Role of Ligand Exchange Processes in the Reaction Kinetics of the Antitumor Drug cis-Diaminedichloroplatinum(II) with Its Targets

Evelyne Segal and Jean-Bernard Le Pecq

ABSTRACT

The kinetics of a model reaction between the antitumor drug cis-diaminedichloroplatinum(II) (cis-DDP) and the signal nucleotide diadenosine 5',5''-P'1,P'3-triphosphate (Ap3A) has been investigated by spectrophotometry. The formation of the reactive platinum aqurated species was first analyzed by potentiometry using a chloride-specific electrode. Both equilibrium and rate constants were measured. The rate constants for the release of the first and second chloride were found 1.1 ± 0.5 (S.D.) x 10^-4 and 4.2 ± 0.2 x 10^-5 sec^-1, respectively, at 37°. It was shown that anions such as acetate, phosphate, and pyrophosphate were able, in some conditions, to exchange with chloride to form acetato, phosphato, or pyrophosphate complexes. The reaction of cis-DDP with Ap3A or other targets involves at least four steps, which have been analyzed separately. The values of rate constants deduced from the analysis of the overall reaction are in agreement with those determined independently from the separated steps. The second-order rate constants for the reaction of acetato, phosphato, and pyrophosphate complexes with Ap3A (0.2 ± 0.02, 0.20 ± 0.02, and 0.16 ± 0.03 m^-1 sec^-1, respectively) are close to that of monoaqua-monochloro (0.16 ± 0.1 m^-1 sec^-1) and lower than that of the diqua species (0.94 ± 0.06 m^-1 sec^-1). At a high concentration of Ap3A, the reaction kinetics is slowed down. The formation of a complex of cis-platinum with the Ap3A phosphate groups is suggested. The intracellular concentration of phosphates, pyrophosphates, and carboxylates is large enough to displace chloride from cis-DDP. Inside cells, therefore, ligand exchange processes have to be taken into account to account for the in vivo reactivity of cis-DDP with its potential targets.

INTRODUCTION

The mechanism of action of the antitumor drug cis-DDP has been studied by many investigators (16, 18). It has been shown that cis-DDP must first exchange its chloride ligands with water to yield the aqurated forms (8), which then react covalently with various intracellular molecules bearing nucleophilic groups. In extracellular fluids, the aquation reaction is inhibited by the high chloride concentration, whereas the low intracellular chloride concentration favors the formation of the reactive aported species. Although cis-DDP reacts with many different molecules inside the cell (proteins, nucleic acids) (23, 24), the formation of DNA adducts is thought to be the main cause of cytotoxicity (16). However, a reaction of cis-DDP with other cellular targets cannot be excluded and could contribute to cellular toxicity. Ap3A has been characterized recently as a signal nucleotide possibly involved in the control of DNA replication initiation (2, 6, 7, 13, 14, 22). This nucleotide could therefore represent a potential target for antitumor drugs such as cis-DDP. In order to investigate this possibility, a study of the reaction of cis-DDP with Ap3A was initiated. It was shown that cis-DDP reacts readily with Ap3A to form a single adduct with unusual conformation (20); it is a N7-N7 chelate of the metal with the 2 adenosines in a head-to-head arrangement and an anti-anti conformation of the adenosines. Ap3A-cis-DDP reaction appears as a good model to understand the mechanism of reaction of this drug with cell components.

The results underline the role of the exchange of chloride with intracellular ligands in the control of the reactivity of cis-DDP with its intracellular targets.

MATERIALS AND METHODS

Nucleotides. Ap3A and Ap4A lithium salts were from Boehringer. Ap3A ammonium salt was from Sigma. GppG, GppG, and GppG were from P. L. Biochemicals. cis-DDP was a gift of the Roger Bellon Laboratories, and trans-DDP was prepared as described previously (9); the aque derivatives. (cis-[Pt(NH3)2(H2O)]NO3 or trans-[Pt(NH3)2(H2O)]NO3) were prepared according to the method of Scovell (19).

HPLC Analyses. The HPLC analyses were carried out on a Waters chromatograph with a C18-Bondapak column with 254 nm detection, using an isocratic elution of CH3CO2NH4 (10 mM) in 8.5% aqueous CH3OH, pH 5.5.

Potentiometric Measurement of Chloride Release. The chloride ion concentration was measured by potentiometry at 37° using a specific electrode (PCL 3; Tacussel, France). The reference electrode was a Hg/HgSO4/K2SO4 saturated electrode (S8, Tacussel). A digital millivolimeter (Minis 20000, Tacussel) was used to determine the voltage between the 2 electrodes. The signal was recorded on a LKB tracer. Calibration was performed with solutions of NaCl of known concentrations. Measurements could be performed in the 0.1 to 50 mM range. For the measurements of the auration kinetics, solid cis-DDP was freshly dissolved in the various buffers, and the concentration of CT release was recorded over time. Gentle stirring was maintained during all of the measurements.

Spectrophotometry. The difference spectra were measured in 1-cm double-compartment cells (Hellma) with a Kontron Uvikon 810/820 spec-
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The dinucleotide and platinum solutions were placed in the 2 separated compartments. The base line was recorded. The solutions of both compartments were then mixed by stirring the sample cell, and difference spectra were recorded at different time intervals for 24 hr.

For kinetic studies, the spectrophotometer was controlled by a Minc Digital PDP 11/23 computer. Absorbance data measured to 5 significant digits were stored in the Minc computer; these were later analyzed by nonlinear regression using the Marquardt algorithm (11). A FORTRAN program was kindly provided by Dr. Rigler and Dr. Nilsson from Karolinska Institut (Stockholm) and adapted to the Minc Computer.

RESULTS

Potentiometric Measurement of Chloride Release. The various equilibria to be considered for the aquation reaction of cis-DDP are:

\[
\begin{align*}
H_2N & \text{Pt} \quad \text{H}_3N & \text{Cl} & \text{H}_3N & \text{Cl} & \text{H}_2O & \text{OH}_2 & \text{Cl} \\
\text{Pt} & \text{Cl} & \text{Pt} & \text{Cl} & \text{Pt} & \text{Cl} & \text{Pt} & \text{Cl} \\
\text{cis-PtCl}_2 & \text{cis-Pt(Cl)(H}_2O) & \text{cis-Pt(H}_2O)_2
\end{align*}
\]

with \( K_{d1} = \frac{k_{-1}}{k_1} \) and \( K_{d2} = \frac{k_{-2}}{k_2} \).

The release of chloride ion from cis-DDP was followed directly by potentiometry with a Cl\textsuperscript- specific electrode. Cis-DDP was incubated at 37\textdegree at various concentrations for 24 hr, and free Cl\textsuperscript- was then measured. Chart 1 shows the variation in the number of Cl\textsuperscript- released per molecule of cis-DDP as a function of concentration. Because of the poor solubility of cis-DDP, measurements could not be performed at concentrations greater than 3 mM.

These results are in agreement with the isotopic exchange data reported by Reishus and Martin (15), which indicated that the values of dissociation constants \( K_{d1} \) and \( K_{d2} \) for the release of the first and second chloride at 35\textdegree were 3.3 and 0.2 mM, respectively. These results show that, in the absence of added chloride, at a high concentration of cis-DDP (~1 mM), the monoaqua-monochloro derivative [cis-Pt (Cl)(H\textsubscript{2}O)] is predominant whereas, at a low concentration (<0.1 mM), the diaqua form is the main species. Furthermore, in solutions containing Cl\textsuperscript- at concentrations close to that found inside cells (3 to 5 mM), cis-DDP will yield mainly the monoaqua-monochloro form.

Chart 2 shows the rate of chloride ion release when cis-DDP (1 mM) is diluted at 37\textdegree in NaClO\textsubscript{4} (10 mM, pH 5.3), or CH\textsubscript{3}COONa (10 mM, pH 5.3) buffers. In NaClO\textsubscript{4}, at this concentration, cis-DDP releases only one chloride ion per molecule of cis-DDP. As expected, at a lower concentration (<0.2 mM), 2 chlorides are released.

At 1 mM, the rate constant for the release of the first chloride anion is found to be \( k(cis)_{Cl} = 1.1 \pm 0.5 \) (S.D.) \times 10\textsuperscript{-4} sec\textsuperscript{-1}. After the release of the first chloride, CH\textsubscript{3}COONa (10 mM, pH 5.3) is added; then the release of a second chloride ion is observed. The corresponding rate constant is \( k(cis)_{Cl} = 4.2 \pm 0.2 \times 10\textsuperscript{-5} \) sec\textsuperscript{-1}. Chart 2 also shows that, when cis-DDP is directly diluted in CH\textsubscript{3}COONa, 2 chloride ions are released according to a single exponential process with a rate constant of \( k(cis)_{Cl} = 0.61 \pm 0.02 \times 10\textsuperscript{-4} \) sec\textsuperscript{-1}. It appears that CH\textsubscript{3}COO\textsuperscript- ion is able to displace the chlorine atom from the monoaqua-monochloro form of cis-DDP. In order to determine the effect of other anions on this chloride displacement, the number of Cl\textsuperscript- ions released at equilibrium in the presence of various concen-
trations of several anions was measured. Results are shown in Chart 3. The weak platinum ligands $\text{NO}_3^-$, $\text{ClO}_4^-$, and $\text{SO}_4^{2-}$ are unable to displace the second chlorine atom, whereas acetate, phosphate, and pyrophosphate anions clearly cause the displacement of the second chloride atom at concentrations greater than 1 mM. Interestingly, trimetaphosphate is unable to cause this displacement.

It must be noted that, with the nonantitumoral isomer trans-DDP, only one chloride ion is released \[
[k (\text{trans}) \text{Cl}_1 = 1.9 \pm 0.3 \times 10^{-4} \text{ sec}^{-1}],
\]
whatever the concentration of the anion in solution. This phenomenon could be related to the ability of acetate, phosphate, and pyrophosphate anions to form only cis-platinum chelates.

**Kinetics Analysis of Ap\textsubscript{A}-cis-DDP Reaction.** The following scheme can represent the reaction of cis-DDP with Ap\textsubscript{A}, at pH 5.3.

\[
\begin{align*}
\text{cis-PtCl}_2 & \xrightarrow{k_1 - k_2} \text{cis-Pt(Cl)(H}_2\text{O)} + \text{Cl}^- \\
\text{Ap}_\text{A} & \xrightarrow{k_3} \text{Ap}_\text{A}[\text{Pt}] + \text{Cl}^- \\
\text{Ap}_\text{A}[\text{Pt}] & \xrightarrow{k_4} \text{Ap}_\text{A}[\text{Pt}] \\
\end{align*}
\]

Scheme A

Under these conditions, there is no significant dimerization of the diaqua and aqua-hydroxo species (10, 17). The cis-DDP itself is supposed to be unreactive. One or 2 chlorides must first be exchanged with water to give the monoaqua-monochloro or the diaqua forms, which are then able to react with Ap\textsubscript{A}. In order to analyze this rather complicated situation, first the rate constants $k_3$ and $k_4$, which correspond, respectively, to the reaction of the mono-aqua-monochloro and pure diaqua species with Ap\textsubscript{A}, shall be directly measured. This can be performed with accuracy using difference absorbance spectroscopy because a single adduct is formed with Ap\textsubscript{A} (20). Accordingly, well-resolved isosbestic points are registered in the various difference absorbance spectra (Chart 4). Next, the overall kinetics of cis-DDP with Ap\textsubscript{A} shall be analyzed. The results shall be compared with the various independently measured rate constants for the separated steps.

**Reaction Kinetics with the Diaqua Species [cis-Pt(H\textsubscript{2}O)\textsubscript{2}].** The reaction kinetics were measured with either one of the 2 reactants [cis-Pt(H\textsubscript{2}O)\textsubscript{2} or Ap\textsubscript{A}] in excess, so that pseudo-first order analysis could be used. The reaction kinetics follow, as expected, a single exponential process, whichever the reactant is in excess (Chart 5). If the pseudo-first order model applies, the apparent rate constant must be proportional to the concentration of the reactant which is in excess. As seen in Chart 6, this is indeed observed when cis-Pt(H\textsubscript{2}O)\textsubscript{2} is in excess. The rate constant for adduct formation, deduced from these data, is found to be $k_4 = 0.94 \pm 0.06 \text{ M}^{-1} \text{ sec}^{-1}$. The same analysis was performed using HPLC to detect the formation of the Ap\textsubscript{A}[Pt] adduct.\textsuperscript{4} The rate constant is found to be not significantly different from that determined by absorbance variation. In contrast, when Ap\textsubscript{A} is in excess, the apparent rate constant levels off at high concentrations of Ap\textsubscript{A}, so that the rate of the reaction becomes much slower in excess Ap\textsubscript{A} than in excess cis-Pt(H\textsubscript{2}O)\textsubscript{2}. This unexpected behavior will be related to a phosphate anion effect of Ap\textsubscript{A}, as discussed later.

The rate constants of the reaction of cis-Pt(H\textsubscript{2}O)\textsubscript{2} with dinucleoside analogues of Ap\textsubscript{A} were also measured. The values are reported in Table 1. One can notice that the reactivity of the diguanosines is 6 to 7 times higher than the reactivity of the diadenosines. This is in agreement with the specificity observed for the G base in the interaction of cis-Pt(H\textsubscript{2}O)\textsubscript{2} with DNA (21).

**Reaction Kinetics with Monoaqua-Monochloro Species [cis-Pt(Cl)(H\textsubscript{2}O)].** The same kinetic analysis was repeated in the presence of 2 mM Cl\textsuperscript{-}. Under these conditions, only one chloride is released (Chart 1), and the mono-aqua-monochloro is the main species in solution. It was found that the kinetics follow a single exponential process as in the previous case (Chart 5). However,

\textsuperscript{4} E. Segal and J-B. Le Pecq, unpublished results.
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The term \((1 - e^{-\alpha t})\) represents the pre-equilibration step leading to the monoaqua-monochloro species.

(b) cis-DDP reaction is measured in solution containing no added \(\text{Cl}^-\), at platinum concentration leading to almost complete hydrolysis (0.25 mM). In this case, the following scheme can be considered.

\[
\text{cis-PtCl}_2 \xrightarrow{k_{-1}} \text{cis-Pt(Cl)(H}_2\text{O)} + \text{Cl}^- \xrightarrow{k_2} \text{cis-Pt(H}_2\text{O)}_2 + \text{Cl}^- \\
\text{Ap}_4\text{A} \xrightarrow{k_4} \text{Ap}_4\text{A}[\text{Pt}] \\
\]

**Scheme C**

This simplified model is considered because \(\text{cis-Pt(Cl)(H}_2\text{O)} \) concentration remains low during hydrolysis and because \(k_3 < k_4\). Therefore, the reaction from \(\text{cis-Pt(Cl)(H}_2\text{O)}\) can be neglected. At this concentration, the \(\text{Cl}^-\) reassociation steps \((k_1, k_2)\) can also be neglected.

The following equations are derived for Scheme C:

\[
\frac{A_t - A_\infty}{A_0 - A_\infty} = \exp(-k_{\text{app}}t) \\
\]

with \(k_{\text{app}}(t) = \frac{k_4}{k_2 - k_1} \biggl[ (k_2 - k_{-1})t - k_{-1} \biggr] \) \(\frac{k_1}{(1 - e^{-\alpha t}) + \frac{k_1}{k_2 - k_1} \biggl[ (1 - e^{-\alpha t}) \biggr]} \)

and \(c = [\text{Pt(H}_2\text{O)}_2]\) at equilibrium.

In this model, the pre-equilibration step is a 2-exponential process.

The kinetics observed in the presence of \(\text{Cl}^-\) are shown in Chart 7A. The results are fitted to Equation A, corresponding to Scheme B. An excellent agreement between the best fit and the data is obtained. The observed lag in the reaction corresponds to the cis-DDP hydrolysis step which leads to the monoaqua-monochloro reactive species. This pre-equilibration step is accelerated, and the lag progressively disappears as \(\text{Cl}^-\) concentration increases as expected from Equation B (Table 2; Chart 7A). The study of the apparent rate constant \(k_{\text{app}}\) versus the \(\text{Cl}^-\) concentration permits the determination of the aquation equilibrium constant \(K_a\):

\[
K_a = \frac{[\text{Pt(Cl)(H}_2\text{O)}][\text{Cl}^-]}{[\text{PtCl}_2]} \tag{E}
\]

combining equations and knowing that \(k_{\text{app}} = k_3[\text{Pt(Cl)(H}_2\text{O)}]:\)

\[
\frac{1}{k_{\text{app}}} = \frac{[\text{Cl}^-]}{k_3[\text{PtCl}_2]} + \frac{1}{k_3[\text{PtCl}_2]} \tag{F}
\]

with \([\text{PtCl}_2]\) the initial concentration of cis-DDP.

If \(\frac{1}{k_{\text{app}}} = 2.3\) is plotted as a function of \([\text{Cl}^-]\), one gets the dissociation constant \(K_a = 2.3\) mm. This value is to be compared to the value reported by Reishus and Martin (15) (3.3 mm).

---

**Table 1**

| Diadenosines | Diad constants measured by spectrophotometry for the reaction of cis-|  
| Pt(NH₃)₂(H₂O)²⁺ |  
| Ap₄A | 0.80 ± 0.05  
| Ap₂A | 0.94 ± 0.06  
| Ap₄A | 0.35 ± 0.05  

* Mean ± S.D.

the corresponding rate constant \(k_3\) was found to be significantly smaller than that of the diaqua species (0.16 versus 0.94 M⁻¹ sec⁻¹), indicating that the monoaqua-monochloro is less reactive than is the diaqua species.

**Reaction Kinetics with the Dichloro Species (cis-PtCl₂).** Two situations will be considered: (a) cis-DDP reaction is measured in solution containing more than 2 mM \(\text{Cl}^-\). In this case, cis-monoaqua-monochloro is the main reacting species formed after hydrolysis, and the simplified following scheme can be applied.

\[
cis-PtCl₂ \xrightarrow{k_{-1}} cis-Pt(\text{Cl})(H₂O) + \text{Cl}^- \\
Ap₄A \xrightarrow{k_2} Ap₄A[\text{Pt}] + \text{Cl}^- \\
\]

**Scheme B**

When cis-DDP is in excess, a pseudo-first order approximation can also be used, as in the preceding model. Using the usual analysis of kinetic reactions, the following equations are derived for Scheme B:

\[
\frac{A_t - A_\infty}{A_0 - A_\infty} = \exp(k_3[\text{Pt(Cl)(H}_2\text{O)}]1 - e^{-\alpha t} - at) \\
\]

\(A_0, A_t, and A_\infty\) are the absorbance at 0, \(t\), and infinite times, respectively, with:

\[
a = k_{-1} + k_1([\text{Pt(Cl)(H}_2\text{O)}] + [\text{Cl}^-]) \tag{B}
\]
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Chart 7. Variation of the absorbance at \( \lambda = 280 \text{ nm} \) during cis-DDP-Ap4A reaction in solutions containing increasing \( \text{Cl}^- \) concentrations (A) and in solutions containing no \( \text{Cl}^- \) (B). All measurements were done at the same concentrations (Ap4A, 0.01 mM; cis-DDP, 0.25 mM in 10 mM NaClO\(_4\), pH 5.3; 37°). The 2 lower curves were shifted down by a small absorbance value to obtain a clearer representation. The dots represent the experimental data. The continuous lines are the best fits computed (A) using Equation A and Scheme B when Cl is present; and (B) using Equation C and Scheme C when Cl is absent.

The kinetics measured under conditions where Scheme C can apply is shown in Chart 7B. It is observed that the data are much better fitted by Equation C than by Equation A. The values of the different rate constants deduced from the analysis of the data using the 2 different schemes (Equation A or Equation C) are compared in Table 3 to those determined separately for the various steps (iii).

Furthermore, when cis-DDP was preequilibrated for 24 hr\(^{-1}\) in its buffer before reaction with Ap4A, lag periods were never observed, confirming that this lag results from the hydrolysis step(s).

The same study was also performed with trans-DDP. In this case, as shown by the potentiometric measurements, the mono-octa-monochloro species is the predominant hydrolyzed form under all conditions studied. Therefore, the data can be analyzed using Scheme B and Equation A.

In the absence of added \( \text{Cl}^- \), the lag period was smaller, giving \( 48 \times 10^{-4} \text{ sec}^{-1} \) and a rate constant of \( k'3 = 0.8 \pm 0.04 \text{ M}^{-1} \text{ sec}^{-1} \).

As soon as chlorides ions are added, latency disappears. As the release rate constant of \( \text{Cl}^- \) from trans-DDP was found to be about the same as that from cis-DDP, \( k'1 \) must be at least 10 times higher than the corresponding value \( k1 \) with cis-DDP. This is consistent with the fact that the dissociation equilibrium constant of \( \text{Cl}^- \) from cis-DDP (3.3 mM) is smaller than that from trans-DDP (0.32 mM) (1).

Effect of Acetate, Phosphate, and PP on cis-DDP Reaction Kinetics. The results shown in Chart 2 indicate that acetate, phosphate, and PP, are able to displace \( \text{Cl}^- \) from cis-DDP. The effect of these anions on the reactivity of cis-platinum was therefore investigated.

The kinetics of the cis-PtCl\(_2\) reaction with Ap4A were measured: (a) with cis-PtCl\(_2\) or cis-Pt(H\(_2\)O)\(_2\) preequilibrated for 24 hr in acetate, phosphate, or PP, before addition of Ap4A. (b) with

Table 2

Values of the rate constants, derived from Equation A, for the reaction of cis-[Pt(NH\(_3\)Cl\(_4\)] (0.25 mM) with Ap4A (0.01 mM) in solution containing various concentrations of NaCl (37°).

<table>
<thead>
<tr>
<th>( \text{Cl}^- ) (mM)</th>
<th>10(^{-4} \times k_{epp} \text{ sec}^{-1} )</th>
<th>10(^{-4} \times k_3 \text{ sec}^{-1} )</th>
<th>10(^{-4} \times k_{Apt} \text{ sec}^{-1} )</th>
<th>10(^{-4} \times k_{Apt} \text{ sec}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 ± 0.04</td>
<td>0.12 ± 0.06</td>
<td>0.10 ± 0.04</td>
<td>0.10 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>2.0 ± 0.14</td>
<td>2.5 ± 1.4</td>
<td>2.3 ± 1.2</td>
<td>2.3 ± 1.2</td>
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</tr>
<tr>
<td>3.3 ± 0.15</td>
<td>3.8 ± 0.12</td>
<td>3.5 ± 0.12</td>
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</tr>
<tr>
<td>5.0 ± 0.21</td>
<td>5.5 ± 0.21</td>
<td>5.2 ± 0.21</td>
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</tr>
<tr>
<td>7.0 ± 0.25</td>
<td>7.5 ± 0.25</td>
<td>7.2 ± 0.25</td>
<td>7.2 ± 0.25</td>
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</tr>
<tr>
<td>9.0 ± 0.29</td>
<td>9.5 ± 0.29</td>
<td>9.2 ± 0.29</td>
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<td></td>
</tr>
<tr>
<td>11.0 ± 0.35</td>
<td>11.5 ± 0.35</td>
<td>11.2 ± 0.35</td>
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<td></td>
</tr>
</tbody>
</table>

(a) Rate constants deduced from the fit of the kinetics reaction of Ap4A (0.01 mM) with cis-DDP (0.25 mM) using Scheme B, Equation A.

(b) Mean ± S.D.

(c) \( k_3 \) was determined with cis-PtCl\(_2\) (1 mM).

Result of the reaction of Ap4A (0.01 mM) with cis-PtCl\(_2\) (0.25 mM) in 10 mM NaClO\(_4\) containing 2 mM NaCl, directly deduced from spectrophotometric measurements.

Result of the reaction of Ap4A (0.01 mM) with cis-Pt(H\(_2\)O)\(_2\) (0.25 mM) in 10 mM NaClO\(_4\) containing 2 mM NaCl, directly deduced from spectrophotometric measurements.

Table 3

Rate constants deduced from the analysis of the overall reaction of cis-DDP with Ap4A

<table>
<thead>
<tr>
<th>Reduced ( x^2 ) for data fit</th>
<th>( 10^4 \times k_1 \text{ sec}^{-1} )</th>
<th>( 10^4 \times k_2 \text{ sec}^{-1} )</th>
<th>( 10^4 \times k_{Apt} \text{ sec}^{-1} )</th>
<th>( 10^4 \times k_{Apt} \text{ sec}^{-1} )</th>
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<tr>
<td>0.08 ± 0.02</td>
<td>2.3 ± 1.4</td>
<td>2.3 ± 1.2</td>
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<tr>
<td>1.5 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>3.3 ± 0.15</td>
<td>3.5 ± 0.21</td>
<td>3.5 ± 0.21</td>
<td>3.5 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

(a) Rate constants deduced from the fit of the kinetics reaction of Ap4A (0.01 mM) with cis-DDP (0.25 mM) using Scheme A.

(b) Mean ± S.D.

(c) \( k_3 \) was determined with cis-PtCl\(_2\) (1 mM).

(d) Rate constants deduced from the fit of the same reaction as in Footnote a but using Scheme C, Equation C (see Chart 7B).

(e) Rate constants directly deduced from the potentiometric measurements of chloride release from cis-PtCl\(_2\) (1 mM).

(f) Rate constant of the reaction of Ap4A (0.01 mM) with cis-Pt(H\(_2\)O)\(_2\) (0.25 mM) in 10 mM NaClO\(_4\), containing 2 mM NaCl, directly deduced from spectrophotometric measurements.

The preequilibration step becomes so fast that the corresponding rate constant can no longer be determined.

Effect of Acetate, Phosphate, and PP on cis-DDP Reaction Kinetics. The results shown in Chart 2 indicate that acetate, phosphate, and PP, are able to displace \( \text{Cl}^- \) from cis-DDP. The effect of these anions on the reactivity of cis-platinum was therefore investigated.

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cis-PtCl₂ extemporaneously diluted in buffer containing the anion to be studied and Ap₄A.

The results obtained for cis-PtCl₂ and cis-Pt(H₂O)₂ preequilibrated in the same buffer are very similar, as expected. The data obtained for preequilibrated cis-PtCl₂ are shown in Chart 8 (Curves 1 and 3). There is no lag period, and the corresponding rate constants are significantly lower than those measured for the diaqua form, as shown in Table 4.

The result obtained for cis-PtCl₂ extemporaneously diluted in buffer containing Ap₄A is shown in Chart 8 (Curves 2 and 4). Clearly, there is a lag period. The data are well fitted by Equation A according to the following scheme:

\[
cis-PtCl₂ + X^- \xrightarrow{k_{-1}} cis-PtX + 2Cl^- \\
\]

\[
\text{Ap₄A} \xrightarrow{k_{\text{rel}}} \text{Ap₄A}[Pt] \\
\]

\[
\text{where } X^- \text{ is acetate, phosphate, or PP₃.} \\
\]

The deduced rate constants are reported in Table 4. This Table shows that the lag period seems independent of the anion in solution and corresponds to the time of Cl⁻ release measured by potentiometry.

The values of \(k_{\text{rel}}\) which are deduced from the kinetics with and without preequilibration are very similar. Furthermore, \(k_{\text{rel}}\) is to be compared to \(k_2\) measured for the monoaqua-monochloro species (Table 3). This indicates that, immediately after the release of Cl⁻, an acetato, phosphato, or pyrophosphato complex is formed which then becomes the reactive species.

The competition between acetate and Cl⁻ is further evidenced by the effect on kinetics of various Cl⁻ concentrations in acetate buffer. The results are shown in Table 2.

DISCUSSION

Because cis-DDP can react with several intracellular targets, the formation of the various adducts would depend on the kinetics of the different reactions. The understanding of these kinetics is therefore of importance.

Potentiometric measurements using a chloride-specific electrode permitted both the direct measurement of the rate of chloride release upon cis-DDP acid hydrolysis and the determination of the corresponding equilibrium dissociation constants. The results are in good agreement with those reported by Rheius and Martin (15), which were deduced from the measurements of chloride isotopic exchange. These results underline that the main intracellular form of cis-DDP, in the absence of other anion, will be the monoaqua-monochloro species. The appearance of the diaqua form would require the chloride concentration to be below the millimolar range. Such a low concentration might exist in the nucleus if the number of free negative charges born on nucleic acids were large enough to cause a significant Donnan effect.

Furthermore, the potentiometric measurements show that P₃, PP₃, and acetate anions are able to displace the chloride from cis-DDP, implying that these anions are able to exchange with chloride on platinum to form the corresponding phospho, pyrophospho, and acetato complexes. Direct evidences for the formation of phospho, pyrophospho, and triphospho complexes have just been reported. The triphospho complexes are bidentate chelates between α and β or α and γ phosphate groups (3). Accordingly, we suggest that the phosphate groups of Ap₄A are able to form a chelate with cis-platinum rather than a ion-pair complex. The concentration of P₃, PP₃, triphosphate, and other polyphosphate compounds in living cells deduced from ³¹P-NMR spectroscopy (4, 5) are in the concentration range which will cause chloride displacement from cis-DDP. This is especially apparent for PP₃, which displaces the chloride at concentration close to millimolar (Chart 3). The intracellular concentration of substances bearing carboxyate groups, such as citrate, glutamate, and succinate, determined by ¹³C NMR (4, 5) is also in the range which allows the formation of carboxylato platinum complex. Therefore, inside cells, cis-platinum might well exist complexed with phosphate, PP₃, or carboxylate groups. Interestingly, the chlorides from trans-platinum complex are not displaced by these anions. It was therefore of interest to study the reactivity of such cis-platinum complexes.

The kinetic study of the reaction of cis-DDP with Ap₄A allows...
the following conclusions to be drawn:

In medium containing no other anions than Cl\(^-\), the monoaqua and diaqua species are the only reactive derivatives, the diaqua being almost 6 times more reactive than the monoaqua form. The chloride concentration in solution is therefore critical in controlling the reactivity of cis-DDP, and it determines the rate of aquation and the nature of the reacting species.

In medium containing other anions such as acetate or phosphate at concentration close to 10 mM or PP, at 1 mM, cis-DDP will first exchange its chlorides with acetate, phosphate, or PP.

The rate of the exchange process is limited by the rate of aquation of cis-DDP. Direct chloride displacement by phosphate ligands is only significant above the 10 mM range (3). The resulting acetato, phoshato, or pyrophosphate complexes are able to react at a rate much lower than that of the diaqua form but not very different from that of the monoaqua-monochloro form (Tables 3 and 4).

The results also illustrate the effect of phosphate groups on the cis-platinum targets. It was observed that, when cis-DDP is in excess and Ap\(_4\)A is at low concentration, a simple pseudofirst order analysis applies. On the contrary, when Ap\(_4\)A is in excess and its concentration is larger than 0.1 mM, the apparent rate constant for the reaction is independent of the Ap\(_4\)A concentration and close to 2 \(\times\) 10\(^{-4}\) sec\(^{-1}\). Such results suggest that when the reaction is performed with Ap\(_4\)A under these conditions, the phosphate groups of Ap\(_4\)A first exchange with the chloride on platinum. The reaction with cis-DDP is therefore limited by the rate of the intramolecular ligand exchange.

Such a mechanism probably applies also in the case of DNA, when DNA is in excess over cis-DDP. In order to study this possibility, the ability of DNA to compete with Ap\(_4\)A for Ap\(_4\)A-[Pt] adduct formation was analyzed under conditions where both Ap\(_4\)A and DNA were in excess over cis-DDP. It was observed that, under these conditions, the cis-DDP-DNA adduct could not form faster than the Ap\(_4\)A adduct. Furthermore, it was reported that cis-DDP-DNA reaction kinetics (12) are not very different from those found here for Ap\(_4\)A. Under intracellular conditions, DNA concentration is in large excess over that of cis-DDP. Therefore, this could cause a considerable apparent slowing down of the DNA-cis-DDP reaction, as observed in vitro for Ap\(_4\)A (Chart 6).

The present results illustrate the particular complexity of the reaction of cis-DDP with its potential intracellular targets. The nature and the concentration of the various anions inside the cell could be critical in determining the rate of appearance and the nature of the reactive platinum species. In addition, the existence of charged phosphates on the potential cellular targets, such as DNA, might be also determinant.

Finally, it appears that, from a kinetic point of view, Ap\(_4\)A can represent a cellular target of cis-DDP. Because of the potentially important role of this signal nucleotide in controlling cell division, the biological significance of such a reaction merits further study.

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Role of Ligand Exchange Processes in the Reaction Kinetics of the Antitumor Drug cis-Diamminedichloroplatinum(II) with Its Targets

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