KINETIC STUDY OF THE REACTION OF CISPLATIN WITH THIOLS

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ABSTRACT:

The reactions of cisplatin [cis-diaminedichloroplatinum(II), CDDP] with glutathione (GSH) and drug thiols were investigated at 37°C in 100 mM Tris-NO₃, pH ∼7.4, using a clinically relevant concentration of CDDP (33 µM), a large excess of GSH (16.5 mM), and [NaCl] of 4.62 mM. The conditions were designed to mimic passage of CDDP through the cytosol. The reactions were studied by UV-absorption spectroscopy, high-pressure liquid chromatography (HPLC), and atomic absorption spectroscopy. The initial rates, detected by UV absorbance, confirmed that the reactions are first order in [CDDP]. The HPLC peak corresponding to CDDP was analyzed for platinum content by atomic absorption spectroscopy, which decreased exponentially with time, confirming that the reactions are first order in [CDDP] and allowing determination of the pseudo first order rate constants (kᵢ). For reaction of the dichloro form of CDDP with GSH, the kᵢ value was ~2.2 × 10⁻⁴ s⁻¹ (t₁/₂ of ~53 min), giving the second order rate constant value (k₂) of ~1.3 × 10⁻² M⁻¹ s⁻¹. Reaction of a mixture of the aquated forms of CDDP with GSH gave a lower kᵢ value (~0.9 × 10⁻⁴ s⁻¹). Reaction of CDDP with sodium 2-mercaptoethanesulfonate (mesna) gave a kᵢ of ~1.8 × 10⁻⁴ s⁻¹ (t₁/₂ of ~65 min and kₐ of ~1.1 × 10⁻⁸ M⁻¹ s⁻¹). Reaction of CDDP with S-2-(3-aminopropylamino)ethanethiol (WR-1065) gave a kᵢ value of ~12.0 × 10⁻⁴ s⁻¹ (t₁/₂ of ~10 min and k₂ of ~7.3 × 10⁻² M⁻¹ s⁻¹). The relatively slow reaction rate of CDDP with GSH is consistent with the efficient DNA platination by CDDP in the presence of millimolar concentration of GSH in the cytosol.

CDDP exerts its antitumor activity by binding to cellular DNA (Rosenberg, 1971; Gelasco and Lippard, 1999). When the drug enters the cell, it passes through the cytosol, enters the nucleus envelope, binds to nitrogen atoms on the bases of DNA, and promotes cell death by apoptosis (Bellon et al., 1991; Demarchi et al., 1994). The cytotoxic activity of CDDP thus seems to correlate with the amount of platinum (Pt) bound to DNA (Zwelling et al., 1979; Lindauer and Holler, 1996).

Biological thiols such as GSH, metallothionein, and other protein thiols defend the cell against CDDP (Kraker et al., 1985; Pattanaik et al., 1992; Ishikawa and Ali-Osman, 1993; Reedijk and Teuben, 1999). Pt ions entering the cell may preferentially bind to GSH and metallothionein (Dedon and Borch, 1987), both present in millimolar concentrations in the cytoplasm (Souid et al., 1999, 2001; A.-K. Souid personal observation). Because Pt-thiol adducts interact less well with DNA (Volkova et al., 2002), formation of these complexes limits the amount of drug available for binding to DNA. Furthermore, continued exposure to CDDP can increase the cytosolic sulfhydryl level (Schilder et al., 1990; Eastman 1991; Godwin et al., 1992), which may produce CDDP resistance.

The sulfhydryl agents WR-1065 and mesna are often administered to mitigate toxicities of Pt-based compounds and alkylating agents, such as cyclophosphamide (Brock et al., 1982; Souid et al., 2001; Tacka et al., 2002). The protective mechanism of WR-1065 and mesna involves reaction of the thiolate ion with CDDP in a manner analogous to that of GSH (Leeuwenkamp et al., 1991; Treskes et al., 1991). Previously, we measured the rate of CDDP binding to DNA in peripheral blood mononuclear cells and ovarian cancer cells (Sadowitz et al., 2002). By blocking the cellular thiol groups and by adding known concentrations of WR-1065 or mesna, we showed how the amount of CDDP reaching the DNA is affected by thiol concentration.

Although CDDP reaction with thiols has been studied extensively (Barnes-Price and Kuchel, 1990a,b; Ishikawa and Ali-Osman, 1993; Bernareggi et al., 1995; Perez-Benito et al., 1995; Bose et al., 1997; El-Khateeb et al., 1999), there is little kinetic data under clinically relevant conditions. In this work, we use UV absorption, HPLC, and atomic absorption spectroscopy (AAS) to measure the rates of CDDP reaction with GSH, WR-1065, and mesna, using a therapeutic con-
concentration of CDDP and conditions that mimic passage of CDDP through the cytosol. The data obtained improve our understanding of how cellular GSH and drug thiols influence DNA platination by CDDP.

Materials and Methods

Chemicals. CDDP (1 mg/ml, −3.3 mM in 154 mM NaCl) was obtained from American Pharmaceutical Partners (Los Angeles, CA); mesna (mol. wt. 164.18, 100 mg/ml solution) was obtained from Bristol-Myers Squibb Co. (Princeton, NJ); WR-1065-2HCl (mol. wt. 207.16) was obtained from U.S. Bioscience (West Conshohocken, PA); Tris was purchased from Fluka Chemical Corp. (Ronkonkoma, NY); GSH, 5,5′-dithio-bis(2-nitrobenzoic acid), dihexylamine, glacial acetic acid, NaCl, and pH Test Strips (4.5–10.0) were purchased from Sigma-Aldrich (St. Louis, MO); and Pt atomic spectroscopy standard (H2PtCl6, 1 mg/ml in 10% HCl) was purchased from PerkinElmer Instruments (Norwalk, CT).

Solutions. GSH, WR-1065, and mesna solutions were prepared in dH2O and stored at −70°C in small aliquots; their concentrations were determined by titration with 5,5′-dithio-bis(2-nitrobenzoic acid) (Soud et al., 1998). Dihexylammonium acetate (DHAA, 2.5 mM) HPLC solvents were prepared in the hood by the addition of 590 µl of 4.24 M dihexylamine and 144 µl of 17.4 M glacial acetic acid to each liter of dH2O or methanol. The pH was adjusted to 7.0 by small additions of dihexylamine or acetic acid.

Reaction of CDDP with Thiol (GSH, WR-1065, or Mesna). Reactions were carried out in the dark at 37 ± 0.1°C in a total volume of 1.0 ml. In a typical experiment, the mixture contained 100 mM Tris-NO3, pH 7.4; 33, 66, or 99 µM CDDP (from a 3.3 mM stock solution in 154 mM NaCl); and 16.5 mM thiol. For all reactions, the final concentration of NaCl was adjusted to 4.62 mM, the approximate concentration of NaCl in the cytoplasm, by the addition of appropriate amount of 154 mM NaCl solution. Reactions were initiated by mixing CDDP with the buffer and NaCl followed by immediate addition of the thiol. The [CDDP]:[GSH] ratio in the reaction mixture is 1:500, which mimics that of cells exposed to a therapeutic concentration of CDDP (see Discussion).

The effects of “aging” CDDP on the reaction with GSH were also investigated. In this case, 38.6 µM CDDP, in a medium containing 117 mM Tris-NO3, pH 7.4, and 5.4 mM NaCl (total volume, 856 µl) was allowed to stand at room temperature (RT, −21°C) in the dark for 4, 24, and 48 h before the addition of GSH. Aging CDDP in this manner causes a decrease in the affinity of the drug (Miller and House, 1990; El-Khateeb et al., 1999) to the chloro-aquo complex (2) and some of the diaquo complex (3) (Scheme 1). Reactions with aged CDDP were carried out using the following final concentrations and conditions: 33 µM aged CDDP, 16.5 mM GSH, 4.62 mM NaCl, and 100 mM Tris-NO3, pH 7.4, at 37°C.

UV Absorbance. The absorbance at 260 nm as a function of time (reflecting formation of Pt-thiol bonds and disulfides) was measured using a single beam spectrophotometer (model DU 640B; Beckman Coulter, Inc., Fullerton, CA). Samples were in a 1-cm path length quartz cuvette with a Teflon stopper, which was thermostatted at 37 ± 0.1°C. The reaction contained 33 µM CDDP, 16.5 mM thiol, 4.62 mM NaCl, and 100 mM Tris-NO3, pH 7.4, at a final volume of 1.0 ml. The spectrophotometer was “zeroed” immediately after thiol addition, which initiated the reaction. The control experiments for determining the rate of disulfide formation (thiol oxidation) contained the exact same reaction mixture without CDDP.

HPLC-UV. Analysis was performed on a reversed-phase HPLC system (Beckman Coulter, Inc.), which consisted of an automated injector (model 507e), a pump (model 125), and a UV detector (model 166). UV detection at 260 nm was used; at this wavelength the GS-Pt adducts absorb strongly (Ishikawa and Ali-Osman, 1993; Perez-Benito et al., 1995). Solvent A was 2.5 mM DHAA in dH2O and solvent B 2.5 mM DHAA in HPLC-grade methanol. The column, a 4.6 × 250-mm Ultrasphere IP column (Beckman Coulter, Inc.), was operated at RT at a flow rate of 0.5 ml/min.

For the reaction of CDDP with GSH, the chromatography procedure used linear gradients as follows: 0 min, 0% B; 5 min, 10% B; 20 min, 75% B; 40 min, 100% B; 45 min, 100% B; 46 min, 10% B; and 60 min, reinject. The injection volume was 50 µl. For the CDDP reaction with WR-1065 or mesna, the chromatography procedure used linear gradients as follows: 0 min, 0% B; 5 min, 0% B; 15 min, 10% B; 20 min, 100% B; 21 min, 0% B; and 30 min, reinject. This modified program was necessary to separate CDDP from the Pt-drug thiol products. The injection volume was also 50 µl.

AAS. The Pt analysis was performed using the graphite furnace of an AAS (model AA-6800; Shimadzu, Kyoto, Japan), with an inte (Imaging and Sensing Technology, Horseheads, NY) hollow cathode Pt lamp, deuterium arc background correction, and pyrolytically coated graphite tubes (Sadowitz et al., 2002). A calibration curve (using H2PtCl6) was generated before each measurement and proved to be linear from 0 to 10 pmol (r > 0.99). The lower limit of detection was ~20 pg of atomic Pt (~0.1 pmol). A background reading of ~0.0060 optical density (for dH2O) was subtracted from each of the determinations containing Pt. The injection volume was 20 µl.

Results

Reaction of CDDP with GSH as Monitored by UV Absorption. UV absorption at 260 nm of solutions containing different concentrations of CDDP, 16.5 mM GSH, 100 mM Tris-NO3, pH 7.4, and 4.62 mM NaCl (final volume of 1.0 ml) as a function of time is shown in Fig. 1. The increased absorption with time resulted partially from the reaction of CDDP with GSH (formation of Pt-sulfur bonds) and partially from the oxidation of GSH (formation of the disulfide GSSG), as shown by the increased absorption with time for GSH alone (Fig. 1, dashed lines). Because the rate of disulfide formation is slow (Fig. 1, dashed lines), the concentration of GSH was assumed not to change much over the time course of the GSH-CDDP reaction (several hours). This assumption was also verified by HPLC, which showed a large excess of GSH at the end of each incubation period. Because the GSH concentration is 500 times that of the concentration of CDDP, the reaction of GSH with CDDP likewise does not deplete GSH.

To obtain the absorbance associated with CDDP reaction with GSH, the absorbance due to the disulfide formation (essentially linear over 1200 min) was subtracted from the observed absorbance (Fig. 1). The difference, represented by the solid curves in Fig. 1, shows the change in absorption for the reaction of CDDP with GSH (because the rate of the disulfide formation, as determined by HPLC, was not significantly changed by the presence of CDDP). Although both CDDP and Pt-thiol products absorb at 260 nm, the extinction coefficients of the products are much greater than those of CDDP (Ishikawa and Ali-Osman, 1993; Perez-Benito et al., 1995). Thus, the absorbance at 260 nm is mainly due to the formation of Pt-thiol products.

To establish the order of the initial reaction with respect to CDDP, each difference curve was fitted to the following equation:

\[ I_t = C + A_1 \exp(-b_1t) + A_2 \exp(-b_2t) \]

and the initial slope (\(S_{in}\)) was calculated as \(-A_1b_1 + A_2b_2\). The results are shown in Table 1. The \(S_{in}\) values plotted versus [CDDP] fit \((r^2 = 0.993)\) a straight line going through the origin; \(S_{in} = -7.48 \times\)
TABLE 1

Initial rates of reactions of CDDP with thiols obtained from UV absorption data

For GSH and mesna, the absorption at 260 nm as a function of time (0 ≤ t ≤ 1200 min) for CDDP concentration = 0 was subtracted from the absorption for each CDDP concentration. The difference was fitted to C + A₁ exp(-b₁t) + A₂ exp(-b₂t), and the initial slope (Sₐ) was calculated as -(A₀b₁ + A₂b₂). For WR-1065, the difference of absorptions for each CDDP concentration was fitted to a linear function of time (0 ≤ t ≤ 30 min) to obtain the slope. The concentrations and condition were: Thiol, 16.5 mM; NaCl, 4.62 mM; and Tris-NO₃, 100 mM, pH 7.4 at 37°C.

<table>
<thead>
<tr>
<th>[CDDP]</th>
<th>Initial Rates (Slopes)</th>
<th>GSH</th>
<th>Mesna</th>
<th>WR-1065</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>min⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>0.001046</td>
<td>0.000415</td>
<td>0.0010</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>0.001955</td>
<td>0.001682</td>
<td>0.0021</td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>0.003182</td>
<td>0.004382</td>
<td>0.0058</td>
<td></td>
</tr>
</tbody>
</table>

260 nm was measured. The absorption of GSH alone was subtracted, the difference was fitted to eq. 1, and the initial slopes (rates) were calculated as indicated above.

For the 24-h aged CDDP, the Sₐ value was 0.000755 and for the 48-h aged CDDP, the Sₐ value was 0.000355. These values are much lower than Sₐ determined for unaged 33 µM CDDP, 0.001046 (Table 1). This shows that aging converts CDDP into a form(s) that reacts more slowly with GSH than CDDP itself. It also shows that the conversion is a slow process, because it is incomplete in 24 h.

Reactions of CDDP with WR-1065 and Mesna as Monitored by UV Absorption. UV absorption at 260 nm was also observed during 20-h incubations at 37°C of 0 to 99 µM CDDP, 16.5 mM mesna, 100 mM Tris-NO₃, pH 7.4, and 4.62 mM NaCl. With no CDDP present, the UV absorption of mesna varied with time in a more complicated way than that of GSH. Nevertheless, when the absorption for mesna alone is subtracted from the absorption for mesna plus 33, 66, and 99 µM CDDP, we obtained plots similar to those in Fig. 1. Fitting these difference plots to eq. 1, we calculated the initial slopes as Sₐ = -(A₀b₁ + A₂b₂), giving the results in Table 1. Plotted versus [CDDP], these Sₐ values give a fit (r² = 0.96) to a straight line, Sₐ = -0.00181 + 6.01 × 10⁻⁵ [CDDP]/µM, showing that the initial reaction is first order in [CDDP].

The UV absorption of 16.5 mM WR-1065 without added CDDP increased with time fairly linearly up to ~200 min and then became extremely large, suggesting a colloid may be forming. The mixtures of CDDP and WR-1065 also behaved similarly. We subtracted the profiles for short times only and fitted the differences to linear functions of time for t ≤ 30 min. The slopes are given in Table 1. Plotted versus [CDDP], these Sₐ values give a fit (r² = 0.91) to a line Sₐ = -0.00181 + 7 × 10⁻⁵ [CDDP]/µM, consistent with first order kinetics in [CDDP].

Reaction of CDDP with GSH as Monitored by HPLC-AAS. A typical chromatogram of the reaction of CDDP with GSH is shown in Fig. 2. The reaction mixture (at 37°C) contained 33 µM CDDP, 16.5 mM GSH, 100 mM Tris-NO₃, pH 7.4, and 4.62 mM NaCl (final volume of 1.0 ml). It shows about 45 peaks on a curved baseline, with retention times (tᵣ) between 4 and 48 min. Values of tᵣ in minutes for some peaks are as follows: Tris-NO₃, ~21.0; GSH, ~25.0; and GSSG, ~26.5. The chromatogram also contained a significant number of peaks from DHAA, which do not change with reaction time.

The area of the peak with tᵣ = ~4.5 min diminishes with reaction time, suggesting that it corresponds to CDDP. Peaks whose areas increase with reaction time, and thus correspond to Pt-sulfur products, are 21 min < tᵣ < 25 min and 26.5 min < tᵣ < 31 min. To confirm this, we collected fractions throughout the chromatogram and analyzed them for Pt by AAS. Some of the AAS absorptions are given in Table 2.
The reaction of aged CDDP with GSH was measured using AAS, similarly to the results above (Fig. 3). In one experiment, the CDDP was aged for 4 h before being mixed with GSH under the exact conditions described above. After reaction times of 0, 0.27, 0.8, 1.33, 1.85, 2.38, and 2.92 h, samples were subjected to HPLC. The Pt content of elutes 3.0 min ≤ t_R ≤ 6.5 min, which includes the CDDP peak, was measured by AAS. The results are shown in Fig. 3 (circles). The logarithm of AAS absorption intensity is not linear in time, indicating that more than one reaction is taking place. The initial rate was estimated in two ways (see Discussion). First, we fitted the logarithm of the absorption A to an exponential function of time, i.e., \( \ln(A) = a + bc^{-rt} \) (long-dashed line in Fig. 3). The initial rate is then \( bc = 0.767 \text{ h}^{-1} = 0.0128 \text{ min}^{-1} (\sim 2.1 \times 10^{-4} \text{ s}^{-1}) \), slightly below the average rate determined for unaged CDDP. Second, we fitted the first three values of \( \ln(A) \) to a linear function of time, which gave a slope of 0.0115 min\(^{-1}\) (\( \sim 1.9 \times 10^{-4} \text{ s}^{-1} \)).

In another experiment, CDDP was aged for 24 h at 25°C before reaction at 37°C. The measured AAS absorption intensities are given in Table 3. For the 4.5-min ≤ t_R ≤ 5.75-min peak, the intensities fit \( (r^2 = 0.998) 0.649 e^{-0.00547t} \). There is no evidence for more than one reaction, and the \( k_1 \) is 0.00547 min\(^{-1}\) (\( \sim 0.9 \times 10^{-4} \text{ s}^{-1} \)). This is less than half the value of the rate constant for the standard reaction (i.e., with unaged CDDP). Note that the UV absorption measurements showed that the rate was reduced by a factor of 0.72 by 24 h aging and by a factor of 0.34 by 48 h aging at 25°C.

The other three elutes, for 21.25 min ≤ t_R ≤ 22.75 min, 26.5 min ≤ t_R ≤ 28.0 min, and 28.0 min ≤ t_R ≤ 29.5 min (Table 3), show the Pt contents increasing with reaction time, indicating that, as for unaged CDDP, they represent Pt-sulfur products. Although the absorption intensities for the last elute (28.0 min ≤ t_R ≤ 29.5 min) are linear in reaction time \( (r^2 = 0.991) \), this is not the case for the other two. For these, the absorption increased faster at larger reaction time, suggesting that the Pt-sulfur products corresponding to these peaks are not formed directly from CDDP, but produced from an intermediate.

**Reaction of CDDP with WR-1065 and Mesna as Monitored by HPLC-AAS.** The reaction rate constants for CDDP with WR-1065 and mesna (Fig. 4) were determined in the same way as for the reaction of CDDP with GSH. After establishing that the chromato-
graphic peak for CDDP corresponded to the reaction of CDDP with WR-1065 and mesna (Fig. 4), were determined in the same way as for the reaction of CDDP with WR-1065 and mesna (Fig. 4), were
growth of reaction time, the reason for the incomplete product recovery is not clear, but it may be due to retention of some of the Pt-containing products (possibly polymeric in nature) on the column.

Figure 3 shows the Pt-AAS absorption for elutes from the CDDP peak as a function of reaction time. Two experiments (experiments 1 and 2; Table 2), using 33 μM CDDP, were conducted. In the first experiment (experiment 1 in Table 2 and triangles and dashed line in Fig. 3), AAS-Pt absorptions were measured for the CDDP peak at reaction times of 0, 0.5, 2.6, and 3.6 h. The natural logarithms of the absorbance, after background subtraction, fit \( (r^2 = 0.999) \) the line \( -0.264 - 0.758t \), showing that the reaction is first order. The \( k_1 \) value is 0.758 ± 0.017 h\(^{-1}\) or 0.0126 ± 0.0003 min\(^{-1}\) (\( \sim 2.1 \times 10^{-4} \text{ s}^{-1} \)).

The absorption \( t_{1/2} \) is 54.9 ± 1.2 min. In the second experiment (2 in Table 2 and squares and solid line in Fig. 3), the Pt content (absorption) of the CDDP peak was measured for reaction times of 0, 1, 2, and 3 h. After subtraction of background, the logarithms of the absorption were plotted versus reaction time. It fits the line \( -0.086 - 0.831t \), with \( r^2 = 0.991 \), also showing the reaction is first order. The \( k_1 \) value is 0.831 ± 0.057 h\(^{-1}\) or 0.0138 ± 0.0010 min\(^{-1}\) (\( \sim 2.3 \times 10^{-4} \text{ s}^{-1} \)). The \( t_{1/2} \) is 50.0 ± 3.4 min. The \( k_2 \) value, obtained by dividing \( k_1 \) value by the GSH concentration (16.5 mM), averages \( \sim 1.33 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1} \).

The reaction is first order, giving \( k_1 = 0.072 \pm 0.008 \text{ min}^{-1} (\sim 12.0 \times 10^{-4} \text{ s}^{-1}) \).
For the 4-h aging time, the reaction is biphasic; the initial $k_1$ curves) for the unaged CDDP reaction average

1995; Perez-Benito et al., 1995; Bose et al., 1997; El-Khateeb et al., Kuchel, 1990a,b; Ishikawa and Ali-Osman, 1993; Bernareggi et al.,

CDDP that is available for binding to DNA (Berners-Price and

mixtures were separated on HPLC and fractions were collected for various t

AAS-Pt absorptions of elutes from reaction of 33 μM CDDP with GSH determined using HPLC-

of 60 and 290 min, respectively.

Clinically, typical CDDP doses are 20 to 120 mg/m², administered

about 5 times as large as the rate constant for CDDP reaction with

GSH. The $t_{1/2}$ is $9.6 \pm 1.1$ min and $k_2$ value is $7.3 \times 10^{-2}$ M⁻¹ s⁻¹.

Discussion

The reactions of CDDP with GSH and thiols affect the amount of CDDP that is available for binding to DNA (Berners-Price and Kuchel, 1990a,b; Ishikawa and Ali-Osman, 1993; Bernareggi et al., 1995; Perez-Benito et al., 1995; Bose et al., 1997; El-Khateeb et al., 1999). For example, blocking the cellular thiol groups with N-ethylmaleimide increases the amount of DNA-Pt adducts by ~8-fold (Sadowitz et al., 2002). Although previous work provides valuable information on the structure, rate, and mechanism of product formation, most studies were not performed using biologically relevant conditions. For example, the concentration of CDDP was in the millimolar range (too high to be clinically relevant) and/or the concentration of thiol was relatively low.

Clinically, typical CDDP doses are 20 to 120 mg/m², administered intravenously over 1 h. In a recent trial, after infusion of 30 mg/m² of CDDP, the maximum concentration of unbound Pt in the plasma was [mean ± S.D.(n)] $4.5 \pm 1.6(19)$ μM and the $t_{1/2}$ was 25.4 ± 5.4 min; Souid et al., 2003]. Other studies showed peak plasma Pt levels of ~10 μM after infusion of 70 mg/m² CDDP (Corden et al., 1985;
Korst et al., 1998). Thus, CDDP concentrations of ~5 to 30 μM mimic most conditions encountered in the clinical administration of the drug.

Therefore, we selected the following conditions for measuring the rate constants of the reactions of CDDP with GSH and drug thiols: 1) The source of the drug used in our work is platinol, the formulation used for treating patients. Platinol contains 3.3 mM cis-diaminedichloroplatinum(II) in a slightly acidic solution, pH ~5.5, which also contains 154 mM NaCl. 2) The concentration of CDDP used is 33 μM, which is at the high end of the therapeutic range and still provides sufficient Pt to be detected by AAS. 3) The ratio of CDDP to GSH approximates that in the cell. Because the concentration of GSH in the cell is 1 to 3 mM (Eastman, 1991; Souid et al., 1999, 2001) and the low end of the therapeutic range for CDDP in the cell is ~5 μM, the ratio of thiol to drug in the cytoplasm is ~500:1. In most of our studies, the mixture contained 33 μM CDDP and 16.5 mM thiol, making the reaction pseudo first order with rate proportional to [CDDP]. The low CDDP concentration and the large excess of GSH would favor formation of mononuclear thiolate complexes. 4) The pH of the study was maintained at 7.4, using either 100 mM Tris-NO₃ (or 50 mM NaNO₃; data not shown). These buffer concentrations were the minimum for maintaining the pH at ~7.4 during the course of the reaction. The nitrate ion was chosen because it binds to Pt²⁺ only weakly. Nevertheless, both buffers gave the same results. 5) Reactions were studied at 37 ± 1°C.

It is well known that CDDP (1) reacts in water to produce the chloro-aquo (2) and diaquo (3) forms of the drug, shown in Scheme 1 (Miller and House, 1990). In platinol, the clinical formulation of CDDP, the aquation is suppressed by a high chloride concentration (154 mM), and the drug exists mainly as the dichloro form (1). When the chloride content in the medium is reduced, e.g., to 4.62 mM, CDDP begins to aquate but the time to produce 2 and 3 is relatively long (hours). Because the t₁/₂ for the reaction of unaged CDDP with GSH is ~50 min (Fig. 3), the major species reacting with GSH is the dichloro form (1). Aging solutions of CDDP at low chloride concentration (4.62 mM) before the addition of GSH allows the drug to aquate to the chloro-aquo (2) and diaquo (3) forms (Scheme 1), with more 2 than 3. Because the pKₐ for 2 is 6.85 and the pH of the reaction is 7.4, the major form present is the chloro-hydroxo (4) (Miller and House, 1990). Hydroxide ion, being a poor leaving group compared with water (4), should be much less reactive than 2 in a displacement reaction with GSH. As shown in Fig. 3, the aging clearly affects the kinetics of CDDP reaction with GSH. For a 4-h aging time, the reaction is biphasic: initially the k₁ ≈ ~0.012 min⁻¹ (~2.0 × 10⁻⁴ s⁻¹) and later (using the last 4 points) ~0.0024 min⁻¹ (~0.4 × 10⁻⁴ s⁻¹), corresponding to t₁/₂ of 60 and 290 min, respectively (Fig. 3, circles). Because 4 h at RT would be insufficient time for equilibrium between 1 and 2 (or 3), a significant amount of 1 is present, corresponding to the short t₁/₂ GSH reaction observed in Fig. 3 (circles). At longer aging times, only the slower reaction of GSH with the chlorohydroxo 4 is observed. From the value of t₁/₂, this compound reacts with GSH more slowly than 1 (Table 3). The absorbance versus time curves for solutions containing both CDDP and thiol (Fig. 1) could be fit very accurately by a constant plus (or minus) two exponentials of the form A exp(−bₓt), j = 1 or 2. This indicates that at least two reactions take place. However, one can fit the data as well with a number of other 5-parameter functions, so it is not justified to assume that b₁ and b₂ represent rate constants for two first order reactions. Only an explicit kinetic model, based on evidence from HPLC as well as UV absorption, can indicate the proper interpretation of the UV data. We have used the fit to exponentials only to calculate the initial slope (Table 1), which was proportional to [CDDP], proving that the reaction is first order in CDDP (as proven by HPLC-AAS).

The ability of GSH and other thiols to bind Pt may help explaining how cells resist CDDP therapy (Richon et al., 1987; Eastman, 1991; Mistry et al., 1991). CDDP binding to thiols in the cytoplasm diminishes the concentration of drug capable of binding to DNA. However, the results presented herein show that the reaction of 1 with a large excess of GSH is relatively slow (t₁/₂ ~50 min). For comparison, the k₂ for GSH binding to 4-hydroperoxycyclophosphamide (~38 M⁻¹ s⁻¹) is ~3 orders of magnitude higher than that for GSH binding to CDDP, ~1.3 × 10⁻² M⁻¹ s⁻¹ (Tacka et al., 2002). This fact and the expected rapid diffusion of CDDP, suggest that a significant amount of CDDP, as 1 and 4, can reach the nuclear envelope unchanged by reaction with GSH. Further work on the rate of CDDP transfer across the cellular membrane and the rate of its reaction with other cellular thiols (e.g., metallothionein and large molecular weight protein thiols) will provide a better picture of the amount of the drug available for binding to DNA under clinical conditions.

The thiol drugs WR-1065 and mesna exhibit different reactivity toward CDDP (Fig. 4). The higher kₚ for WR-1065 is probably related to the fact that, of the three thiols studied, WR-1065 has the lowest pKₐ value (7.7) for the thiol group: mesna, 9.1, and GSH, 8.7 (Shaked et al., 1980; Newton et al., 1992). Thus, at pH 7.4, WR-1065 would have the highest concentration of the thiolate ion, the attacking nucleophile in the substitution reaction.

Other studies have reported the reaction rates for CDDP with mesna and WR-1065 by measuring the amount of UV absorption of the CDDP peak from HPLC. In one study, the kₜ value decreased from 1.5 × 10⁻⁴ to 0.7 × 10⁻⁴ M⁻¹ s⁻¹ for 200 μM CDDP reacting with 5 to 500 mM mesna in the presence of 150 mM NaCl (Leeuwenkamp et al., 1991); our kₜ value of 1.08 × 10⁻² M⁻¹ s⁻¹ is in the middle of the range.

In another study, kₜ decreased from 2.8 × 10⁻² to 1.1 × 10⁻² M⁻¹ s⁻¹ for 100 μM CDDP reacting with 5 to 20 mM WR-1065 (NaCl concentration was not given) (Treskes et al., 1991); our kₜ value of 7.3 × 10⁻² M⁻¹ s⁻¹ is larger. However, in our hands, measurements of UV absorption of the HPLC-CDDP peak produced inconsistent results, partly from difficulty in establishing the baseline. Note also that the peak probably contains species other than CDDP, whereas measurement of Pt content by AAS is unambiguous.

Mesna and WR-1065 (the active metabolite of WR-2721) are used to reduce the cytotoxicity of CDDP and other anticancer drugs (Dedon and Borch, 1987; Leeuwenkamp et al., 1991; Treskes et al., 1991; Souid et al., 1999, 2001; Reedijk and Teuben, 1999). Unlike mesna, which is an anion, WR-1065 is a cation, which can readily cross the cellular membrane and distribute in the nuclear matrix. Because it can bind to DNA (Smoluk et al., 1986) and it has the largest rate constant for reaction with CDDP, WR-1065 may be more efficient in decreasing the concentration of CDDP reaching the DNA.

The rate constants reported herein for the reactions of CDDP with thiols are important for accurate modeling of cellular DNA platination by CDDP (Sadowitz et al., 2002). Although we have previously constructed a simple model, describing complex processes with simple rate laws, it involves several undetermined kinetic parameters. With values for more parameters fixed by measurement, the model should lead to more reliable values for the others. This would enable a serious test of the model and possibly make it useful as a guide to clinical practice.

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