STUDIES ON *cis*-DDP, $[Pt(Dach)(MePhSO)Cl]^+$ AND $[Pt(NH_3)_2(N\text{-}Py)Cl]^+$ BINDING TO FUMARASE

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SUMMARY

Two platinum analogs which have suitable physical properties and show antineoplastic activities comparable or greater than that obtained with *cis*-DDP were synthesized: [*cis*-Pt A_2 (Am) Cl] NO_3 and [Pt (dach) (RR'SO) Cl] NO_3 (A: ammonia, Am: pyridine, dach: *trans*-diaminocyclohexane, RR'SO: methylphenylsulphoxide). Their interactions with fumarase were studied. The inhibition of fumarase activity by the platinum compounds was followed kinetically by a spectrophotometric method. These two platinum compounds generally inhibited fumarase less than *cis*-DDP at the concentrations and reaction media (phosphate buffer or NaCl-buffer) studied.

KEY WORDS

cis-dichlorodiamineplatinum(II), [Pt(MePhSO)(diaminocyclohexane) Cl] NO_3, *cis*-[$Pt(NH_3)_2$(pyridine)Cl]NO_3, fumarase, inhibition

INTRODUCTION

The square planar complex *cis*-dichlorodiammineplatinum (*cis*-DDP) (**I**) is an antineoplastic drug with proven effectiveness against numerous animal and human tumors /1/. Despite significant antineoplastic activity, its clinical use is limited due to severe side effects /2,3/, especially nephrotoxicity. The mechanism underlying the nephrotoxicity and other side effects is unclear /3-5/.

Recently two cationic classes of platinum compounds were described which showed activity in primary screening comparable to or greater than *cis*-DDP (**I**), with higher solubility and stability than *cis*-DDP which is neutral. They have the general formulas [Pt (diam)

(RR'SO) Cl]$^+$ (**II**), in which diam = bidentatamine and RR'SO = substituted sulfoxide, and [*cis*-Pt (NH_3)$_2$ (Am) Cl]$^+$ (**III**), in which Am = a derivative of pyridine, pyrimidine, purine or aniline /6,7/ (Fig. 1).

The first group of these new complexes showed activity in *cis*-DDP-resistant cells; like *cis*-DDP they have a bifunctional mechanism of action /6,8/; the second group contains only one leaving ligand and their cytotoxicity most likely arises from the formation of monofunctional DNA adducts /9/.

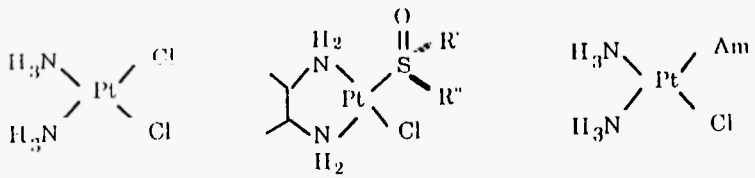

Fig. 1: The structures of the platinum compounds discussed in the text.

Although DNA is the most important target for platinum, it is also known that sulfur-containing nucleophiles coordinate to platinum in plasma or cytosol by a variety of mechanisms /10-14/. Binding characteristics and toxicity of *cis*-DDP may differ between extracellular and intracellular fluids due to the differing chloride ion concentrations /15/. Some authors have shown that platinum has an affinity for the thiol groups of biomolecules /16-19/, while others have shown that platinum has a high affinity for the methionine groups of certain enzymes /20-22/ and also for methionine itself /21/. It is still unclear whether or not certain protein-Pt complexes contribute to the toxicity observed with *cis*-DDP.

MATERIALS AND METHODS

Materials

Pig heart fumarase was obtained from Boehringer Mannheim. Methylphenylsulfoxide, (1R,2R)-(-)diaminocyclohexane and L-malic acid were obtained from Aldrich. K_2PtCl_4 was obtained from Johnson Matthey as a gift.

The platinum compound [Pt (dach) (MePhSO) Cl] NO_3 was prepared according to Farrel *et al.* /6/, the other platinum compound [*cis*-Pt $(NH_3)_2$ (N-Py) Cl] NO_3 was prepared according to Hollis *et al.* /7/, and the resulting product was recrystalized from 4:6 methanol: ethanol. *cis*-DDP was prepared according to Dhara /23/. The compounds were characterized by ^{1}H NMR, IR and elemental analysis. These three platinum compounds are shown in Figure 1.

Instrumentation

Inhibition reactions were followed on a Shimadsu 2100P UV spectrophotometer; IR spectra (KBr pellets) were obtained on a Perkin Elmer 580 spectrophotometer and ^{1}H NMR spectra were obtained on a Bruker WM 300 spectrometer. Elemental analysis was carried out by Mikr. Lab. Pasher, Remagen, Germany.

Assay of fumarase

Fumarase was assayed by modification of the method of Racker /24/. A substrate solution of 0.05 M L-malic acid in 0.1 M potassium phosphate buffer was adjusted to pH = 7.4 with 0.1 M NaOH. Aliquots (20 μl) of 0.3 x 10^{-6} M enzyme solution were mixed with 2.98 ml aliquots of substrate solution in a 1 cm cuvette, and the rate of formation of fumarate was measured at 240 nm using a Shimadsu 2100P spectrophotometer at 25°C.

Inactivation of fumarase

The concentration of fumarase for the inhibition reactions was 1.2 x 10^{-5} M in 0.08 M phosphate buffer. The molar ratios of platinum compounds to the enzyme were adjusted to give measurable inhibition. At 20:1 the inhibition could not be measured, thus 200:1 and 600:1 were used. By adding 0.125 ml of platinum compound solution to 0.125 ml of enzyme solution the desired platinum:enzyme ratio was obtained. To obtain a final concentration of enzyme of 0.3 x 10^{-6} M the mixture was diluted with 0.1 M phosphate buffer. The initial activity of the system was measured as quickly as possible after the above-mentioned mixture was prepared and measurements were continued up to about 8 hours. This time was chosen because a significant amount of the enzyme was inhibited by *cis*-DDP within 8 hours, and after 24 hours incubation the activity was almost zero. In a previous study it

has been shown that the activity of fumarase was inhibited by the compound [*cis*-Pt $(NH_3)_2$ $(H_2O)_2$] $(NO_3)_2$ within 6 hours /25/.

To study inhibition in 0.9% NaCl solution, the platinum complexes were dissolved in 1.8% NaCl solution and added to the enzyme solution.

Appropriate blanks containing only fumarase in phosphate buffer were measured in all cases and these were used as the reference in calculation of the percentage of remaining activity. All experiments were run in duplicate.

RESULTS AND DISCUSSION

Calculation of kinetic rate constants

Kinetic data were recorded as ΔA versus time (minutes). Because inhibitor concentrations were several hundred times greater than the enzyme concentrations, they were considered constant. Thus, the reaction is pseudo-first order. In these conditions, the rate would be dependent only on the concentration of free enzyme, where k' is the second order rate constant, E is the enzyme, I is the inhibitor (Pt compound), and EI is the enzyme-Pt complex:

$$E + I \xrightarrow{k'} EI \qquad [1]$$

$$\frac{d[EI]}{dt} = k'[E_0]-[EI][I] \qquad [2]$$

By integrating equation [2]:

$$\ln \frac{[E_0]}{[E_0]-[EI]} = k'[I]t \qquad [3]$$

E_0 is proportional to the initial activity at time t=0 and $[E_0]$ - [EI] is the concentration of uninhibited enzyme and proportional to the remaining activity at time t. Equation [3] may be written:

$$\ln \frac{\text{initial activity}}{\text{remaining activity}} = k'[I]t \qquad [4]$$

The results shown in Table 1 are the second order rate constants of the related reactions. The rate constants in the table are the mean values of two measurements.

Because A_0, which is the initial absorbance at t = 0, is proportional to the initial activity and ΔA, which is the absorbance at time t, is proportional to the remaining activity, the plot of ln A_0 / ΔA versus time gives linear plots with a slope of k'[I]. Percentage remaining activities versus time (minutes) are shown Figures 2 and 3. Second order rate constants are given in Table 1.

In this study, the reactions of the enzyme fumarase with *cis*-DDP (**I**), [Pt (dach) (MePhSO) Cl] NO_3 (**II** - MePhSO) and [*cis*-Pt $(NH_3)_2$ (N-Py) Cl] NO_3 (**III** - Py) were compared, and the binding rate

TABLE 1

Second order rate constants for the inhibition of fumarase by *cis*-DDP, [Pt(dach)(MePhSO)Cl]NO_3 and [*cis*-Pt$(NH_3)_2$(N-Py)Cl] NO_3

Pt compound	Pt : fumarase	Condition of inhibition reaction	k' s^{-1}
Cis - DDP	200 : 1	Phosphate buffer	2,80
		0.9% NaCl - buffer	1,80
[Pt (dach) (MePhSO) Cl]+	200 : 1	Phosphate buffer	2,86
		0.9% NaCl - buffer	1,48
[Pt (dach) (MePhSO) Cl]+	600 : 1	Phosphate buffer	2,69
		0.9% NaCl - buffer	1,48
[Cis- Pt $(NH_3)_2$ (N - Py) Cl]+	200 : 1	Phosphate buffer	1,22
		0.9% NaCl - buffer	1,07
[Cis- Pt $(NH_3)_2$ (N - Py) Cl]+	600 : 1	Phosphate buffer	1,21
		0.9% NaCl - buffer	1,02

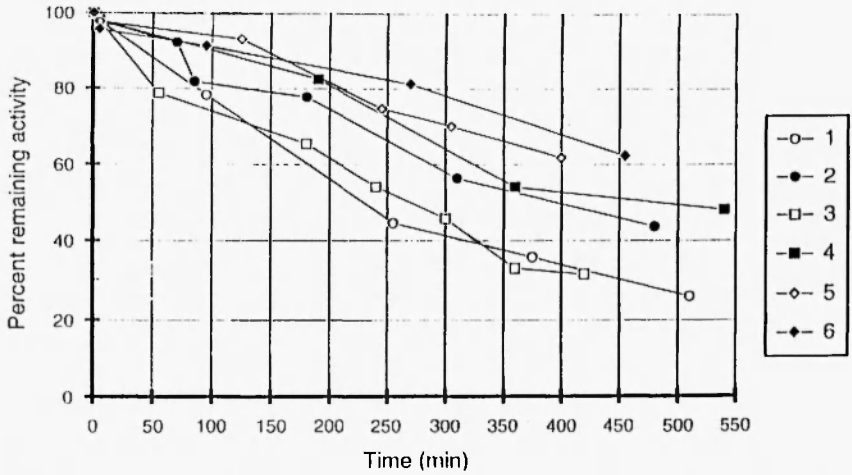

Fig. 2: Inhibition of fumarase. The ratio of Pt:fumarase was 200:1. (1) *cis*-DDP in buffer; (2) *cis*-DDP in 0.9% NaCl-buffer; (3) Pt(dach)(MePhSO)Cl] NO_3 in buffer; (4) [Pt(dach)(MePhSO)Cl]NO_3 in 0.9% NaCl-buffer; (5) [*cis*-$Pt(NH_3)_2$(N-Py)Cl] NO_3 in buffer; (6) [*cis*-$Pt(NH_3)_2$ (N-Py)Cl] NO_3 in 0.9% NaCl-buffer.

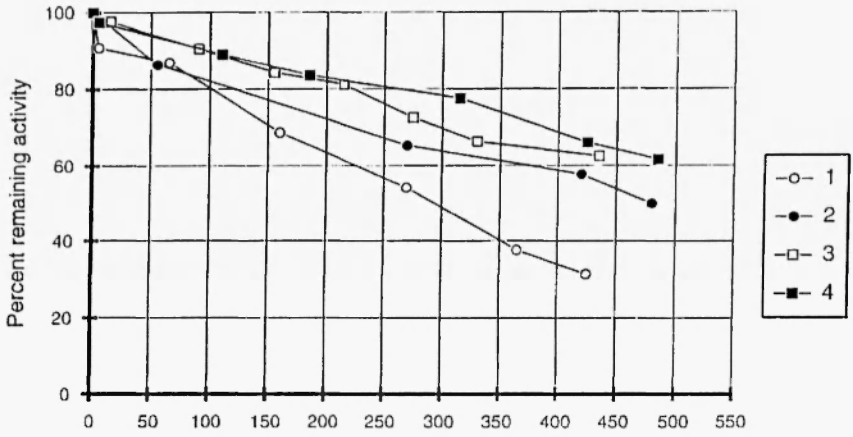

Fig. 3: Inhibition of fumarase. The ratio of Pt:fumarase was 600:1. (1) [Pt(dach) (MePhSO)Cl] NO_3 in buffer; (2) [Pt(dach) (MePhSO)Cl] NO_3 in 0.9% NaCl-buffer; (3) [*cis*-$Pt(NH_3)_2$(N-Py)Cl] NO_3 in buffer; (4) [*cis*-$Pt(NH_3)_2$(N-Py)Cl] NO_3 in 0.9% NaCl-buffer.

constants were determined. The reaction medium used was phosphate buffer or 0.9% NaCl-buffer. The concentrations of Pt-compounds were 200-fold and 600-fold those of the fumarase concentration, but *cis*-DDP was only 200-fold. The results were almost the same with the different concentrations.

Fumarase has 12 cysteine groups which are not at the active side, but are buried in hydrophobic regions of the enzyme, so it is unlikely to react via its cysteine residues. However there are four active sites per molecule, presumably one site per unit, and the total amount of histidine plus methionine per molecule corresponds to one residue per active site /26-28/. It is known that platinum has a high kinetic affinity for methionine, and Appleton and colleagues have shown that *cis*-DDP binds to methionine by its S, N donor atoms and forms $[Pt(NH_3)_2$ (met-S,N)$]^+$ chelate at pH 5 and 7 /21/. Platinum also binds to the $-SCH_3$ groups of some enzymes /20,22/. It has been suggested that fumarase is inhibited by platinum complexes probably through complexation with the essential methionine and/or histidine, and platinum complex inhibition of fumarase is probably dominated by methionyl-platinum reactions /16,17/.

It is known that the aqua species of *cis*-DDP reacts much faster with nucleic acids than the dichloro species /29/. It would be expected that it would react much faster with the nucleophilic groups in proteins.

All hydrolysis schemes of *cis*-DDP to date involve the replacement of the labile chlorine atoms with various water functions, ultimately producing a hydroxylated molecule, $[Pt\ (NH_3)_2\ (OH)_2]^\circ$ /15,29,30/ which would react with DNA bases /31/. For the compound II, Farrel and colleagues /6/ have proposed a similar hydrolysis scheme, producing a hydroxylated molecule; but they have shown that the chloride ion is much more labile than the RR'SO ligand. After forming [Pt (dach) (RR'SO) (OH)], it is very probable that the sulfoxide would be displaced by the guanine base intracellularly /6,8/. Thus, these two compounds interact with DNA in a bifunctional manner.

Although the aqua species of *cis*-DDP and compound II would be expected to react much faster with the enzyme fumarase, higher reaction rate constants have been found for both compounds in phosphate buffer compared to 0.9% NaCl-buffer solution, which is meant to mimic the extracellular medium (Table 1). The data presented in this paper have also demonstrated that the two above-mentioned compounds bind to fumarase at almost the same rates and the

remaining activities after 6 hours are almost the same in phosphate buffer (Fig. 1). The two compounds might be expected to have different reaction rates because of different non-leaving and leaving groups. A discussion of possible hydrolysis products and their interaction with the enzyme is beyond the scope of this study /8,27,32/.

In previous studies, it has been shown that the lability of the chlorine atoms is related to the chloride concentration in the solvent in which the drug is dissolved /33/. The kinetics of inhibition by *cis*-DDP was significantly affected by the addition of chloride ion (rate constant 1.80 s^{-1}; without chloride 2.80 s^{-1}). For compound II the chloride effect was also significant (rate constant 1.48 s^{-1}; without chloride 2.86 s^{-1}) (Table 1). As can be seen the rate constant of compound II is less than that of *cis*-DDP in 0.9% NaCl-buffer solution. It is probable that *cis*-DDP and compound II are in the forms *cis*-$Pt(NH_3)_2Cl_2$ and [Pt (dach) Cl_2] in 0.9% NaCl and they might react with the enzyme by their their chlorine atoms.

On the other hand, for the monofunctional compound III the rate constants found were almost the same with or without NaCl with a value of around 1.00 s^{-1}, although there was a slight difference between the constants in the two different reaction media (Table 1). As can be seen in Figures 2 and 3 the remaining activities with this compound were the highest, with above 60% in the two reaction media. Inhibition was therefore slowest with compound III.

In a previous study it was shown that *cis*-DDP is more toxic when not administered in physiological saline, which may imply toxicity of the charged aqua species /34/. If so, the two new Pt compounds, especially compound III, may be promising.

In conclusion the monofunctional compound III seems to have less of an inhibitory effect on the enzyme fumarase as compared with *cis*-DDP and compound II, and it is possible that compound III is less toxic. Future studies with this compound are necessary to investigate this in detail.

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