Studies on the Inhibition of Fumarase and Malate Dehydrogenase by Second Generation Platinum Antitumor Drugs

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Introduction

The discovery by Rosenberg *et al.* that *cis*-Pt $(NH_3)_2Cl_2$ could regress Sarcoma 120 tumors and L1210 leukemia in mice [1, 2] has led to rapid clinical investigation of this compound. It has been found to be efficacious in trials on head and neck cancers [3–5], cancers of the urinary tract [6], disseminated testicular cancer [7], ovarian cancer [8], and it has enhanced effectiveness in combination chemotherapy with other more established antitumor drugs such as adriamycin, vinoblastin and bleomycin [5, 8].

However, *cis*-$Pt(NH_3)_2Cl_2$ has considerable unpleasant side-effects, most notably nausea, hearing loss, vomiting, diarrhoea, bone marrow damage, neuropathy, ototoxicity and acute nephrotoxicity. In addition to these disadvantages low solubility has also made the search for new drugs with reduced toxicity and a higher aqueous solubility imperative. Amongst these new agents are the Pt(1,2-diaminocyclohexane)$(H_2O)(SO_4)$, Pt(N,N′-diethylenediamine)$(H_2O)(SO_4)$, Pt(iso-propylamine)$(CH_2(COO)_2)$, $Pt(NH_3)_2$(1,1-cyclobutanedicarboxylate), and *cis*-$(NH_3)_2(ClCH_2CO_2)_2$ [9–13].

While the search for new drugs continues there has been a great deal of effort aimed at elucidating the mechanism of action, and including radiopharmaceutical distribution studies [14, 15]. The diverse types of cancer towards which *cis*-$Pt(NH_3)_2Cl_2$ shows activity suggests a very general mechanism. Studies have been aimed at elucidating changes in the platinum coordination sphere on aquation [16–18], interactions with DNA [19–29] and protein [30, 31]. We have been particularly interested in studies of enzyme inhibition by these compounds. We have studied the effects of platinum drugs on malate dehydrogenase [32–37] and leucineaminopeptidase [38, 39]. Observations involving yeast or liver alcohol dehydrogenase with *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ show that the latter inhibits the enzyme 3.5 times more than the former [39].

In this present study we have examined the activities of malate dehydrogenase (EC 1.1.1.37) and fumarase (EC 4.2.1.2) in the presence of the new generation of platinum drugs, mentioned above, in physiological saline solution or sulfate solution.

Materials and Methods

Pig heart fumarase (Lot No. 27C-8760) and pig heart mitochondrial L-malate dehydrogenase (Lot. No. 56C-95003) were both purchased from the Sigma Chemical Co. Chromatography on carboxymethylcellulose (CMC) obtained from Pharmacia produced no further purification. Fumaric acid, L-malic acid, oxidized nicotinamideadeninedinucleotide (NAD^+) were also obtained from Sigma, while glycine was purchased from Fisher Scientific.

The following complexes were prepared by Dr. C. A. McAuliffe and Dr. M. J. Cleare (Johnson Matthey Research Center, Reading) and will not be described here [40]; *cis*- and *trans*-$Pt(NH_3)Cl_2$*, *cis*-$Pt(NH_2Pr^i)_2(OH)_2Cl_2$, *cis*-$Pt(NH_2EtOH)_2Cl_2$, Pt(1,2-diNH_2-c-hex)$(H_2O)(SO_4)$, Pt(N,N′-dieten)$(H_2O)(SO_4)$, Pt-$(NH_2Pr^i)_2$(mal), $Pt(NH_3)_2$(1,1-c-budicarb) and Pt-$(NH_2Pr^i)(Clac)_2$. All the rest of the compounds were of reagent grade.

*For footnote see overleaf.

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Assay of Fumarase

Fumarase was assayed by a modification of the method of Racker [41]. A substrate solution of 0.1 *M* phosphate buffer and 0.025 *M* L-malic acid was adjusted to pH 7.0 ± 0.1. Ten μL aliquots of enzyme solution (0.5 μ*M*) were mixed with 3 ml of the substrate solution in a 1 cm-path cuvette, and the rate of formation of fumarate was measured at 240 nm, on a Gilford Model 250 recording spectrophotometer.

Assay of Malate Dehydrogenase

Malate dehydrogenase was assayed under conditions described by Friedman *et al.* [32]. The substrate solution of 0.1 *M* L-malic acid, 0.1 *M* glycine and 0.2 m*M* NAD^+, was adjusted to pH 9.5 ± 0.1. The enzyme concentration in all experiments was 0.5 μ*M*. All assays were done on the Gilford recording spectrophotometer.

Protein concentrations were determined by the Lowry method [42] and by the absorptivities of the enzymes; 6.22×10^3 M^{-1} cm^{-1} at 340 nm for malate dehydrogenase and 2.4×10^3 M^{-1} cm^{-1} at 240 mm for fumarase.

24 Hours Inhibition Studies

The following concentrations (0.3 m*M* to 20 m*M*) of platinum complexes were used in order to partially inhibit malate dehydrogenase and fumarase. Enzyme solutions were prepared at pH 7 in phosphate buffer. Fumarase studies were done in 0.1 *M* phosphate, but malate dehydrogenase activity was better maintained in 0.2 *M* phosphate. Platinum complexes were prepared by dissolving a few mg in a few ml of the appropriate pH 7 phosphate buffer, with gentle heating to 70 °C. These solutions were then diluted to give the desired platinum/protein ratio. All of the complexes were sufficiently soluble in the aqueous solutions for the purpose of this study. The process of dissolving the platinum complexes took a few minutes only, and 0.50 ml of each diluted solution was immediately added to 0.50 ml of the enzyme solution and the initial enzyme activity of the system was at once determined. In the experiments involving the effects of chloride or sulfate ions on platinum inhibition, the platinum complexes were first dissolved in 0.2 *M* NaCl or Na_2SO_4, in phosphate buffer at pH 7, and the final Cl^- or SO_4^{2-} were adjusted to 0.1 *M*.

The enzyme–platinum systems were then incubated at 25 °C for 24 h. Appropriate blanks containing the enzyme in phosphate, phosphate–chloride and phosphate–sulfate buffer were always included. Assays were made afer 24 h, and in one experiment the activity was measured after 48 h to ensure that no further significant inhibition had occurred.

The molar ratio of platinum complex to protein was adjusted to give measurable inhibition, and it was usually quite high, between 6×10^2 and 4×10^4. The binding constants (Ke) were calculated as previously described [32]. All experiments were run in duplicate.

Kinetic Studies

Where appropriate, rapid inhibition was studied kinetically. *Cis*-$Pt(NH_2Pr^i)_2(OH)_2Cl_2$ solutions were prepared as before except a 1.4 m*M* solution (which was 8 times higher than those used in the equilibrium studies) was employed in this study. The platinum complex solutions were prepared as follows. One sample was dissolved in 0.1 *M* phosphate buffer, pH 7.0, a second sample was dissolved in the phosphate buffer containing 0.1 *M* Cl^-, while a third sample was allowed to aquate in the phosphate buffer for 24 h. A 0.5 ml aliquot of the complex solution was then rapidly mixed with 0.5 ml of fumarase solution and the first measurement was recorded within 40 s. Recordings were taken at regular intervals thereafter until the activity was negligible or constant. A control of enzyme and buffer was employed and comprised the 100% activity level in the calculations.

Results

It is well known that $Pt(ammine)_2X_2$ type complexes hydrolyze in aqueous solution, *viz*:

$$[Pt(ammine)_2X_2] + 2H_2O \longrightarrow [Pt(ammine)_2(H_2O)_2]^{2+} + 2X^- \quad (1)$$

(the rate for aquation of *trans* species is *ca.* four times that for *cis* compounds) [43]. This process is, of course, severely inhibited by the presence of *e.g.* chloride ion or sulfate ion. We have thus followed inhibition of the malate dehydrogenase and fumarase enzymes by (*a*) freshly prepared solutions of the platinum complexes, (*b*) these freshly prepared platinum drug solutions in the presence of 0.1 *M* Cl^- or SO_4^{2-}, where either of these anions was a liquid in the complex, and (*c*) platinum drug solutions

*Abbreviation: (1), *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ = *cis*- and *trans*-dichlorodiammine platinum(II); (2), *cis*-$Pt(NH_2Pr^i)_2(OH)_2Cl_2$ = *cis*-dichlordihydroxy bis(isopropylamine) platinum(IV); (3), *cis*-$Pt(NH_2EtOH)_2Cl_2$ = *cis*-dichloro bis(ethanolamine) platinum(II); (4), Pt(1,2-diNH_2c-hex)$(H_2O)(SO_4)$ = sulfatoaquo(1,2-diaminocyclohexane) platinum(II); (5), Pt(N,N'-dieten)$(H_2O)(SO_4)$ = sulfatoaquo(N,N'-diethylethylenediamine) platinum(II); (6), $Pt(NH_2Pr^i)_2$(mal) = malonato bis(isopropylamine) platinum(II); (7), $Pt(NH_3)_2$(1,1-c-budicarb) = (1,1-cyclobutyldicarboxylato)diammineplatinum(II); (8), $Pt(NH_2Pr^i)$ (Clac) = bis(chloroacetato)-bis(isopropylamine) platinum(II).

TABLE I. Equilibrium Constants for the Inhibition of Fumarase by Platinum Complexes under Various Conditions.

Complex	Ke[a]	K_e (aquated)	K_e (in 0.1 *M* Cl^-)
cis-$Pt(NH_3)_2Cl_2$	$3.1(\pm1.0) \times 10^3$	$5.8(\pm2.0) \times 10^2$	$3.1(\pm1.0) \times 10^3$
trans-$Pt(NH_3)_2Cl_2$	$2.5(\pm1.0) \times 10^3$	$9.4(\pm3.0) \times 10^2$	$1.7(\pm0.6) \times 10^4$
cis-$Pt(NH_2Pr^i)(OH)_2Cl_2$	$1.5(\pm0.8) \times 10^3$	$1.9(\pm0.9) \times 10^2$	$6.7(\pm1.2) \times 10^3$
$Pt(NH_2EtOH)_2Cl_2$	$1.9(\pm0.5) \times 10^2$	$1.5(\pm0.4) \times 10^2$	$2.3(\pm0.3) \times 10^2$
$Pt(1,2\text{-diNH}_2\text{c-hex})(H_2O)(SO_4)$	$2.45(\pm1.2) \times 10^3$	$3.6(\pm1.0) \times 10^2$	$2.05(\pm0.6) \times 10^3$
$Pt(N,N'\text{-dieten})(H_2O)(SO_4)$	$2.3(\pm1.2) \times 10^2$	$2.7(\pm0.4) \times 10^2$	$3.45(\pm1.0) \times 10^2$
$Pt(NH_2Pr^i)_2(mal)$	$5.0(\pm2.0) \times 10^2$	$3.0(\pm1.0) \times 10^2$	
$Pt(NH_3)_2(1,1\text{-c-budicarb})$	$3.9(\pm1.5) \times 10^1$	$4.2(\pm1.0) \times 10^1$	
cis-$Pt(NH_2Pr^i)(Clac)$	$2.6(\pm0.6) \times 10^2$	$2.5(\pm0.7) \times 10^2$	

[a]Units of K_e are M^{-1}.

which had been allowed to 'aquate' for 24 h before the inhibition study. In this way we could draw some broad correlation between the more active inhibitor and its structure.

The sensitivity of the two enzymes studied to platinum complexes may be explained in terms of the known essential side-chain groups and their affinity for platinum. Fumarase has at its active site both an essential methionine and histidine [44, 45]. It is likely that the –SMe group will have high affinity for platinum, and the histidine will also probably bind strongly. Fumarase has twelve thiol groups, but they appear to be too buried in the hydrophobic regions of the molecule to be of importance under anything but perturbing conditions. Thus, fumarase is probably inhibited by the platinum drugs largely by their binding to the essential methionine and/or histidine. Malate dehydrogenase, or at least the mitochondrial form with which this study is concerned, is a very different case. M-malate dehydrogenase has two essential thiol groups, and its activity is very sensitive to thiol modifiers. These thiols appear to be near the co-enzyme binding site [45]. There is also a histidine side-chain that can be modified to produce an inactivated enzyme, which is involved in the catalytic mechanism [46]. There is no evidence of sensitivity to methionine reagents.

This, platinum inhibition of the two enzymes is probably dominated by methionine–platinum reactions in fumarase and thiol platinum interactions in malate dehydrogenase. It was generally found that fumarase was more strongly inhibited (sometimes by a considerable factor) then malate dehydrogenase by individual complexes.

Fumarase

Cis-$Pt(NH_2Pr^i)_2(OH)_2Cl_2$ is a good inhibitor of fumarase, as is the *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ (Table I), and pre-aquation diminishes it considerably. It

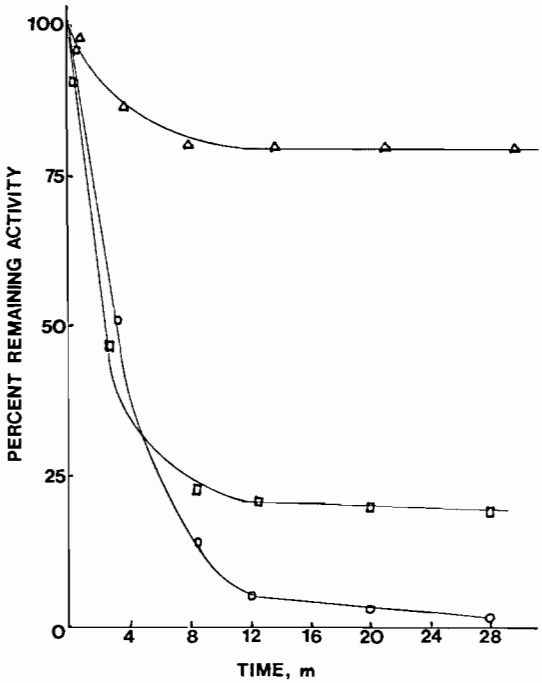

Fig. 1. Inhibition of fumarase by *cis*-$Pt(NH_2Pr^i)(OH)_2Cl_2$ under a variety of conditions. The concentrations of the platinum complex and the enzyme were 1.0 m*M* and 0.5 μM, respectively. –□–, 0.1 *M* phosphate buffer pH 7.0, –○–, 0.1 *M* Cl^- in 0.1 *M* phosphate buffer, pH 7.0; and –△–, 24 h aquation of complex in 0.1 *M* phosphate buffer, pH 7.0.

is also seen in Fig. 1 that prevention of the aquation reaction by exogenous chloride also produces good inhibition. It is observed in Fig. 1 how weak the inhibition is using preaquated *cis*-$Pt(NH_2Pr^i)_2(OH)_2Cl_2$, and how close the curves are for freshly prepared complex and complex plus 0.1 *M* chloride. The difference in the levels of activities in the presence and

TABLE II. Equilibrium Constants for the Inhibition of Malate Dehydrogenase by Platinum Complexes under Various Conditions.

Complex	Ke[a]	K_e (aquated)	K_e (in 0.1 *M* Cl^-)
cis-$Pt(NH_3)_2Cl_2$	$2.8(\pm0.3) \times 10^2$	$2.2(\pm1.0) \times 10^2$	$1.82(\pm1.0) \times 10^2$
trans-$Pt(NH_3)_2Cl_2$	$3.1(\pm1.5) \times 10^2$	$9.0(\pm1.5) \times 10^1$	$5.3(\pm1.0) \times 10^3$
cis-$Pt(NH_2Pr^i)(OH)_2Cl_2$	$5.6(\pm1.0) \times 10^2$	$5.5(\pm3.0) \times 10^2$	$3.1(\pm2.0) \times 10^3$
cis-$Pt(NH_2EtOH)_2Cl_2$	$6.7(\pm1.5) \times 10^2$	$2.3(\pm1.0) \times 10^2$	$2.4(\pm1.0) \times 10^2$
$Pt(1,2\text{-diNH}_2\text{c-hex})(H_2O)(SO_4)$	$8.85(\pm1.5) \times 10^2$	$5.7(\pm2.0) \times 10^2$	$2.6(\pm1.0) \times 10^3$
$Pt(N,N'\text{-dieten})(H_2O)(SO_4)$	$1.9(\pm1.5) \times 10^2$	$4.2(\pm1.5) \times 10^2$	$4.2(\pm1.5) \times 10^1$
$Pt(NH_2Pr^i)_2(mal)$	$2.15(\pm0.5) \times 10^2$	$1.1(\pm0.3) \times 10^2$	
$Pt(NH_3)_2(1,1\text{-c-budicarb})$	$2.3(\pm1.0) \times 10^2$	$3.3(\pm1.0) \times 10^2$	
cis-$Pt(NH_2Pr^i)(Clac)$	$2.1(\pm0.2) \times 10^2$	$1.6(\pm0.6) \times 10^2$	

[a]Units of K_e are M^{-1}.

absence of Cl^- suggests that the neutral halide species is the more active form of inhibitor, rather than a positively charged aquo species. The other di-chloro complex, *cis*-$Pt(NH_2EtOH)_2Cl_2$ mimics the *cis*-$Pt(NH_3)_2Cl_2$, (Table I), although the overall level of inhibition is an order of magnitude smaller as compared to the previously discussed complexes. Inhibition is slightly suppressed by aquation and slightly enhanced by exogenous chloride, suggesting that both forms of the complex are almost equally inhibitory after 24 h of mixing. The lowered level of inhibition with this complex as compared to the previous three di-chloro complexes is difficult to explain. Perhaps the ethanolamine exerts a weaker *trans* effect than ammonia or propylamine, although this seems unlikely to be the reason for so large a difference. Equilibrium inhibition results for the sulphato complexes also are shown in Table I. The presence of exogenous sulfate ion increases inhibition by the complexes, while aquation decreases the inhibition of the 1,2-diaminocyclohexane complex considerably. The diaminocyclohexane complex is about ten times more inhibitory to fumarase than is the N,N'-diethylethylenediamine complex. The reason for this could reside in the influence of the relatively inert diamine ligands over the binding affinity of the platinum for nucleophiles on the protein molecule. The more inhibitory complex is a primary amine, the diethylethylenediamine complex is a secondary amine ligand. However, the experimentally determined '*trans* effect' series has not been studied in relation to the different types of amines, except to observe that they are all close. The fact that 1,2-diaminocyclohexane is a rigid chelating agent, more so than the less restricted diethylethylenediamine group, may give its complexes greater stability.

The remaining three complexes all contain carboxylate ligands. Aquation makes little difference to their capacities to inhibit, and they were not studied in the presence of exogenous ligand. The malonate complex is a better inhibitor than the dichloroacetate, but the 1,1-cyclobutanedicarboxylate complex is a much weaker inhibitor than either of these and is the weakest inhibitor of fumarase among the complexes studied. Of these complexes it is hard to say whether their individual inhibitory properties are more affected by leaving group stability or by the influence of the amino groups on the complex-protein stability.

Malate Dehydrogenase

Malate dehydrogenase does not differentiate between the isomers of $Pt(NH_3)_2Cl_2$ in fresh solution (Table II). Aquation suppresses inhibition by both isomers, the *trans* one more markedly. In the presence of Cl^- the *cis* inhibition is slightly suppressed, but the *trans* inhibition shows a very large, 17-fold enhancement.

Cis-$Pt(NH_2Pr^i)_2(OH)_2Cl_2$ follows the standard pattern suggesting that the undissociated complex is more active against malate dehydrogenase than the aquated one. Aquation has little effect on the inhibition while in the presence of chloride there is six-fold enhancement. Again it follows the pattern of the *trans*-$Pt(NH_3)_2Cl_2$ inhibition of the enzyme rather than the *cis* isomer pattern.

Cis-$Pt(NH_2EtOH_2)_2Cl_2$ is a fairly good inhibitor of malate dehydrogenase, more so than with fumarase. Both aquation and exogenous chloride suppress its inhibition. Again it mimics the behaviour of the *cis*-$Pt(NH_3)_2Cl_2$ system in everything but the actual values of the equilibrium constants. The fresh complex seems to be the best inhibitor of malate dehydrogenase.

Of the two sulfate complexes, again the 1,2-diaminocyclohexane complex is the stronger inhibitor by a factor of about 5. Aquation suppressed, while exogenous sulfate enhanced the inhibition by this

complex, suggesting that the undissociated form is the better inhibitor. But inhibition by the N,N'-diethylethylenediamine complex is greatly suppressed by exogenous SO_4^{2-} suggesting that there is a difference in the way that these complexes inhibit malate dehydrogenase.

The three carboxylate complexes are all moderate inhibitors of malate dehydrogenase and there is little difference between them. Aquation does suppress inhibition by the malonate and chloroacetate complexes, but inhibition by the 1,1-cyclobutanedicarboxylate is slightly enhanced.

Discussion

This study shows that a group of diamine platinum complexes are slow inhibitors of fumarase and malate dehydrogenase, and that the enzymes are sensitive to differences in the steric and chemical nature of the complexes, as well as the ionic state.

The nature of the relatively inert amine ligand has a pronounced effect on the inhibitory properties of the complex. *Cis*-$Pt(NH_3)_2Cl_2$ and *cis*-$Pt(NH_2EtOH)_2HCl_2$ both show the same inhibition patterns, generally, suggesting similar mechanisms of inhibition, although the levels of inhibition are different; the ammonia complex being a much stronger inhibitor of fumarase. Of the two sulfato complexes the diaminocyclohexane complex is a much better inhibitor of both enzymes than the N,N'-diethylethylenediamine complex. These differences can only be due to the influence of the amine ligands on the Pt affinity for the enzyme binding sites.

By contrast the nature of the reactive ligands seems to be less important; for example, $Pt(NH_2Pr^i)_2$(mal) and $Pt(NH_2Pr^i)$(Clac) inhibit very similarly. This seems logical if we consider that the inhibition process entails a substitution of the labile ligands by groups bound to protein; the nature of the leaving group will not affect the equilibrium primarily, but the rate of its attainment. The reaction of some of the complexes seems to be facilitated or mediated by an aquation step. Exogenous concentrations of the leaving ligand often affect the inhibitions markedly.

Malate dehydrogenase and fumarase were observed to distinguish between the *cis* and *trans* isomers of $Pt(NH_3)_2Cl_2$ in the presence of Cl^-, the *trans* inhibition being strongly enhanced while the *cis* inhibition was not much affected [46]. It is proposed that both *cis* and *trans* isomers bind the protein at first mono-functionally, and these reactions do cause similar inhibition. However, once a strongly *trans* affecting ligand such as $-SCH_3$ or $-SH$ is bonded to either a *cis* or *trans* complex, two different situations arise concerning further reaction of the complexes. The remaining chloride position, in the *trans* complex will become more labile, and its binding affinity for any near-by nucleophile on the protein will greatly increase. The *trans* ligand in the *cis* complex is an unreactive amine, and there should be little, if any, tendency for it to react or become labile. In this way, the *trans* complex may have a much greater tendency to bind bi-functionally, and thus inhibit any enzyme more strongly, than the *cis*. It would be necessary to extend enzyme inhibition studies to a wider range of *trans* complexes to substantiate this idea. This type of effect may also account for the strong inhibitions observed with $PtCl_4^{2-}$.

A model such as this seems preferable to the idea that there is simply a specific binding site on the enzyme that 'fits' the *trans* isomer better than the *cis*. Several cases have now been reported where the *trans* isomer of $Pt(NH_3)_2Cl_2$ is a stronger inhibitor than the *cis*: the two in this study, yeast alcohol dehydrogenase [39] (*trans* is 3½ times better), lactate dehydrogenase [39] (*trans* is twenty times better), and thymidylate synthase [46]. Rather than postulate a stereospecific binding site on each of these enzymes, which implies a series of coincidences in favor of the *trans* isomer, it is suggested that an intrinsic property of the *trans* complex itself is involved. Therefore, this first reaction is possible in any enzyme that can bind platinum through an activating group, the second, enhanced reaction can bind platinum through an activating group, the second, enhanced reaction can occur with any convenient nucleophilic group on the enzyme and the resulting bi-functional binding should restrict the platinum complex and inhibit the enzyme strongly.

It was generally found that fumarase is more strongly inhibited than malate dehydrogenase by seven of the ten complexes. Thus, it is concluded that platinum inhibition of fumarase is dominated by interaction with the $-SCH_3$ of methionine, which has a very high affinity for platinum, while inhibition of malate dehydrogenase is caused by reaction with the less reactive thiol groups.

There is no apparent relationship between enzyme-platinum interactions and antitumor activity. However, there is a correlation between high toxicity in complexes and their ability to inhibit relatively more strongly than the low toxic complexes [13]. This suggests that toxicity could be a result of general inhibition of sensitive enzymes (mainly those containing essential methionine or cysteine) in an organ that concentrates the complexes, leading to cell death and organ dysfunction.

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