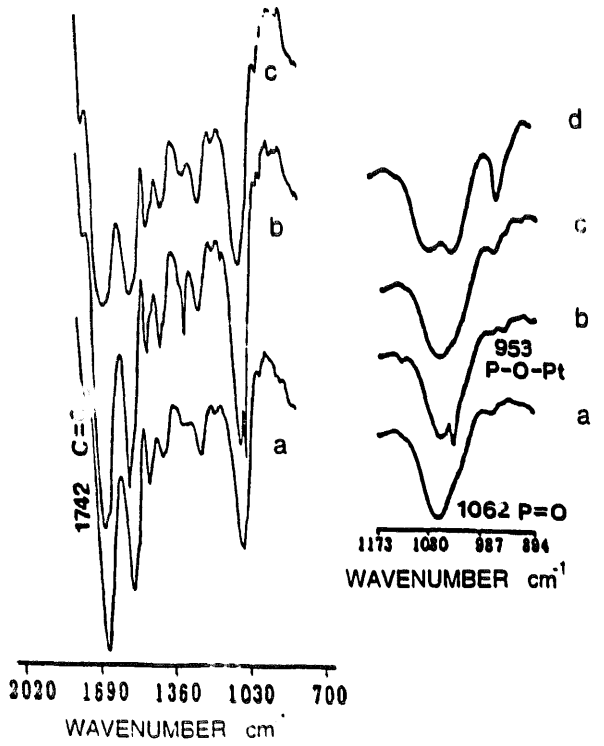


Fig. 1. IR spectra of human erythrocyte membrane (2 mg protein ml^{-1}) in the presence of *cis*-DCDP (1 mmol l^{-1}) and *trans*-DCDP (1 mmol l^{-1}) after incubation (4 °C) with *cis*-DCDP for 0 h (spectra a), 2.5 h (spectra b) and 12 h (spectra c) and with *trans*-DCDP for 0.5 h (spectrum d).

than with the other complexes. This is probably due to the relatively high Cl^- concentration which inhibits the hydrolysis of cisplatin. The ^{31}P NMR spectrum of human erythrocyte membrane at 37 °C (Fig. 2) has a peak B at −10.77 ppm and a sharp peak A at 3.11 ppm, which is overlapped with the broad shoulder C at lower field. It was known that in the ^{31}P NMR of the biological membrane both peaks B and C may be considered as the result of anisotropy of chemical shifts, while a sharp peak A is presumably an isotropic component to overlap it. The reaction with cisplatin leads to high field shifts $\Delta\delta_A = 1.2$ ppm and $\Delta\delta_B = 0.72$ ppm of A and B and disappearance of C. This is probably due to the change of membrane structure from bilayer to hexagonal II [26].

Although the binding of cisplatin on membrane phospholipids is transient and recoverable, its influence can be propagated and amplified. The changes in the conformation and dynamic state of lipids induced by cisplatin have been studied by measuring the fluidity of different segments of the fatty acid chain [27]. The *N*-doxylstearic acid spin labels containing ^{14}N nitroxide moieties at carbon 5, 7, 12 and 16 positions (5DS, 7DS, 12DS and 16DS) were used to label the membrane phospholipid molecules at different depths. Based on electron spin resonance (ESR) spectra the membrane fluidity was estimated from the order parameter *S*. The *S* values were calculated from the parallel and perpendicular parts $A'_{\parallel}$ and $A'_{\perp}$ of the hyperfine splitting in a spin-labelled ESR spectrum. The changes in fluidity of different segments caused by platinum binding can be estimated as a function of time. As shown in Fig. 3, the effects of platinum binding to phospholipids were observed; starting from the head groups, the reduction in fluidity begins from 5DS and is transmitted to the deeper sites in the bilayer. All the perturbations are recovered within several hours. The transmittance from surface to the hydrophobic region is sequential and the influence becomes less pronounced with increasing depth.

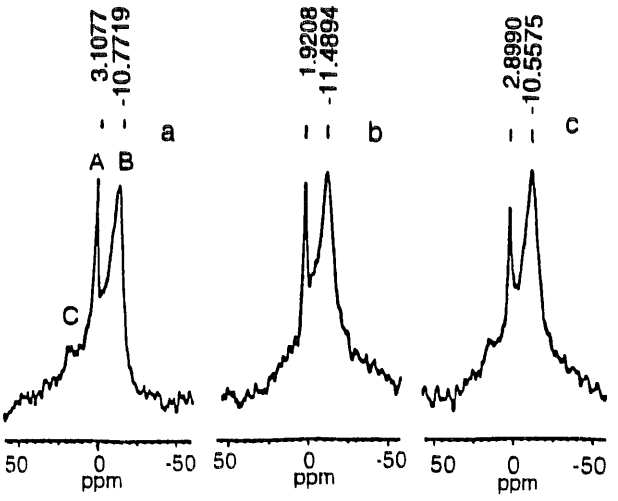


Fig. 2. ^{31}P NMR spectra (81 MHz) of human erythrocyte membrane (15 mg protein ml^{-1}) at 37 °C: (a) control, membrane in tris buffer (20 mmol l^{-1}, pH 7.4 and 400 mmol NaCl l^{-1}); (b) after 2.5 h incubation; (c) 12 h incubation with cisplatin (1.6 mmol l^{-1}).

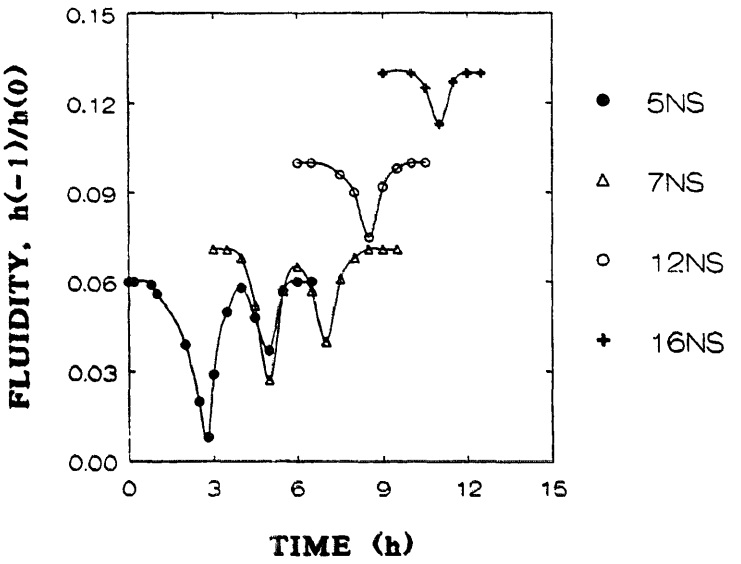


Fig. 3. The temporal change of the fluidity of human erythrocyte membrane (2.5 mg protein ml^{-1}) induced by cisplatin (1 mmol l^{-1}). ESR measurement conditions: X band with 2 mW microwave power and 100 kHz field modulation of 2 G.

2.3. *Membrane-bound proteins as target molecules*

It is evident that, in the course of cisplatin–membrane interactions, the most important events are related to membrane-bound proteins. The decrease in thiol levels of cell membranes is the most straightforward and most simple evidence for platinum binding to membrane proteins [28,29]. Cisplatin quenches the fluorescence of FMA-labelled protein–thiol groups of human erythrocyte membranes, and the concentrations of thiol groups of erythrocyte [19] and hepatocyte [29] membranes are reduced by cisplatin in a dose-dependent manner. This provides strong support for platinum-thiol binding.

The quenching effect caused by cisplatin of the intrinsic fluorescence of membrane proteins in human erythrocyte "ghosts" indicates that a conformational change was induced, leading to the exposure of buried fluorophoric groups to the aqueous environment. The conformations are expressed in terms of the ratio S:W of the strongly and weakly immobilized thiol groups, which can be estimated from MSL spin labelled ESR spectra. By determining the change with time in the S:W ratio of MSL-labelled ghosts after the addition of cisplatin, it was shown [30] that the induced conformation change is a complex slow process. In the initial stage, a rapid increase indicates that the thiol groups become less mobile. A decrease in S:W ratio follows, which reflects the exposure of the buried thiol groups. Transplatin causes the S:W ratio to drop abruptly from the beginning. The effects of both isomers are reversible, but to different extents. The effect on the S:W ratio is different for different cells and is dependent on cisplatin concentration. The α-helix content of the membrane decreases in parallel with the change in environment of thiol groups and tryptophan residues, as shown in circular dichroism (CD) spectra. The mitochondrial

membrane is attacked by intracellular cisplatin and its hydrolytic products in the same way. A rapid loss of protein thiol groups, followed by a decrease in the uptake of Ca^{2+} by mitochondria, was observed [31].

A Tb^{3+} label fluorescence method has been used to study the reactions of metal complexes with membrane proteins. The quenching of Tb^{3+} fluorescence can be interpreted on the basis of the substitution of Ca^{2+} from calcium-binding proteins, or binding to a site close to, or intimately associated with, the Ca-binding site. By this method, it has been shown that cisplatin binds to a site associated with a certain Ca-binding protein on the plasma membrane of GH3/B6 pituitary tumour cells [32]. The interaction of cisplatin, *cis*-dichloro-*trans*-dihydroxybis(isopropylamine)Pt(IV) (CHIP) and *cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II) (CDCDP) with Tb^{3+}-labelled mouse thymocytes was studied. Cisplatin and CHIP quench the fluorescence significantly, but CDCDP quenches it only slightly. This difference can be explained by the higher lipophilicity of CDCDP. The nature of CDCDP binding is possibly due to the effect of charge, because the quenching effect is related to ionic strength [33]. In these experiments, a conformational change in the membrane was also observed.

In their recent work, Canada and coworkers [34] focused their studies on platinum binding to a thermosensitive calcium-binding protein in the plasma membrane of human MCF-7 breast tumour cells. An increased affinity of platinum to the calcium-binding protein at higher temperature was observed and was proposed to be the basis for why the cytotoxic action of cisplatin increased during hyperthermia.

2.4. *Effects of platinum complexes on the phase transition and fluidity*

The transition from liquid crystal phase to gel phase is characterized by the phase transition temperature t_p [35]. To be exact, the behaviour in phase transition at different portions of the phospholipid bilayer is different and can be measured in order to clarify the transmittance of this action. The results of spin-labelling ESR studies (Fig. 4) show that the binding of platinum complexes results in an elevation of transition temperature. It is quite interesting to note that the t_p value for 5DS is essentially the same as that for the control, but t_p increases abruptly for 7DS. This is probably due to cisplatin hydrolysis, which occurs just after it enters the membrane bilayer. It is likely that the chloride concentration in the membrane is already sufficiently low for hydrolysis to occur.

It would be reasonable that, in parallel with the alteration in phase transition temperature, the membrane fluidity changes as a result of the effect of platinum complexes on the motion of the fatty acid chain. The order parameter S of 5DS in the membrane is used as the criterion of the fluidity. A higher S value reflects a lower fluidity. The S values shown in Table 4 indicate that platinum binding increases the degree of order in the assembly of the bilayer aliphatic chains.

By a fluorescence bleaching method, the lateral diffusion D of membrane phospholipids of ascites liver cancer cells was found to be enhanced and the recovery rate R was lowered after treatment with dichlorodimethyldopamideplatinum(II) (MDP). The increase in D values is related to an increase in diffusion rate, while the lower

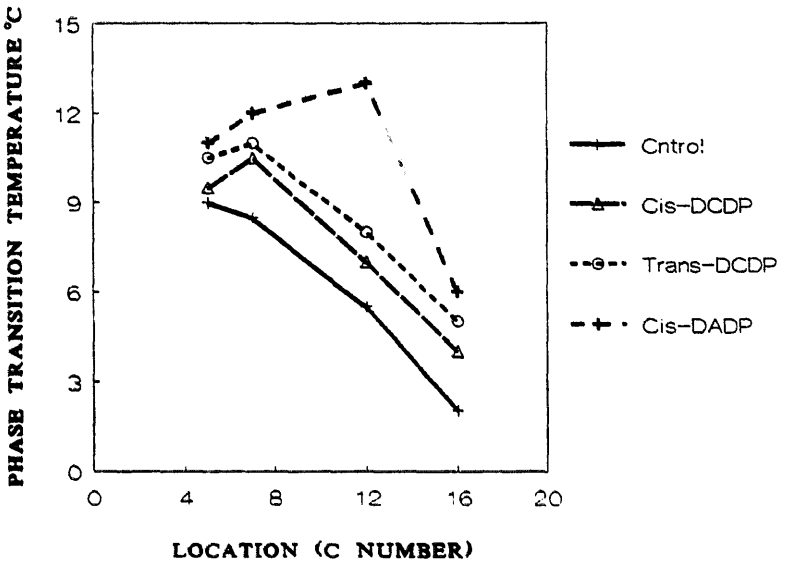


Fig. 4. The effect of platinum complexes (1 mmol l^{-1}) on the phase transition temperature at different depths of human erythrocyte membrane (2 mg protein ml^{-1}): control, *cis*-DCDP, *trans*-DCDP, *cis*-DADP. The phase transition temperature was calculated *via* τ_c from the ESR parameters, $h(-1)$, $h(0)$ and $h(1)$ (the heights of peaks at high, centre and low magnetic field respectively) and ΔH_0 (peak-to-peak width at centre field).

Table 4
The order parameter S of 5DS spin label in human erythrocyte in the presence of platinum complexes[a] (from Ref. [36])

	Control	*cis*-DCDP	*cis*-DBDP	*cis*-DIDP	*trans*-DCDP	*cis*-DADP
S	0.746±0.016	0.775±0.014	0.778±0.019	0.787±0.020	0.787±0.030	0.791±0.035

[a] Final concentration of platinum complex, 1.5 mmol l^{-1}; incubation time, 1 h; NaCl concentration, 5 mmol l^{-1}.

R value shows that the molecules become less able to diffuse into the bleached region [37]. The change in membrane fluidity is the overall result of platinum binding to both proteins and phospholipids. Since hydrolysis of platinum complexes favours both reactions the effect of *cis*-DADP, as shown in Table 4 and Fig. 4, was more significant.

2.5. *Effects of platinum complexes on the cell permeability*

The toxic effects of heavy metals are related in several aspects to their effect on cellular permeability to small molecules and ions. This effect is attributable to the inhibition of membrane enzymes, blocking of ionic channels, and the alteration of membrane structure. From the results on phase transition and fluidity given above, we can expect that cisplatin binding might induce alterations in the permeability of cell membranes.

A few studies have been reported in this area. Frog skin has been used as a representative transporting epithelium by van den Berg *et al.* to investigate the effects of cisplatin on the transport of NaCl and urea. In this case, cisplatin elevates the permeability of the mucosal surfaces, and the effects were reported to be related to the *cis*-leaving groups [38].

The permeability of human erythrocyte membrane was studied by measuring the rate of inward diffusion of ascorbate anion from outside to various depths of membrane labelled by stearic acid spin labels 5DS, 7DS, 12DS and 16DS [36]. Fig. 5 shows the ESR spectra of 5DS spin labelled human erythrocyte membrane as a function of time after addition of ascorbate, which reduces the amplitude of the ESR signal. The arrival of ascorbate anion at a certain site is monitored by the diminishing ESR signal at that site, as a result of the reaction between ascorbate and the spin label. The outward diffusion to the extracellular space was determined by a similar method involving efflux of spin label TEMPO. The effects of various platinum complexes were compared. The results (Table 5) are displayed as the half-times T_{in} and T_{out} for ascorbate influx and TEMPO efflux respectively.

All the platinum complexes studied increased the membrane permeability and

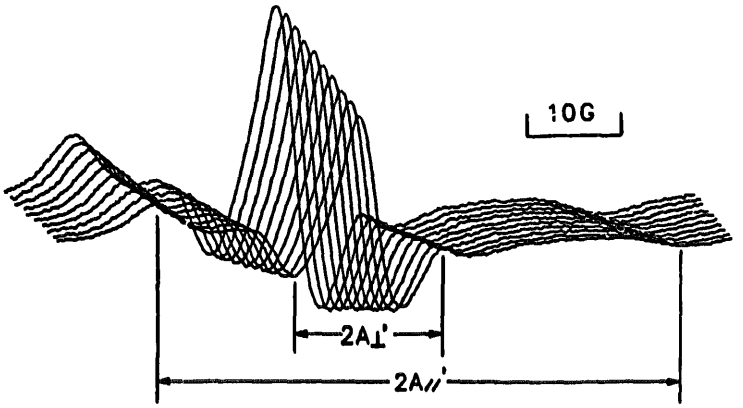


Fig. 5. Three-dimensional ESR spectra of 5DS spin labelled human erythrocyte membrane (2 mg protein ml^{-1}) as a function of the time after adding ascorbate (10 mmol l^{-1}). The time interval was 2 min and the step change of center magnetic field was 1 G automatically. $2A'_{\parallel}$ and $2A'_{\perp}$ are the parallel and perpendicular parts of the hyperfine splitting in the spectra.

Table 5
Half-time T_{out} for spin label TEMPO leaking out through membrane and being reduced by extracellular ascorbate at 0 °C and half-time T_{in} for ascorbate anion entering the membrane bilayer and interacting with spin label 5DS in the lipid bilayer in the presence of Pt(II) complexes (from Ref. [36])

	Control	*cis*-DCDP	*cis*-DBDP	*cis*-DIDP	*trans*-DCDP	*cis*-DADP
T_{out} (min)[a], TEMPO	13.11±0.32	12.24±0.31	11.58±0.25	11.20±0.28	10.72±0.33	10.21±0.26
T_{in} (min)[a], ascorbate	15.89±0.25	14.12±0.32	12.65±0.24	12.10±0.30	10.25±0.27	8.78±0.31

[a] Results are mean±SD, $n=5$.

Table 6
Half-time T_{in} of reduction by ascorbate anion of spin labels (*n*DS) at different depths of the lipid bilayer of human erythrocyte membranes in the presence of Pt(II) complexes[a] (from Ref. [36])

	T_{in} (min)[b]			
	Control	*cis*-DCDP	*trans*-DCDP	*cis*-DADP
5DS	14.08±0.70	13.28±0.54	12.10±0.45	9.79±0.44
7DS	28.31±1.27	25.20±1.13	24.86±1.02	22.33±0.98
12DS	66.81±3.34	60.02±2.41	77.10±3.44	55.08±2.31
16DS	34.21±1.39	25.29±1.28	49.49±2.02	24.81±1.28

[a] Membrane concentration, 2.5 mg protein mg^{-1}; final platinum concentration, 1.5 mmol l^{-1}.
[b] Results are mean±SD, $n=3$.

facilitated the passage of small molecules and ions. The largest effects were observed for *cis*-DADP, because it is more reactive to both membrane lipids and proteins than other complexes. The influence of the *trans* isomer is more pronounced when compared with cisplatin, probably because of its higher reactivity in ligand exchange reactions and its higher lipophilicity.

To follow the path of ascorbate ion, the T_{in} values of ascorbate to different depths of the aliphatic chain were determined in the presence of various Pt(II) complexes. Since the motions of segments at different depths are different intrinsically, the changes in T_{in} indicate the influences of Pt(II) complexes at different depths of the lipid bilayer (Table 6).

The behaviour of transplatin is quite different from that of cisplatin. It facilitates the influx of ascorbate to 5DS and 7DS sites but hampers the further inward diffusion. The difference cannot be interpreted simply by differences in reactivity. The aquo species gives stronger effects than cisplatin, and the difference becomes pronounced just beneath the membrane surface.

2.6. Lipid peroxidation

Toxicological studies have shown that the hepato- and nephrotoxicity of cisplatin to liver, kidney and arteries are all probably related to the oxidative stress, as reflected by the enhanced lipid peroxidation. The supporting evidence derives from the increasing reports on the efficacy of antioxidants and reactive oxygen species (ROS) scavengers in the depression of the toxicity [39]. However, it is still controversial as to whether the free radicals are involved in the toxicological mechanism. The early works of Sugihara et al. showed that cisplatin causes kidney damage via a free radical mechanism [40]. Their evidence was limited to the increase in MDA concentration, *p*-aminohippurate accumulation and the inhibitive effects of superoxide dismutase and α-tocopherol. No direct evidence for the generation of ROS has been reported. On the contrary, Vermeulen's group [41] showed that no MDA increase was observed in experiments on rat kidney microsomes and an NADPH-generating system. Nevertheless, there are a number of works supporting this postulation. The

Table 7
Levels of electron spin resonance signals of lipid radicals and MDA in rabbit liver microsomes in the presence of various Pt(II) complexes[a]

Agents	Levels of lipid radicals (arbitary units)	Increase[b] (%)	MDA (nmol (mg protein)$^{-1}$)
Buffer	4.54±0.70	0	1.62±0.08
cis-DCDP	6.86±0.82	51.5	2.88±0.12
cis-DBDP	7.48±0.76	64.7	4.15±0.35
cis-DIDP	7.50±0.88	65.1	4.27±0.31
trans-DCDP	7.90±0.62	74.0	4.96±0.42
cis-DADP	8.38±0.56	84.6	5.77±0.48
$H_2O_2+Fe_4SO_4$	8.82±0.64	94.3	

[a] Results are mean±SD, $n=3$; microsome, 18 mg protein ml^{-1}; platinum concentration, 0.5 mmol l^{-1} in tris buffer. The mixture of microsome and platinum complex was incubated with POBN (deionized water solution) for 3 min at 37 °C and ESR spectra were measured immediately. Instrument conditions: 10 mW microwave power, 100 KHz field modulation at 2 G.

[b] Compared with control, $p<0.05$.

studies on the damage in mesenteric arteries after transarterial infusion of cisplatin indicated also that the degenerative changes in the epidermal and medial smooth muscular cells are related to free radical mediated lipid peroxidation [42]. Several sulphur-containing compounds, ranging from simple thiosulphate to glutathione and WR2721 and WR1065, have been reported to be effective in the prevention of the damage, but their mechanism is still ambiguous. Our recent ESR studies give convincing evidence to show that free radical generation by rabbit liver microsomes is promoted by cisplatin and its analogues. The lipid radical L$^{\cdot}$ was determined by spin-trapping ESR spectroscopy with 4-(pyridyl-1-oxide)-*N*-*t*-butylnitrone (4-POBN) as the spin-trapping agent [43]. The results given in Table 7 revealed that all the platinum complexes studied promoted the free radical formation. The trans isomer and the *cis*-diaquo species are most remarkable. These results indicate that the free radical generation is related to the reactivity of the complexes. The sequence of the effect is as follows:

cis-DCDP < *cis*-DBDP ≈ *cis*-DIDP < *trans*-DCDP < *cis*-DADP

The MDA levels in the microsomes varied in parallel with the level of free radical in these experiments.

2.7. *The uptake*

On reviewing the studies on the cellular uptake of heavy metal complexes, we would realize that they are quite different from that of organic compounds. The most prominent feature of the metal complexes will be their high reactivity with a variety of potential targets in, or on, the membrane. We cannot imagine a complex entering a cell without any interactions with the molecules encountered throughout its journey. Actually the uptake of most metal complexes is just the net result of a

sequence of binding-and-releasing interactions with a series of transferring molecules along the way. Therefore the rate of uptake and the level of intracellular accumulation are determined by the relay of metal complexes through the related acceptor–donors.

For the uptake of cisplatin and its analogues, it has been recognized that the inward diffusion is dominated by the passive diffusion, but there is accumulating evidence in favor of protein-mediated transport. In a recent review, Gately and Howell attempted to put the two mechanisms together [44]. They interpreted the uptake of cisplatin to be both by passive diffusion and *via* a gated channel. The flux through this channel is affected by several pharmacological agents and the activators or blockers of signal transduction pathways. It is also dependent on Na^+–K^+–ATPase, membrane potential, extracellular pH and extracellular osmolality. The current understanding is based mainly on the biochemical and biological studies. Clearly there are a lot of problems that still remain to be interpreted on a chemical basis.

Regardless of whether the transport involves active or passive mechanisms, the interactions between platinum complexes and membrane components will be involved. Without consideration of these interactions, the differences between complexes with different structures cannot be explained.

2.7.1. Transport rate of platinum complexes

The rate of transport and the intracellular accumulation of platinum complexes are doubtless dependent on their reactivity with membrane bound molecules and this reactivity depends on the structure. The rate of uptake of cisplatin analogues by erythrocytes was determined by monitoring the platinum concentration in the cytosol [45]. The uptake *vs.* concentration relationship follows a straight line for *cis*-DCDP, *cis*-DBDP, and *cis*-DIDP and their rates of uptake were found to increase with increasing hydrophobicity, *i.e.* *cis*-DCDP < *cis*-DBDP < *cis*-DIDP. These complexes are likely to be transported mainly by passive diffusion. No significant reactions with proteins were expected. On the contrary, the rate of uptake of hydrolytic products is remarkably lower than for cisplatin, indicating that the binding of platinum to proteins retards the entry of the complexes. A curved uptake vs. concentration relationship was obtained for transplatin and the transport rate was very rapid. A similar, but less pronounced, result was obtained for $PtCl_4^{2-}$ [45]. It is probable that they enter cells by a different mechanism from cisplatin. Although transplatin and $PtCl_4^{2-}$ are both reactive to proteins, their diffusion is not suppressed by the binding. Thus, we may postulate that these two complexes are mainly taken up *via* a carrier-mediated mechanism. Based on these observations, we can speculate that binding to proteins can play two opposite roles in the transport of platinum complexes. The irreversible strong binding leads to retardation whereas the reversible binding to an appropriate carrier protein facilitates the transportation. The reactions with proteins are unavoidable for any complex, but the effect on transport depends on the presence of a carrier.

To clarify the relationship between uptake and reactivity with the structure of the complexes, we have determined the rate of uptake of the complexes with 1*R*,2*R*-, 1*R*,2*S*- and 1*S*,2*S*-dach as carrier ligands [46]. For human erythrocytes the first-

order uptake constants k decrease in the following order where the k (h^{-1}) value is given under each formula:

$$\underset{(0.045)}{PtCl_2(1R,2R\text{-dach})} > \underset{(0.033)}{Pt(ox)(1R,2R\text{-dach})}$$

$$> \underset{(0.028)}{PtCl_2(1R,2S\text{-dach})} \approx \underset{(0.027)}{PtCl_2(1S,2S\text{-dach})}$$

$$> \underset{(0.015)}{Pt(ox)(1R,2S\text{-dach})} \approx \underset{(0.013)}{Pt(ox)(1S,2S\text{-dach})} > \underset{(0.0058)}{cis\text{-DCDP}}$$

It is evident that, for the same leaving group, the rate of uptake of *R,R*-dach complexes is higher than *S,R* and *S,S* complexes. With the same carrier ligand, the chloro complexes diffuse in more rapidly than the oxalato complexes. All the complexes are transported into the cells much more readily than cisplatin. By comparing the extent of decrease in the concentration of thiol groups, it was shown that all the oxalato complexes are less reactive than cisplatin. The higher uptake of the oxalato complexes is likely to be due to less retardation by the membrane, at least partially.

No significant disparities were found among the three isomeric dach complexes in their reactivity with protein thiol groups, but we cannot overlook the contribution of protein binding to the different transport rates of these complexes. The conformational changes of the membrane proteins caused by these complexes were compared. The rotational correlation time τ_c calculated from the ESR data was used to evaluate the freedom of the motion of the labelled thiol groups (Table 8) [46].

The data in Table 8 show that these chiral isomers affect the conformation to different extents and the sequence of alterations is in accordance with that of uptake rate, i.e. *R,R* gives the highest τ_c and S:W values. We have discussed the influence of platinum binding on the permeability of the membrane. The platinum binding and the conformational change might be the reasons leading to the alteration of membrane structure and resulting in the change of permeability. The inward diffusion of ascorbate and the outward diffusion of TEMPO were studied for human erythrocytes as the model system. The results showed that all the complexes studied

Table 8
Rotational correlation time τ_c and S:W ratio of human erythrocyte membrane affected by Pt(II) complexes[a]

Sample	S:W	τ_c ($\times 10^{-10}$ s)
Control	0.115	0.849
cis-DCDP	0.168	3.55
CDCDP	0.116	1.15
Pt(ox)(1*R*,2*R*-dach)	0.146	1.84
Pt(ox)(1*R*,2*S*-dach)	0.120	1.39
Pt(ox)(1*S*,2*S*-dach)	0.119	1.20

[a] Determined by measuring the ESR signal of maleimide spin labelled thiol group.

depressed the transport of ascorbate and the *R,R* isomer gave the least influence. The sequence is consistent with the uptake and the conformational changes. Differing from these results, the efflux of TEMPO was influenced in a different way. The *R,R* and *S,S* isomers cause little influence, and the *R,S* promotes, but cisplatin inhibits the diffusion. In summary, the chirality of the carrier ligand determines the tendency of a complex to be transported. The reactivities to proteins, as shown by the decrease in thiol groups in the membrane, are nearly the same, but the conformational changes are different. For this reason, the difference in uptake is probably related to the chiral recognition by the membrane. We have synthesized a series of complexes of *R,R*-, *S,S*- and *S,R*-dach and their uptakes were compared. Those with *R,R*-dach as ligand are taken up most readily.

2.7.2. Permeability and uptake

The relationship between uptake and the permeability of cellular membranes is obvious. It has been shown that the reversible electropermeabilization of the plasma membrane in cultured human NHIK3025 cells increases the cisplatin uptake [47]. The elevation of temperature or the action of digitonin was demonstrated to enhance the uptake of cisplatin significantly through the interference with the membrane structure [48].

We have mentioned that the binding of a certain foreign molecule to the membrane molecules may induce the changes in membrane structure and permeability. By ESR measurements we found that cisplatin binding to human erythrocyte membrane causes increased inward diffusion of ascorbate anion and outward diffusion of TEMPO [36]. Perhaps related to our result is the report by Suniti and Bhola that, in animal experiments, ascorbate enhances the anticancer activity of cisplatin, if they are administered simultaneously. They interpreted these observations by postulating that ascorbate increases the transmembrane permeability [49]. A positive cooperative effect is possible in the uptake of platinum complexes, i.e. the initial binding to the membrane leads to an alteration in membrane permeability which facilitates the further entry of cisplatin.

2.7.3. Efflux of cisplatin

The rate of cisplatin efflux is an important factor in determining the intracellular accumulation. The cell membrane plays the role of a barrier for not only uptake by the cells but also the efflux from the cells. Based on the studies with 2008 cells, Mann et al. [50] found that the kinetics of the efflux of cisplatin is biphasic in nature, with a very rapid initial phase followed by a much slower phase. However, the mechanism of efflux is seldom studied and the involvement of the interaction of the complexes with the membrane components is expected, but unknown.

By means of spin-labelled *cis*-diamminedichloroplatinum (SLDCDP), *cis*-diamminediaquoplatinum (SLDADP), *cis*-diamminedibromoplatinum (SLDBDP) and *cis*-diammineiodoplatinum (SLDIDP), the kinetics of efflux was investigated by ESR measurements [51]. The spin-labelled platinum complexes were incorporated in the human erythrocyte membrane and their outward diffusion was monitored on the basis of the reduction of the platinum complexes by extracellular ascorbate,

which diminishes their ESR signal. The kinetics was found to be consistent with Mann et al.'s result: a rapid initial phase followed by a slower phase. The time required for half-diffusion of SLDADP (2.3 ± 0.23 min) is less than those of SLDCDP and SI DIDP (2.7 ± 0.22 min and 2.9 ± 0.21 min respectively). In the first stage, the efflux rate decreases in the order SLDADP > SLDIDP > SLDCDP. After a few minutes, in the second stage the sequence is reversed. The difference could be interpreted in terms of three different interactions. The electrostatic interaction between the charged membrane surface and the cationic aquo species accelerates the outward diffusion. The covalent binding of platinum with membrane proteins inhibits the efflux strongly. The hydrophobic interaction between the platinum complexes and the lipid bilayers is relatively weak, but it might also cause some difference between complexes. It is reasonable that in the case of SLDADP the diffusion of the unbound cationic complexes is more rapid than the others under the influence of the electrostatic interaction, but the diffusion of the fraction bound to protein is slower than those kept in the bilayer by the weak hydrophobic interaction. This interpretation is also supported by the difference in temperature of the phase transition after reaction with various platinum complexes.

The transmembrane transport of cisplatin and its analogues was compared by determining the cytosolic platinum concentrations as a function of incubation time. Among the complexes studied, transplatin was transported most rapidly, and, after a continuous increase in cytosol platinum concentration for 3 h, the cytosolic platinum concentration decreased, showing that the efflux surpassed the entry [45].

3. Events occurring in the cytoskeleton

For a cell to be living depends on the maintenance of the normal function of the cytoskeleton, which keeps the cell as an integral system with each component in its proper state, allowing the cell to be ready to move, to respond, to grow, to divide and to differentiate. Nevertheless, it is sensitive to attack by foreign compounds and subject to damage, which is sometimes fatal. The cytoskeleton is a three-dimensional composite system constructed from individual networks, microtubules, microfilaments and intermediate microfilaments, as well as the membrane skeleton. These are interlinked and assembled in a specific and orderly way. Each network is assembled from its own linear polymers with corresponding linking proteins, while the polymers (for instance the microtubules or microfilaments) are in turn the product of the self-association of specific monomers; G-actin for the microfilament, and tubulin for the microtubule, etc. Every polymerization system and the overall cytoskeleton are maintained in a steady state by controlled polymerization–depolymerization. The system is in a dynamic state and ready to be modulated. To such an interwoven, hierarchic structure, even a perturbation to the monomer will lead to the disorganization of the cytoskeleton as a whole.

The association of G-actin to F-actin or tubulin to microtubules can be brought about in the chemical systems actin–$Mg^{2+}(Ca^{2+})$–K^+-ATP and tubulin–$Mg^{2+}(Ca^{2+})$–K^+-GTP. The polymerization consists of a sequence of events; the

sequences are similar in both cases. It starts from the binding of divalent ions to the monomers whose conformation then changes to adapt to the requirements for the association. By binding of ATP or GTP to actin or tubulin, the self-association proceeds in a controlled way. The chemical mechanism of the process can be described as shown in Scheme 2.

It is reasonable to expect that these processes would be liable to be influenced by metal ions, because both the proteins and the nucleotides are potential targets of metal ions. Different mechanisms are proposed for various metal ions as follows.

(1) Some metal ions mimic the ions essential for the association, such as Mg^{2+} or Ca^{2+}, but there are thermodynamic or kinetic disparities. For this reason, the steady state stability and repairability of the polymers, as well as the morphology of the polymers, will be affected.

(2) Some "soft" metal ions are different from the essential ions in their high reactivity to proteins or nucleotides. They may induce anomalous aggregation by covalent or electrostatic interactions etc.

(3) Some metal ions promote the depolymerization of the polymers, and some induce the degradation. Both of these result in a decrease in molecular size, but the latter is not reversible or repairable. The reactions involved in this aspect include the following:

(i) the metal binding changes the conformation to a conformation unfavourable for the appropriate association;

(ii) the metal ions bind to the sites located in, or near, the contact area of the monomers;

(iii) the metal ions induce a hydrolytic or oxidative cleavage of the peptide chain;

(iv) the insertion of metal ions between the binding regions of the neighbouring monomers.

The effects of a variety of metal ions on microtubule systems have been studied in relation to their toxicological mechanism. It is well recognized that the microtubule system is relatively unstable and sensitive to exogenous substances. A few studies have been devoted to the effects on microfilaments. The microfilament, although more stable than the microtubule, is expected to be the critical target of some metals,

Metal binding	$A+M^{n+}\rightarrow M-A$
Conformation change	$MA\rightarrow MA^{*}$
ATP (GTP) binding	$A+ATP\ (GTP)\rightarrow A\text{-}ATP\ (GTP)$
	or $MA^{*}+ATP\ (GTP)\rightarrow MA^{*}\text{-}ATP\ (GTP)$
Self-association	$MA^{*}\text{-}ATP\ (GTP)+A_n\rightarrow A_nA\text{-}ATP\ (GTP)$
Hydrolysis of ATP	$A_nA\text{-}ATP\ (GTP)+H_2O\rightarrow A_{n+1}ADP\ (GTP)$
Length regulation	$A_n\rightarrow A_i+A_j\ (i,j<n)$
	$A_j+A_j\rightleftharpoons A_{2j}$
	$A_i+A_i\rightleftharpoons A_{2i}$
	$A_i+A_j\rightleftharpoons A_{i+j}$

Scheme 2.

as shown in a few investigations. Lithium ions accelerate the association, leading to an increase in the number of polymers, owing to their similarity to the essential ion K^+ [52]. The metal ions of higher softness, Cu^{2+} [53] and Cd^{2+} [54], inhibit the polymerization and destroy the microfilaments, whereas AlF_4^- [55] and BeF_3^- [55] stabilize the actin polymers by a mechanism in which these complexes act as the analogue of PO_4^{3-} in a 5-coordinated bipyramidal intermediate for the hydrolysis of nucleotide ATP. This is the same basis for the stabilizing effect of vanadate to microtubule.

Fumarulo and Aresta proposed that the nephrotoxicity of cisplatin is related to the binding to tubulin and the inhibition of microtubule polymerization [56]. This is just a tentative insight into the cisplatin–cytoskeleton interaction. No reports appeared on the effects of cisplatin and its analogues on the cytoskeleton until Xiao et al. [57] and Kopf-Maier and Muhlhausen [58] published their results of microscopic studies on the cytoskeleton. The morphologic results showed clearly that the platinum complexes attack the cytoskeletal networks. Whether or not this effect plays any role in the cell killing is not clear. It is expected that, in the cisplatin-mediated disturbance of the state of the cytoskeleton, the core events will be the metal binding to the proteins (either monomers or polymers), the conformational changes and the abnormal association or aggregation.

3.1. *Effects on microfilaments*

3.1.1. *Reactions of platinum complexes with G-actin and the subsequent events*

Starting from the monomer, G-actin, we focused our studies on the platinum binding, the conformational change thus induced and the abnormalities in polymerization.

The binding of $PtCl_4^{2-}$ to G-actin was studied by determining the change in NPM label fluorescence as a function of changing $PtCl_4^{2-}$: actin mole ratio R. It was found that as the first step, when $R < 25$, the $PtCl_4^{2-}$ ions are bound to sulphur-containing groups preferentially. These high affinity sites are characterized by the site number $n_1 = 30$ and average binding constant $K_1 = 1.0 \times 10^7\ M^{-1}$ [59].

The results showed that, by platinum binding, $PtCl_4^{2-}$ or *cis*-DCDP or *cis*-DADP caused G-actin to change from a globular, compacted protein to an opened conformation. The *N*-terminal, which is important in association, moves to the bulk aqueous medium and its secondary structure is destroyed to a considerable extent. The conformation evidently does not fit the requirement for monomer–monomer interaction and self-association.

The self-association has been studied generally by turbidity and viscosity measurements. In fact, the information drawn from these experiments is rather macroscopic and less discriminative of the different processes that result in the alteration in polymer size. However, the turbidity data indicate that, in the presence of cisplatin in the polymerization solution of G-actin, the apparent critical concentration decreases and the initial rate of polymerization increases [60]. We use the term polymerization here instead of association, because we cannot discriminate as to whether the increase in turbidity is due to association or other processes leading to

the same results, such as cross-linking and aggregation. The results of viscometric studies shed a little light on the nature of these effects. The viscosity is determined by the shape of the polymers, and thus the viscosities given by the linear polymers are different from those of the aggregates composed from the same number of monomers. The results showed that cisplatin retards the rate of polymerization and depresses the final degree of polymerization. By comparing these results, we postulate that platinum binding inhibits the association, as shown by viscosity, but in contrast causes aggregation or cross-linking of the monomers or fragments.

Thermokinetic studies on the G-actin–Mg^{2+},K^{+}–ATP system provide additional evidence that platination inhibits the association [61]. The thermal effects of these systems were determined as a function of time and compared with those of the corresponding reference systems, actin–Mg^{2+},K^{+}, actin–*cis*-DADP and actin–*cis*-DCDP. By this means the thermal effects of platinum binding, conformation change and association can be discriminated. The endothermic reaction following the initial rapid exothermic process was attributed to the self-association of G-actin, although the contribution from the delayed conformational change could not be completely excluded. The reactions related to the initial exothermic peak are complicated by the overall result from metal binding, conformation change and, in the case of cisplatin, the hydrolysis of this complex. The contribution of self-association in the presence of various concentrations of platinum complexes is reflected in the values of ΔH_2, the enthalpy change of the endothermic peak. As shown in Table 9, ΔH_2 dropped pronouncedly with increasing concentration of cisplatin, showing that cisplatin retards the association. The ΔH_2 values decrease more rapidly with concentration in the case of transplatin. A very low concentration of transplatin exerts a pronounced effect. There are two contributors to ΔH_1, the hydrolysis of cisplatin and the binding of platinum to the protein. The latter would be the origin of cross-linking, another pathway to molecular size increase. The ΔH of hydrolysis can be estimated by comparing the ΔH of cisplatin–actin and *cis*-DADP–actin systems. It is likely that the association is a very slow reaction and during the course of the reaction the linear polymers grow continuously, as characterized by the increasing average length of the population of the polymers. Cisplatin affects the association either *via* simple inhibition or by the induced aggregation and cross-linking. If the side reactions proceed simultaneously but with different rates, we could expect that the increase in the polymer length will be non-linear.

The temporal change in polymerization was measured by means of quasi-elastic light scattering. The results showed that the average length of the population of polymers increases continuously with time, provided that the ATP concentration in the polymerization media is sufficient for the association. However, we observed that a shortage of ATP caused "bifurcation" of the polymers, owing to the lowered stability. The presence of cisplatin and transplatin causes overshooting by cross-linking or aggregation, but on the contrary destabilizes the polymers. Thus the addition of these platinum complexes triggers the "bifurcation" [62].

The results discussed above are limited to the chemical process in the polymerization system including ATP and Mg^{2+} or Ca^{2+} ion. In a simple cisplatin–actin system, only aggregation due to platinum binding was observed.

Table 9
ΔH values of actin polymerization system in the presence of cisplatin and transplatin

System	R (μmol Pt (mg actin)$^{-1}$)[a]	ΔH_1 (kJ mol^{-1})	ΔH_2 (kJ mol^{-1})
Actin	0	−3.93	52.01
Actin–cisplatin	0.038	−2.53	7.60
	0.110	−0.39	6.95
	0.156	—[b]	6.67
	0.363	—[b]	4.78
	0.517	−2.52	—[b]
	0.660	−8.11	—[b]
	1.275	−25.33	—[b]
	2.089	−57.12	—[b]
Actin–transplatin	0.001	−0.311	8.44
	0.003	—[b]	3.01
	0.007	—[b]	1.00
	0.018	—[b]	1.09
	0.089	—[b]	0.82
	0.134	−42.00	—[b]
	0.178	−43.26	—[b]
	0.223	−533.03	—[b]

The thermograms were measured with a Calvet MS-80 microcalorimeter. The actin solutions were mixed in the calorimeter with the polymerization solution (18 mmol $Na_2ATP\ l^{-1}$, 10 mmol $MgCl_2\ l^{-1}$, 450 mmol $KCl\ l^{-1}$) in which various amounts of platinum complexes were added. Measuring temperature, 310.15 K. ΔH_1 and ΔH_2 were estimated on the basis of the areas of the corresponding peaks.

[a] Concentration of actin, 4 mg ml^{-1}.

[b] Not determined.

In summary, platinum(II) complexes with chloride ions as the leaving groups tend to bind with the G-actin, mainly as a result of the formation of Pt—S bonds. The binding induces the change in conformation, but it is different from the Mg^{2+}-induced conformational change. The protein becomes more opened, the secondary structure is partially lost and the *N*-terminal region moves out into the aqueous environment. The conformation is unfavourable to association, but the Pt—S bonding induces cross-linking. These two reactions exert an influence on the polymerization in opposite directions. Under the condition of polymerization, the Pt—S binding occurs as the initial event and it proceeds rapidly, while the association is the slow, secondary reaction. This is why the molecular size increases slightly in the beginning and declines slowly. Both cisplatin and transplatin suppress the final polymerization but the results are different.

3.1.2. Reactions of platinum complexes with F-actin and the subsequent events

As observed by fluorescence microscopy, incubation of Ehrlich ascitic cancer cells with platinum complex causes disorganization of the cytosolic and nuclear microfilament systems. The mechanistic interpretation is not yet clear. It is not known

whether the effect is due to the direct reaction with F-actin and what chemical events have occurred.

The binding of platinum to F-actin is different from that to G-actin, because several binding sites have been blocked in the polymers, especially those adjacent to the contact region, i.e. met 44, met 325 and perhaps cys 374. If the concentration of platinum is relatively low, the complexes are bound to the accessible sites on the lateral surfaces only. In cases of high platinum:actin ratios, once the unmasked sites are fully occupied, the excess platinum would cut through the interacting sites in F-actin and bind to the exposed sites.

By fitting the parameters of metal-to-ligand charge transfer bands of cisplatin-bound F-actin with those calculated by a molecular orbital (MO) approach, it was shown that the square planar platinum(II) atoms bind to sulphur and amino nitrogen with ammonia ligands remaining in the coordination sphere. Pt–OR binding was identified also [63]. Fourier transform Raman spectra of these systems support the occurrence of Pt—S binding by the appearance of a Pt—S vibration accompanied by an increase in the intensity of the C–S vibration, and they are both dependent on cisplatin concentration. A similar feature was observed in spectra of transplatin–actin, but an additional Pt–N stretch was observed (Tables 10 and 11). This was

Table 10
Frequency of Pt–S stretch in the cisplatin–F-actin system[a]

Cisplatin concentration (mmol l^{-1})	0	0.098	0.145	0.283	0.349
Pt–S stretch (cm^{-1})	0	473	480	472	477
Relative intensity[b]		0.84	0.86	0.92	0.98

[a] The solutions contain 1.05×10^{-4} mol F-actin l^{-1}, 0.2 mmol Na_2ATP l^{-1}, 0.2 mmol $CaCl_2$ l^{-1} (pH 8, D_2O).

[b] Relative to band at 185 cm^{-1}.

Table 11
Frequencies of Pt–S and Pt–N stretches of transplatin–F-actin system[a]

Transplatin concentration (mmol l^{-1})	Pt–S stretch frequency (cm^{-1}) (intensity)	Pt–N stretch frequency (cm^{-1}) (intensity)
0	None	None
0.014	470 (0.87), 427 (0.92)	380 (0.94)
0.029	470 (0.90), 427 (0.92)	371 (0.86)
0.073	471 (0.89), 426 (0.89)	394 (0.86)
0.101	470 (0.89), 428 (0.91)	382–403 (0.87)
0.129	470 (0.90), 426 (0.89)	374 (0.85), 381–401 (0.87)
0.169	470 (0.91), 427 (0.89)	377–382 (0.88)
0.272	470 (0.91), 428 (0.91)	387 (0.88)

[a] Same conditions as for Table 10; intensities relative to band at 185 cm^{-1}.

not observed in the case of cisplatin. This difference might be interpreted by the lower specificity of transplatin to proteins.

The changes in conformation are evidently the most important alteration mediated by platinum binding. The changes are different for cisplatin and transplatin owing to the different binding modes. As reflected in the IR and Raman spectra (Table 12), more features were observed for transplatin. In both cases, the amide I bands (about 1651 cm^{-1}) are intensified by platinum binding, but cisplatin gives a more marked effect. A notable difference is the appearance of a very sharp band at 1698 cm^{-1} in the transplatin system. It is likely to be due to the exposure of the primary amide group, probably gln 41, in the contact region. The α-helix content is reduced by both cisplatin and transplatin and more significantly by the latter, as shown in the decreased intensity of the approximately 1650 cm^{-1} band (amide I) in Raman scattering spectra (Table 10). Similar conclusions were obtained from CD spectra.

In summary, cisplatin binds to F-actin by formation of a Pt—S bond, which is strong and specific. The binding causes a pronounced conformation change. On the contrary, transplatin binds to S donors in the protein with less affinity, but binding to N donors becomes important. The disassembly caused by transplatin is thus much greater than that of cisplatin.

Actually, the polymerization state of F-actin is altered in quite different ways by cisplatin and transplatin, although the final results are both towards depolymerization. Turbidity measurements during the course of the reaction showed that, for the cisplatin–F-actin system, a slow increase in turbidity (measured as A_{232}) is followed by a very slow decrease, and the final state is approached beyond 15 h. In the case of transplatin, a very fast and large increase occurred at the start and a rapid drop in turbidity appeared within 1–2 h.

The change in turbidity as a function of the Pt:actin ratio has a similar feature in the difference between cisplatin and transplatin, a slight increase and then a slow decrease for cisplatin, but simply a rapid decrease for transplatin. The viscosity changes in a similar way with either time or Pt:actin ratio. It is likely that cisplatin cross-links the actin molecules at a low Pt:actin ratio and in the initial stage of the reaction. This is the result of the lateral binding. A longer incubation time, or a

Table 12
The features of Fourier transform Raman and IR spectra of cisplatin- and transplatin–F-actin

	Fourier transform Raman		IR	
	ν (cm^{-1})	Assignment	ν (cm^{-1})	Assignment
F-actin	1620		1621 (w)	
	1650	α-helix		
F-actin–cisplatin	1675	Antiparallel β-pleated sheet	1621 (w)	Amide I
			1654 (s)	Amide I
F-actin–transplatin	1625		1620 (w)	Amide I
	1650 (w)	α-helix	1654 (s)	Amide I
			1698 (vs)	Amide I

higher Pt:protein ratio, enables the platinum to "squeeze" in between the neighbouring monomers. After the conformation change, the platinum tends to bind to the newly exposed binding sites. Transplatin attacks the intermolecular binding more readily because of a higher propensity for ligand exchange reactions and a lower tendency for cross-linking, and thus the F-actin molecules are depolymerized and cross-linked from the beginning. This disparity is also reflected in the thermograms. The exothermic reaction is attributed to depolymerization. In the absence of platinum complexes the F-actin tends to depolymerize spontaneously and slowly after a long induction period (more than 30 h), and cisplatin shortens the induction period (about 3 h); in the transplatin–F-actin system only an initial large exothermic peak appears in the thermochemical process. This is consistent with simultaneous cross-linking and depolymerization, both of which proceed rapidly [61]. The quasi-elastic light scattering measurements discussed above showed that cisplatin initiates the depolymerization and cross-linking of both the polymers and the fragments.

We have only talked about the simple F-actin system. The cross-linking of F-actin with other biopolymers cannot be ignored, for instance, the binding of F-actin to DNA [64].

3.2. The effects on other skeletal proteins

3.2.1. Spectrin and tubulin

The membrane skeleton of erythrocytes is composed of the spectrin network, in which spectrin tetramers, as the building blocks, are interwoven together through binding with the ends of microfilaments and the linking protein, band 4.1 protein. The tetramer is in turn built up from dimers and the molecules are characterized by their high molecular weight, the slender morphology (100 nm in length and 5 nm in diameter) and the richness in α-helix. The linear dimers differ from microfilaments and microtubules in that they are not the polymerization products of a monomer; thus their formation is not susceptible to chemical disturbance. However, they are susceptible to cross-linking, with either another spectrin molecule or other proteins. As mentioned above, the sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) studies on the reaction of platinum complexes with erythrocyte membrane revealed that, after incubation with cisplatin, a high molecular weight band was observed and the spectrin bands diminished [28]. The binding of cisplatin to spectrin tetramer was studied by means of a fluorescence method. Binding to thiol groups and amino groups was postulated, since, when the thiol and amino groups of platinated spectrin tetramer were labelled with FMA and fluorescamine respectively, the fluorescence intensities of the two labels were suppressed and these effects were dependent on the concentration of the platinum complex. The platinum binding leads to a conformational change, as shown in the quenching of the intrinsic fluorescence. Meanwhile, at low Pt:spectrin ratios, the α-helix content decreases, but it increases on further increasing cisplatin concentration [65].

Although Kopf-Maier's microscopic studies showed that the microtubule system is sensitive to cisplatin, no extensive studies on this topic have been reported. Our preliminary study showed that cisplatin and its analogues inhibit the polymerization

of tubulin, and their effects decrease in the sequence *cis*-DCDP > *cis*-DBDP > *cis*-DIDP. Further studies are in progress.

3.2.2. The extension of cytosol cytoskeleton to membrane and nucleus

The microfilamental system is not an isolated network confined in the cytosol. It joins with the membrane lipids or membrane proteins to build up a pathway for signal transduction which is involved in a number of cell functions. In the other direction, the microfilaments extend into the nucleus. Therefore, the effects on F-actin are possibly transmitted to, or from, the membrane or nuclei. Luo et al. have examined the changes in the microfilament assembly in ascitic liver cancer cells under the action of laminine and dichlorodimethyldopamidoplatinum(II) (MDP). The results showed that, after laminine binds to its receptor, the assembly of microfilaments is promoted, but it is inhibited by platinum. This indirect effect is probably important in cell function [66].

The events in the cell nuclei are not simply the formation of DNA adducts. Platinum-mediated protein–DNA cross-linking is also important. Among these reactions, actin-DNA cross-linking has been reported [63].

4. Other events

4.1. Apoptosis

It has long been understood that cisplatin kills the cells by inhibiting DNA synthesis as the result of binding to DNA. This is actually an oversimplified interpretation because it gives no information on the process of cell death. A cell progresses to death by two different pathways, necrosis or apoptosis, characterized by entirely different events. Apoptosis is known as physiological cell death and is characterized by events including shrinkage of the cell and condensation of the chromatin, followed by membrane blebbing and release of apoptotic bodies into the surrounding environment. Biochemically, apoptosis is characterized by DNA double-strand breaks at specific sites in the chromatin. Being a heavy metal the toxicity of platinum has traditionally been recognized as being due to the induction of necrosis, which is characterized by swelling of the cell, membrane damage, leaking of enzymes from lysosomes and cell lysis. Sorenson and Eastman, basing on their work of the action of cisplatin on the cell lines CHO/UV41 and CHO/AA8, claimed that there is no simple relation between cell death and inhibition of DNA synthesis. They found that 50% of the CHO/UV41 cells are killed by a 2 h incubation with only 0.06 $\mu g\ ml^{-1}$ of cisplatin, but during the first 12 h after cisplatin treatment no inhibition of DNA synthesis was observed even up to 0.25 $\mu g\ ml^{-1}$. They noted the characteristics of apoptosis in the cells [67]. In a recent work on human carcinoma CH1 cells, Ormerod *et al.* concluded that cisplatin kills these cells by inducing apoptosis. Their results indicated that a DNA double-strand break at a site in the chromatin loops is a critical event [68]. We can describe the cisplatin-induced apoptosis by the sequence of events shown in Scheme 3.

cisplatin treatment
↓
G2 phase arrest
no changes in RNA and protein synthesis, no changes in the pools of ATP and NAD
↓
DNA double-strand break, inhibition of transcription and translation
depletion of ATP and NAD, shrinkage and blebbing
↓
blebs burst, membrane integrity lost
↓
formation of apoptotic bodies

Scheme 3.

The biological events call for interpretation at a molecular level. In their earlier works, Sorenson and coworkers emphasized the role of endonuclease in the cleavage of DNA, but recent work showed that programmed death is a complicated process, with the involvement of several factors and several chemical events in the cells [17]. There are several missing links in the intervention of cisplatin in the process. How does it arrest the cells in G2 phase? How does the signal transduced activate the cleavage of DNA? Does the platinum complex interfere with the gene expression of certain regulatory peptides? As Nishio and Saijo postulated, the effect of cisplatin on arresting the cells in G2 phase was explained by inhibition of p34cdc2 dephosphorylation [69]. A noteworthy development made by Zalewski et al. [18] indicated that zinc ions play a critical role in the suppression of apoptosis. Removal of Zn(II) was found to enhance apoptosis. As one of the early events, reactive oxygen species are involved as the key factors in the apoptosis. These two facts are interrelated. For instance, hydrogen peroxide induces apoptosis and causes a rapid increase in intracellular Zn, detectable by Zinquin. The blocking of ROS generation tends to suppress the apoptosis [70]. The mechanism of metal-induced apoptosis has not been studied extensively. It is quite an attractive topic for bioinorganic studies of cells.

4.2. Glutathione, metallothioneins and drug resistance

The major side effects limiting the clinical effect of cisplatin are its nephro- and hepatotoxicities and the drug resistance. They are related to the level of unbound platinum complexes. It has been postulated that both glutathione (GSH) and metallothionein (MT) play important roles in the development of both toxicity and drug resistance. GSH, together with a number of S donors, is able to reduce the nephrotoxicity [71]. Bismuth [72] and zinc [73] salts have been suggested for use as suppressors of toxicity by promoting the expression of apometallothionein, which immobilizes the platinum, by the formation of metallothionein complexes. However, accumulating evidence indicates a relationship between drug resistance and intracellular GSH and MT levels. It has been indicated that the cisplatin resistance of several cell lines and by the induction of cisplatin has been interpreted in terms of MT

binding. A high MT pool has been shown to be related to drug resistance [74], but it is controversial [75]. The high sulphur affinity of platinum and the stable complexes thus formed, especially those in form of the robust clusters of MT (the apparent equilibrium constants for the reaction $CdMT + Pt^{2+} \rightleftharpoons PtMT + Cd^{2+}$ were determined to be 1.72×10^{23} for *cis*-DCDP and 1.78×10^{23} for *trans*-DCDP [76]), will mean that GSH and MT will compete effectively with the DNA or protein targets. The result will be the formation of complexes of GSH or MT and inhibition of the reactions with the targets. In addition, a platinum-bridged ternary complex, such as GSH–Pt–nucleotide, can be formed [77]. Free cisplatin, or its hydrolysis products, is thus kept at a certain low level, depending on the concentrations of GSH and MT. If most of the platinum complexes are sequestered and thus deactivated, both the anticancer activity and the toxicity are reduced. In contrast, the depletion of GSH, or a shortage of MT, will cause a higher sensitivity and higher activity. The level of free platinum complex can be estimated on the basis of complex formation in thermodynamic terms, but actually it is also determined by the kinetics of the formation and dissociation of the complexes with GSH and MT and the biosynthesis of GSH and apoMTs, which are controlled by the cells and biological factors. Thus the relationship between GSH or MT and toxicity–drug resistance is the net result of a sequence of events that occur in the cells.

The platinum complexes are captured by GSH as soon as they enter the cells and a variety of complexes of different stoichiometry might be formed. Among the species identified $Pt(GS)_2$ is the species pumped out by an ATP-dependent GSH conjugate export pump [44,78]. The GSH-bound platinum species work as the labile pool, from which the platinum is transformed into reactive metabolites under the action of β-lyase and *S*-oxidase [79]. Thus the cisplatin is activated through the binding–releasing process. A relationship between high GSH and MT levels and drug resistance has been reported in some cases [80]. By inducing MT expression, or supplementing GSH levels, the cells are rendered less sensitive in response to platinum complexes, i.e. lower toxicity and higher resistance [81]. On the contrary, the depletion of GSH by inhibiting its synthesis causes lower resistance, but higher toxicity.

An alternative mechanism conceived to explain the toxicity attributes the effects to damage to phospholipids caused by ROS [82]. The lipid peroxidation is regarded as the initial event and the lipid peroxides, and other relevant free radicals, are the likely mediators for the subsequent amplification of the effects. In the modulation of ROS-induced cell damage, both MT and GSH have been shown to be effective in scavenging the peroxides and free radicals. It is reasonable that, by similar considerations, they will be effective in suppressing the platinum toxicity.

Both GSH and apoMT are synthesized under the control of the cells, but the synthesis of apoMT is inducible by sulphur-affinitive metals, such as Zn and Cd, and also platinum complexes. The induction of MT synthesis and platinum binding have been studied, but conflicting results were reported [83]. Sarkar [84] has shown by experiments with IAr (human choriocarcinoma) cells that the hydrolytic product of *cis*-DCDP induces apoMT synthesis, but the unhydrolysed cisplatin, as well as transplatin and its hydrolytic product, is not able to promote MT synthesis. Zhang

et al. reported recently that, following injection of cisplatin and transplatin in rabbits, *cis*-DCDP induced MT synthesis in liver [85]. In contrast, $[PtCl_4]^{2-}$ was effective in elevating MT levels in liver and kidney. However, all the platinum complexes were poor inducers of MT biosynthesis compared with Cd or Zn compounds. When MT was induced by injection of zinc nitrate, followed by *cis*- and *trans*-DCDP, more platinum was found to be incorporated in MT for transplatin than for the cis isomer [73].

Bongers et al. [86] investigated the binding of $PtCl_4^{2-}$ to equine renal MT. They found that at neutral pH, the monomeric Pt_7MT was formed and at pH 2, a polymer with 17 ± 2 mol Pt (mol MT)$^{-1}$ was formed. The polymer is attributed to peptide cross-linking via formation of Pt—S bonds. The formation and dissociation of metallothioneins are different from those of GSH in vivo, in that the platinum complexes of MT are not formed by the direct binding of platinum with the apoMT. Under normal conditions, they exist in the form of ZnMT or CdMT, with the metals incorporated in two clusters of high stability. Depending on the concentration of the metals, the metallothioneins are partially or entirely saturated by binding 1–7 metals per mole of apoMT. The reaction product of $[PtCl_4]^{2-}$ with Cd,Zn MT is different from that formed by direct binding to apoMT, in that only three Pt atoms are captured in the β cluster, and an additional platinum coordinates to the *N*-terminal CH_3S–methionine [85].

5. Concluding remarks

In the present paper, we attempt to discuss some standpoints for the understanding of metal–cell interactions. First of all, the cell responds as a multiple target system to the attacking metal ions. Secondly, the chemical events induced by metal binding should be investigated as a process. We are used to looking at the working mechanism of metal-based drugs (MBDs) with the same viewpoint as for organic drugs. Actually, in principle, there are large differences in their chemicobiological interactions. For metal complexes, there are a number of targets in the cells. The drug–cell interaction is far more complicated as a result of the numerous interlinked reactions. We cannot interpret the destiny of the attacked cell by a single reaction at a single target. We cannot even discern which is the fatal target molecule and which is the fatal reaction. That is why we should put the events together for a description of the working mechanism of these complexes. Any biological effect is merely the manifestation of a process characterized by the sequence of events that occur in specific sites and specific times.

The process is primarily composed of the metal complex induced events. If we focus on what happens in a subsystem of the cell, i.e. the cytoskeleton, the plasma membrane, etc., most processes comprise an initial rapid metal binding followed by a conformation change, which, in turn, mediates the change in supramolecular structure and the change in function. These events might be transmitted to other targets, or other sites, where some new events commence, and so on.

If we look at the events that happen in a whole cell, the competition among the

potential targets will dominate where and when each reaction occurs and how they are organized. Superficially, the metal ions attack the cells with selectivity lower than that of an organic molecule, but, because the affinity and the accessibility of different targets vary markedly, under low concentration of metal ion a selective reaction will happen in the sites of high affinity and high accessibility. If the concentration of the metal complex is increased continuously, the metal will bind to the sites of lower affinity and the selectivity will be reduced. However, we have seen that in the case of platinum complexes, no matter whether the binding is strong or weak, permanent or transient, its perturbation might be transmitted and even amplified further, because the process is proceeding in an open system. We emphasize here the meaning of dose dependence, which turns a metal complex from a drug to a toxicant since the dominant processes are altered. To describe and explain a whole process, and to know where and why an event occurs, requires further experimental results.

Based on the current knowledge, we can trace the biological effects of cisplatin to the following chemical events that occur in the cells.

(1) The first group of events is ascribed to the interaction with the plasma membrane. Platinum binding to phospholipids and proteins induces conformational changes and then changes to membrane structure. Lipid peroxidation aggravates the damage to cell. The changes lead to an increase in permeability and a reduction in flexibility. In addition to these non-specific effects, several membrane enzymes, especially those with sulphydryl groups in active centres, are deactivated and their functions affected.

(2) Once the platinum complexes enter the cells, they are bound by cytosolic thiol compounds, primarily GSH and MT. The reduction in intracellular GSH and MT results in higher levels of ROS which then participate in the peroxidation of membrane lipids.

(3) The GSH-conjugated cisplatin is gradually transformed to reactive metabolites but a fraction of it is pumped out of the cell [78].

(4) The attack on the cytosol cytoskeleton starts from the binding to cytoskeletal protein and cross-linking. The conformational change follows, and the inhibition of polymerization and depolymerization might be one of the factors determining the destiny of the cells.

(5) The platinum complexes start their attack on the nuclei from binding to membrane and also to nucleoproteins. It is not just the well-studied simple binding to DNA. For instance, it is known that platinum binding induces and enhances protein–DNA interactions, and this too causes a lesion in DNA [87]. Anyhow, before or together with the specific binding to DNA, as described elsewhere, the platinum complexes will react with the proteins in the nucleus.

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