KINETICS OF CISPLATIN BINDING TO CELLULAR DNA AND MODULATIONS BY THIOL-BLOCKING AGENTS AND THIOL DRUGS

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ABSTRACT:

DNA platination by cisplatin (CDDP) was investigated in peripheral blood mononuclear cells and ovarian cancer cells using atomic absorption spectroscopy. Plots showing the amount of platinum (Pt) bound to DNA versus the molar concentration of cisplatin in the incubation medium (CDDP) were nonlinear. For [CDDP] < about 5 μM, the amount of Pt bound to DNA increased slowly with added drug. However, for larger [CDDP], the slope of the plot increased significantly. To study the role of thiols in affecting cisplatin binding to DNA, cells were treated with N-ethylmaleimide. A plot of the amount of Pt bound to DNA versus [CDDP] for thiol-blocked cells is linear, with a slope similar to that of unblocked cells at high [CDDP]. Neither S-2-(3 aminopropylamino)ethanethiol (WR-1065) nor mesna, when added at clinically achievable concentrations (i.e., < ~300 μM), affected DNA platination. However, DNA platination was totally abolished by millimolar concentrations of the drug thiols (~ 1.25 mM WR-1065 or ~ 5 mM mesna). Thus, the data show that endogenous thiols intercept cellular cisplatin, but this mechanism is less important at high [CDDP]. Moreover, therapeutic concentrations of drug thiols do not significantly affect DNA platination. A simple model that reproduces the experimental results of the amount of cisplatin binding to DNA as a function of [CDDP], time, and thiol content is proposed. The model takes into account passage of cisplatin and thiols through the cell membrane, binding of cisplatin to cellular thiols, and platination of DNA.

The antitumor drug cisplatin [cis-diaminedichloroplatinum (II)] exerts its effect primarily by interacting with cellular DNA. When cisplatin passes from the blood to the cells, the drug aquates, producing cationic species that bind to nitrogen atoms on the bases of DNA (Zwelling et al., 1979). Although cisplatin can form many types of covalent adducts with DNA, an important lesion is an intrastrand cross-link at two adjacent purine bases, with binding to the sequence guanine-guanine being the most common (Gelasco and Lippard, 1999; Legrenet et al., 2000). Cisplatin binding alters the structure of DNA and affects its ability to act as a template in transcription (Bellon et al., 1991). If the rate of DNA platination DNA exceeds the rate at which Pt adducts are removed by repair, cells enter apoptosis and die (Chu, 1994; Demarcq et al., 1994).

The cytotoxic activity of cisplatin correlates with the amount of Pt bound to DNA (Zwelling et al., 1979; Knox et al., 1986; Lindauer and Holler, 1996). Factors controlling DNA platination include the drug uptake, the rate of Pt adduct formation and repair, and the concentration of cellular thiols. The uptake of cisplatin varies among different cells. For example, in cisplatin-sensitive ovarian cancer cells, the uptake occurs by passive diffusion and active transport. By contrast, in the resistant cells, the uptake occurs by passive diffusion (Sharp et al., 1995). In a clinical study, disproportional increments in the number of Pt adducts were found in patients receiving higher doses of cisplatin (Fichtinger-Schepman et al., 1991), confirming the role of adduct removal by repair (O’Neill et al., 1999).

Thiol (sulfhydryl) groups, such as those on glutathione (GSH)2 and metallothionein (MT), defend the cell against cisplatin (Kraker et al., 1985; Zhang et al., 1995, 2001; Bose et al., 1997). Since the thiolate anion has a high affinity for Pt2+, Pt ions entering the cell may preferentially bind to sulfur atoms rather than the bases of DNA (Dedon and Borch, 1987; Lai et al., 1989; Ishikawa and Ali-Osman, 1993). Although it is easy to overwhelm this protective mechanism in first-time patients receiving cisplatin, continued exposure to the drug ultimately produces resistance due to increased sulfhydryl levels (e.g., GSH and MT) (Schilder et al., 1990; Godwin et al., 1992).

Drug thiols are known to modulate cisplatin toxicity, with WR-

2 Abbreviations used are: GSH, glutathione; MT, metallothionein; WR-2721, S-2-(3-aminopropylamino)ethyl phosphorothioic acid; WR-1065, S-2-(3 aminopropylamino)ethanethiol; PBMC, peripheral blood mononuclear cells; [CDDP], molar concentration of cisplatin; NEM, N-ethylmaleimide; DA, diamide; mBB, monobromobimane; PBS, phosphate-buffered saline; HPLC, high-pressure liquid chromatography; RT, room temperature; nt, nucleotides; AAS, atomic absorption spectroscopy.

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2721 [amifostine; Ethylol; S-2-(3-aminopropylamino)ethyl phosphorothioic acid; \( H_2N-(CH_2)_3-NH_3^+-(CH_2)_3-S-PtH_3^- \)] and mesna (sodium 2-mercaptoethanesulfonate; HS-CH_2-CH_2SO_3Na), being the most commonly used (Brock et al., 1982; Kempf and Ivanovic, 1987; Treskes et al., 1992; Treskes and van der Vijgh, 1993; Reedijk and Teuben, 1999). WR-2721 is a “pro-drug”, which (after hydrolysis by alkaline phosphatase) produces the thiol WR-1065 \[ S-2-(3-aminopropylamino)ethanethiol; \( H_2N-(CH_2)_3-NH_2^+-(CH_2)_3-SH \). The cytoprotective mechanism of WR-1065 and mesna presumably involves formation of Pt-thiolate adducts (Dedon and Borch, 1987; Leeuwen-kamp et al., 1991; Reedijk and Teuben, 1999).

The distribution of WR-1065 and mesna differs markedly. WR-1065 distributes equally between the extra- and intracellular compartments, whereas mesna distributes mostly in the extracellular compartment (Soud et al., 2001). Modeling the kinetics of DNA platination provides a framework for thinking about the reaction of cisplatin with DNA and is helpful in designing effective treatment strategies involving cisplatin.

In this study, we measure the amount of Pt bound to DNA in peripheral blood mononuclear cells (PBMC) and ovarian cancer cells as a function of [CDDP], time of incubation, and thiol content. The data are used to construct a kinetic model, which takes into account the passage of cisplatin through the cell membrane, the transit through the nuclear envelope, the reaction of cisplatin with cellular thiols, and the binding of cisplatin to DNA.

**Materials and Methods**

**Reagents.** Cisplatin [mol. wt. \(-300\); purchased as 1 mg/ml of solution (\(-3.3\) mM); working solution, 0.33 mM; freshly diluted in dH_2O immediately before addition] was purchased from American Pharmaceutical Partners (Los Angeles, CA); mesna [mol. wt. \(164.18\); purchased as 100 mg/ml of solution (\(-609\) mM)] from Bristol-Myers Squibb Co. (Princeton, NJ); WR-1065 2HCl [mol. wt. \(207.16\)] from U.S. Bioscience (West Conshohocken, PA); EDTA, GSH, N-ethylmaleimide (NEM; working solution, 0.1 M; made fresh in dH_2O); diamide [DA; azodicarboxylic acid bis(dimethylamide); working solution, 0.1 M; made fresh in dH_2O]; phenol (prepared as a saturated solution with 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, and stored at \(-20^\circ\)C); proteinase k (working solution, 20 mg/ml; made in dH_2O and stored at \(-20^\circ\)C); ribonuclease A [DNase-free from bovine pancreas; working solution, 10 mg/ml (containing \(-80\) units/mg); made in 10 mM sodium acetate, pH 5.5, and stored at \(-20^\circ\)C]; ribonuclease T1 (from aspergillus oryzae; working solution, 50 units/ml; diluted in ammonium sulfate and stored at \(-20^\circ\)C); SDS (working solution, 10% in dH_2O), and Tris from Sigma (St. Louis, MO); mononobromobimane (mBBr) from Molecular Probes (Eugene, OR); methanesulfonic acid from Fluka BioChemika (Ronkonkoma, NY); perchloric acid from Aldrich (Milwaukee, WI); Pt atomic spectroscopy standard (\(H_2PtCl_6\); purchased as 1 mg/ml of solution in 10% HCl) from PerkinElmer (Norwalk, CT); nitric acid from Teuben, 1999). WR-2721 is a Treskes et al., 1992; Treskes and van der Vijgh, 1993; Reedijk and Teuben, 1999; Dedon and Borch, 1987; Leeuwen-kamp et al., 1991; Reedijk and Teuben, 1999).

**Incubation with Drugs.** In all experiments, \(-1\) to \(2\times10^7\) cells/assay were incubated in RPMI medium in a final volume of 1.0 ml at \(37^\circ\)C. A control sample with no addition was incubated along with the experimental conditions. Mixing was by frequent, rapid inversions. The volume of each addition was 100 \(\mu\)l. Cells were incubated at \(37^\circ\)C with various concentrations of cisplatin for the indicated time before rapid DNA extraction. Cells were also incubated without or with indicated concentrations of WR-1065 or mesna at \(37^\circ\)C for 5 min. Cisplatin was then added to a final concentration of 5 or 7 \(\mu\)M, and the incubation continued at \(37^\circ\)C for 2 h before rapid DNA extraction.

The final pH value in all reaction mixtures was \(-7.2\) and did not change throughout the incubation periods. The free thiols decayed with a \(t_{1/2}\) of \(-16\) min in the WR-1065 reaction mixture and \(-19\) min in the mesna reaction mixture.

**Modification of Cellular Thiol Groups.** In all experiments, \(-2\times10^7\) cells/assay were incubated in RPMI in a final volume of 1.0 ml at \(37^\circ\)C. A control sample without any addition was incubated along with the experimental conditions. PBMC were first incubated with 5 mM NEM or 5 mM DA at \(37^\circ\)C for 15 min. Cisplatin was then added at various concentrations. The mixtures were incubated at \(37^\circ\)C for the indicated time before rapid DNA extraction.

**Repair of Platinated DNA.** The ability of the cells to remove Pt from DNA was measured. PBMC (4.0 \(\times\)10^7/condition in RPMI) were incubated at \(37^\circ\)C for 15 min without or with 5 mM NEM. Cisplatin was then added to a final concentration of 7 \(\mu\)M to normal and thiol-blocked cells, and the incubation continued at \(37^\circ\)C for 2 h (final volume, 1.0 ml). The cells were collected by centrifugation, and the DNA was extracted immediately. In parallel, identical incubations the cells were resuspended in 1.0 ml of RPMI, and incubated at \(37^\circ\)C for additional 2 h to allow for adduct repair before DNA extraction.

**GSH Determination.** Cellular thiols that were not modified by NEM were alkylated with the fluorescent probe mBBr. The low molecular weight alkylated thiols in the acid-soluble supernatants were separated by high-pressure liquid chromatography (HPLC) and detected by fluorescence (Soud et al., 1999, 2001). Briefly, at the end of the 15-min incubation without or with 5 mM NEM, cells (2.1 \(\times\)10^7 cells/condition) were collected and purified with CD45 MicroBeads on a MiniMAC column at-
as above. DNA was precipitated with 2 volumes of cold (−20°C) absolute ethyl alcohol, collected on glass rods, rinsed with 1.0 ml of cold 80% ethyl alcohol, and air-dried at RT for 15 min. After resuspension (in 0.5 ml of 10 mM Tris-Cl, 10 mM EDTA, pH 8, 40 μg of ribonuclease A, and 25 units of ribonuclease T1 were added, and the solutions were incubated overnight at 37°C. DNA was then precipitated, collected, rinsed, and dried as described above. The final DNA pellets were suspended in 200 μl of dH2O and incubated overnight at 37°C to rehydrate. DNA concentrations were calculated on a standard curve of known DNA concentration.

**Fig. 1.** Normal (left panel) and thiol-blocked (right panel) PBMC treated with cisplatin.

In each condition, ~2.5 × 10^7 cells were incubated at 37°C with 1 to 9 μM cisplatin for 2 h. In the thiol-blocked condition, PBMC were incubated at 37°C for 15 min with 5 mM NEM before the addition of cisplatin. At the end of the incubation period, DNA was extracted, and the number of Pt adducts determined using AAS. The values are mean ± S.D. of three separate measurements.

Pt Analysis. Pt analysis was performed using the graphite furnace of a Shimadzu AAS (Model AA-6800; Kyoto, Japan), with an ultra-high current Pt hollow cathode lamp, using AAS. The values are mean ± S.D. of three separate measurements.

**TABLE 1**

Enhancement of DNA platination by NEM and DA

<table>
<thead>
<tr>
<th>Addition</th>
<th>No. of Adducts/10^6 nt</th>
<th>Enhancement</th>
<th>Intracellular GSH μM</th>
<th>% Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP alone</td>
<td>30 ± 11</td>
<td>1</td>
<td>556</td>
<td></td>
</tr>
<tr>
<td>CDP + NEM</td>
<td>240 ± 13</td>
<td>8-fold</td>
<td>6</td>
<td>99</td>
</tr>
<tr>
<td>CDP + DA</td>
<td>110 ± 0</td>
<td>~4-fold</td>
<td>17</td>
<td>97</td>
</tr>
</tbody>
</table>

DNA-Pt Adduct Repair. Incubating PBMC with platinated DNA in cisplatin-free medium for 2 h resulted in substantial removal of Pt from DNA via repair. The numbers of Pt adducts per 10^6 nt for normal and thiol-blocked cells before the 2 h repair period were 75 ± 5 and 185 ± 5, respectively, whereas the number of adducts after repair were 5 ± 2 and 40 ± 18, respectively. Control cells to which no cisplatin was added gave 5 ± 2 adducts/10^6 nt. Thus, in normal cells, 100% of the Pt-bound DNA was repaired in 2 h, and in thiol-blocked cells, ~80% was repaired. Similar results were obtained in cells treated with DA and iodomethane (data not shown).

**Results**

PBMC Treated with Cisplatin. A plot showing the amount of Pt bound to DNA, after a 2 h exposure, as a function of [CDDP] in the incubation medium is shown in Fig. 1 (left panel). Over the concentration range of the study, the plot is nonlinear. However, it appears to consist of two linear portions. For [CDDP] less than about 5 μM, the slope of the plot is ~1.3 adducts/μM cisplatin. For [CDDP] >5 μM, the slope is much larger, with ~9.5 adducts/μM cisplatin. For higher concentrations (up to 80 μM; data not shown), the amount of Pt bound to DNA remains linear in [CDDP].

Thiol-Blocked PBMC Treated with Cisplatin. To study how thiol functions affect Pt binding to DNA, cells were treated with a large excess of NEM or DA; both agents selectively modify thiol groups so that they cannot bind Pt. Analysis using HPLC showed that the amount of unmodified GSH remaining after treatment of cells with NEM or DA was ±3% (Table 1). Treatment with NEM produced 8-fold enhancement in the level of Pt adducts and with DA about 4-fold enhancement (Table 1).

Additionally, thiol-blocked cells (treated with NEM) were exposed to various concentrations of cisplatin for 2 h, and the amount of Pt bound to DNA was determined. The results are shown in Fig. 1 (right panel). The plot of Pt bound to DNA versus [CDDP] is now linear, with a slope of 22 adducts/μM cisplatin. This is similar to the slope for the high [CDDP] of the plot of Fig. 1, with 9.5 adducts/μM cisplatin.

Kinetics of Cisplatin Binding to PBMC DNA. The manner in which the amount of Pt adducts changes with the incubation time for normal and thiol-blocked cells (treatment with NEM) is shown in Fig. 2. Although there is much scatter in the points, each plot shows an initial rapid increase followed by a slower increase up to 2 h. At each time, cells having blocked thiols had increased amount of Pt bound to DNA by a factor of 2 or 3.

**DISCUSSION**

The values are mean ± S.D. of an experiment performed in triplicate. Similar enhancements were obtained in three additional individual experiments. In each condition, 2.1 × 10^7 PBMC (mean ± S.D.; cell volume, 190 ± 50 fL) were first incubated at 37°C for 15 min without or with 5 mM NEM or 5 mM DA. At the end of the incubation period, cisplatin was added to a final concentration of 5 μM, and the incubation continued at 37°C for 2 h. DNA was then extracted, and Pt adducts were determined on AAS. Moreover, in parallel samples, the concentrations of cellular GSH at the end of the 15-min incubations were determined as described under Material and Methods.
thiols are indicated as cisplatin alone. In volunteer 1, the WR-1065 concentration was 300 μM.

Concentration of mesna following incubation (37 °C for 2 h) before DNA extraction. The number of adducts was determined on AAS. The values are mean ± S.D. (n = 2) of two individual experiments. Cells incubated without added thiols are indicated as cisplatin alone.

Table 2
DNA platination in PBMC in the presence of low concentrations of WR-1065 and mesna

<table>
<thead>
<tr>
<th>Additions</th>
<th>Pt Atoms/10^6 nt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volunteer 1</td>
</tr>
<tr>
<td>cisplatin alone</td>
<td>43 ± 2 (2)</td>
</tr>
<tr>
<td>cisplatin + WR-1065</td>
<td>42 ± 10 (2)</td>
</tr>
<tr>
<td>cisplatin + mesna</td>
<td>59 ± 6 (2)</td>
</tr>
</tbody>
</table>

7 μM cisplatin. The number of adducts was unaffected by WR-1065 concentrations ≤ ~300 μM and abolished at ≥ ~1.25 mM. WR-1065 concentrations between ~500 μM and 1.0 mM produced various levels of Pt adduct inhibition. The number of adducts was also unaffected by mesna concentrations of ~300 μM and 1.0 mM produced various levels of Pt adduct inhibition (Table 3).

Similarly, neither WR-1065 nor mesna at 300 μM affected DNA platination in ovarian cancer cells (Table 4). In contrast, at 5 mM WR-1065, Pt adducts decreased by ~90% (Table 4; patient 1).

Cellular Drug Thiol Concentrations. The concentration of WR-1065 in highly purified PBMC (see Materials and Methods) following incubation (37 °C for 15 min) with 2 mM WR-1065 was ~2440 μM (that is, ~112% of that of the incubation medium). In contrast, the concentration of mesna following incubation (37 °C for 15 min) with 2 mM mesna was only ~130 μM (that is, ~6% of that of the incubation medium).

Discussion

The cytotoxicity of cisplatin is related to its ability to bind to DNA (Zwelling et al., 1979; Knox et al., 1986). Competing with DNA for the drug are numerous thiol functions, such as those on GSH and MT (Kraker et al., 1985; Dedon and Borch, 1987; Eastman, 1991; Zhang et al., 1995; Bose et al., 1997; Zhang et al., 2001). If these groups are blocked from binding Pt (by modification with NEM or DA), the amount of cisplatin available to form Pt-DNA adducts is increased. Figures 1 and 2 and Table 1 support this.

The fact that significant cellular GSH depletion does not produce the same level of Pt-DNA adduct enhancement by NEM (Table 1) suggests an important role for other cellular thiol pools in binding cisplatin (e.g., MT and thiol groups on the chromatin) (Zhang et al., 2001).

For normal PBMC, the initial slope of the plot of the number of adducts after a 2-h incubation versus [CDDP] is small (Fig. 1, left panel), with ~1.3 adducts/μM cisplatin (using the first three points). This indicates that much of the cisplatin entering the cell is bound by thiols and unavailable for binding DNA. However, at high [CDDP], the slope of the plot rises sharply (Fig. 1, left panel), with ~9.5 adducts/μM cisplatin (using the last three points). Thus, small increments in cellular cisplatin concentration above 5 μM give substantial enhancements in adduct levels (e.g., increasing [CDDP] from 5 to 9 μM increased the number of bound Pt atoms per 10^6 nt of DNA from 10 to 47). Such enhancement is expected for cisplatin concentrations high enough to saturate the cellular thiols that compete for Pt binding.

One also expects the slope of such a plot to be large when thiols are blocked, so they cannot bind Pt (as by treatment with NEM or DA).
The slope of Fig. 1 (right panel) is in fact about twice the high-[CDDP] slope of Fig. 1 (left panel). However, the latter is calculated from the last three points of Fig. 1 (left panel), whereas the slope is probably still increasing at [CDDP] = 9 μM. It is expected that the plot becomes linear at higher [CDDP]. Once the thiolates are blocked, there should be a simple linear relationship between [CDDP] and the number of Pt atoms bound to DNA for all [CDDP]. Indeed, Fig. 1 (right panel) shows that for thiol-blocked cells the relationship is linear.

Efficient Pt removal from DNA occurs for both normal and thiol-blocked cells (see Results). Thus, treatment with the thiol-modifying agents does not compromise cellular repair mechanisms. The enhancement in the number of Pt adducts in cells treated with NEM or DA (Table 1; Fig. 1, right panel) is, therefore, mostly due to an increase in the amount of Pt available for binding to DNA (Fig. 2).

The extent to which thiols added as cytoprotective agents affect the amount of DNA platination by cisplatin in fresh human cells has not been previously reported, especially at therapeutic cisplatin levels. The data show that neither WR-1065 nor mesna, when added at clinically achievable concentrations (i.e., ≤~300 μM), affected DNA platination (Tables 2–4). In the current Children Oncology Group trial (9970), the peak free plasma Pt levels following a 1-h infusion of 30 mg/m² cisplatin were 4.7 ± 1.6 (15 μM [mean ± S.D. (n)]) (A.-K. Souid, unpublished data). Other investigators reported a peak level of ~10 μM following a 1-h infusion of 70 mg/m² cisplatin (Korst et al., 1998). Thus, the concentrations of cisplatin in Figs. 1 and 2 and Tables 1 to 4 are within the therapeutic range.

Furthermore, we previously reported on the WR-1065 plasma and blood cell levels following intravenous infusion of these agents (Souid et al., 1999, 2001). Briefly, immediately following a 15-min infusion of WR-2721 (850 mg/m² or ~3.1 mmol/m²), WR-1065 peaked in the plasma at 76 ± 31 (6) μM and blood cells at 77 ± 25 (6) μM [mean ± S.D. (n)]. These levels are similar to the previously reported peak plasma WR-1065 value (~50 μM) (Korst et al., 1996, 1997). However, following a 15-min infusion of mesna (400 mg/m² or ~2.4 mmol/m²), mesna level peaked in the plasma at ~340 μM and blood cells at ~85 μM (Souid et al., 2001). The plasma levels are similar to those reported by others (Goren et al., 1998). Thus, the clinical studies demonstrate that patients receiving conventional doses of WR-271 and mesna achieve plasma levels in the micromolar range, and the cellular concentrations do not markedly exceed those found in the plasma (see Results under Cellular Drug Thiol Concentrations).

Both WR-1065 and mesna decay rapidly by oxidation, with a t_{1/2} of ~16 min (Goren et al., 1998 and references therein; Souid et al., 1999, 2001), which is similar to measured decay rates in the incubation medium (see Materials and Methods under Incubation with Drugs).

The data in Tables 2 to 4 show that the number of Pt adducts in PBMC and ovarian cancer cells is not affected by concentrations ≤~300 μM WR-1065 or mesna. This finding confirms the insignificant effect on DNA platination when patients received WR-2721 before cisplatin (Korst et al., 1998). In contrast, millimolar concentrations of WR-1065 or mesna significantly diminish DNA platination (Tables 3–4), presumably due to cisplatin binding to the thiolate ions. The discrepancy in the concentrations of these two thiol agents required to abolish DNA platination (~1.25 mM WR-1065 versus ~5 mM mesna; Table 3) reflects the higher uptake of WR-1065 by PBMC (see Results under Cellular Drug Thiol Concentrations). Furthermore, since the reactive moieties are thiolate anions, the pK\textsubscript{a} of the thiol groups and the pH of the medium play an important role in determining the efficacy of the drug thiols. The experiments are performed at a pH value of ~7.2. The pK\textsubscript{a} value of WR-1065 is ~7.7, GSH ~8.7, and mesna ~9.1 (Shaked et al., 1980; Newton et al., 1992). Thus, WR-1065 is expected to be the most reactive among the three thiols studied (since more of the WR-1065 thiol group is ionized at pH 7.2 and available for nucleophilic attack on Pt).

Similarly, in the two ovarian cancers, WR-1065 and mesna at concentrations ~300 μM produced no effect on DNA platination (Table 4) and at 5 mM produced ~90% inhibition (Table 4; patient 1).

Phenomenological Model. To show the relation between the slopes for normal cells at high or low [CDDP] and thiol-blocked cells (Fig. 1, left and right panels, respectively), we propose a simple model to show the relation between the processes that take place for platination of DNA by cisplatin. For cisplatin to bind to DNA, the following processes must be taken into account: 1) passage of cisplatin into the cytoplasm; 2) passage of cisplatin into the nucleus; 3) reaction of cisplatin with cellular sulphhydryl groups; 4) binding of cisplatin to DNA; and 5) repair (eliminating Pt from DNA). If repair occurs, sites with bound Pt are replaced, but the Pt is not returned to the pool of Pt available for binding to DNA. If drug thiols are present, their passage into the cells must be included in the model.

The diagram in Fig. 3 shows the processes schematically. Each step is certainly the sum of many kinetic processes, so the rate constants used are effective or overall rate constants. For example, Pt, originally in the form of cisplatin, is transformed in several reactions, which are not considered explicitly in this simple model.

Let [P] be the concentration of cisplatin in the cytoplasm and [N] the concentration of cisplatin in the nucleus. The values of [P] and [N] (both at t = 0) change with time because of the following five processes. a) The rate at which cisplatin enters the cells is assumed to be proportional to the difference between [CDDP] in the extracellular ([P]) and intracellular ([P]) compartments (that is, P_e − [P]) and to the area of the cell membrane. For a homogenous cell population, this area is proportional to the cell volume, so this process contributes k_{Ps} ([P] − [P]) to d[P]/dt. b) The reaction of cisplatin with cellular thiols (considered randomly distributed) is assumed second-order and equal to k_s[P][S]. Here, [S] is the effective concentration of free thiols (equal to S_0 at t = 0). If all the thiols are blocked from binding cisplatin, S_0 = 0. c) The transfer of cisplatin through the nuclear envelope is like process a, with the rate assumed to be k_{Pc} ([P] − [N]). d) The reaction of cisplatin with DNA is assumed second-order, equal to k_{Ps}([P] − [P]), where [D] is the effective concentration of free DNA sites capable of binding cisplatin, so the term −k_{Ps}([N][D] appears in d[N]/dt and d[D]/dt. At t = 0, [D] is equal to D_0; at any time, the concentration of Pt-bound DNA sites is D_0 − [D]. e) The repair of DNA, which reverses process c, has a rate k_{Pc}(D_0 − [D]). It contributes to d[D]/dt but not to d[N]/dt, since Pt removed from DNA is not available for binding. f) The rate at which thiols enter the cells, where
their extracellular concentration is maintained at $S_e$, is assumed to be $k_e(S_e - [S])$. This process contributes to $d[S]/dt$. We do not include the reaction between cisplatin and thiols in the incubation medium, since Pt(NH$_3$)$_2$Cl$_2$ reacts slowly with thiols (Bose et al., 1997), and conversion into the active Pt(NH$_3$)(OH)$_2$Cl occurs on passage into the cells.

Taking processes a to f into account, we can write the equations for the rates of change of [P], [N], [S], etc.

$$\frac{d[P]}{dt} = k_e(P_e - [P]) - k_d[P][S] - k_e([P] - [N])$$  \hfill (1)

$$\frac{d[N]}{dt} = k_e([P] - [N]) - k_d[N][D]$$  \hfill (2)

$$\frac{d[S]}{dt} = -k_d[P][S] + k_e(S_e - [S])$$  \hfill (3)

$$\frac{d[D]}{dt} = -k_d[N][D] + k_e(D_0 - [D])$$  \hfill (4)

To simplify, we will assume that [D] is always close to $D_0$ (there is a large excess of potential binding sites on DNA compared with the amount of Pt available), so we neglect the second term in eq. 4 and put $[D] = D_0$ in eq. 2. We also assume that the rate of passage of thiols from the incubation medium is rapid, so that, at $t = 0$, the thiol concentration is $S_e + S_0$. It is then necessary to consider only eqs. 1 to 3, and the amount of Pt bound at the incubation time ($T$) is calculated as:

$$\int_0^T k_e[N][D]dt = k_eD_0 \int_0^T [N]dt$$

If there were experimental evidence of the saturation of DNA sites or of slow passage of drug thiols into the cells, more equations would have to be considered.

The parameters in the model would be $S_0$, $k_a$, $k_b$, $k_c$, $k_d$, $D_0$, and $P_e$. The repair is neglected, $k_e$ does not enter, whereas $k_a$ and $D_0$ enter only as the product, $k_aD_0$, leaving five parameters to be chosen. For $S_0$, we use 2 mM, which seems to be a reasonable estimate for the intracellular thiol concentration (Eastman, 1991). It is not surprising that choosing the remaining four parameters can fit all the experimental data.

Closed-form solutions to the equations are possible under certain circumstances. Thus, if $[S]$ remains close to $S_0$ (which may be 0) for $t \lesssim T$, [D] and [N] are sums of two exponentials in time. Under these circumstances, steady-state solutions may be of interest. If [P] and [N] both reach steady states,

$$\frac{d[P]}{dt} = 0 = k_eP_e - (k_c + k_e + k_0S_0)[P] + k_e[N]$$

$$\frac{d[N]}{dt} = 0 = k_e[P] - (k_c + k_e[D_0])[N]$$

These equations may be solved for the steady-state concentrations of [P] and [N], both of which are proportional to $P_e$, the concentration of Pt in the extracellular medium. The concentration of DNA-bound Pt may then be estimated as $k_eD_0[N]/T$ and is also proportional to $P_e$ in this approximation. The constant of proportionality, or the slope, is larger the smaller the value of $S_0$. This means that a plot of amount of DNA-bound Pt versus $P_e$ starts off linearly, with a relatively small slope and eventually, when $[S]$ has decreased, becomes linear with a larger slope, as is observed in Fig. 1 (left panel).

In general, numerical integration of eqs. 1 to 3 is required. Figures 4 and 5 show the results of numerical integration, using a fourth-order Runge-Kutta method (Mathcad 2000 Professional User’s Guide, 1999, pp 178–182; Mathsoft, Inc., Cambridge, MA). The values of the parameters used were $k_e = 125$ h$^{-1}$, $k_0 = 8 \times 10^3$ M$^{-1}$ h$^{-1}$, $k_c = 150$ h$^{-1}$, and $k_eD_0 = 15$ h$^{-1}$; $S_0$ was taken as 2 mM. These values give rough agreement between calculated and experimental results (compare Figs. 4–5 with Figs. 1–2), and we have not attempted to get the best possible agreement.

Figure 4 shows the expected behavior in the presence of free cellular thiols (assumed initial concentration, 2 mM). For example, for a plot of amount of DNA-bound Pt versus [CDDP] is linear for small [CDDP], with a slope of 1.8 Pt atoms/10$^6$ nm/μM. For larger [CDDP], the slope is larger, about 10.5 Pt/10$^6$ nm/μM at [CDDP] = 9 μM; the slope is still increasing at 9 μM. With thiol groups blocked, so $S_0 = 0$, the plot of amount of DNA-bound Pt versus [CDDP] is close to linear for all [CDDP]. The slope is 25.8 Pt atoms/10$^6$ nm/μM. Calculations with our model show that, for $P_e = 9$ μM and $S_0 = 2.0$ mM, almost 2 h is required to reduce [S] to 1% of $S_0$ (so almost 3 h for cytoplasmic and

![Figure 4](image1.png)

**Fig. 4. Results of calculations using a simple phenomenological model (for values of parameters, see text).**

Incubation time is 2 h. Solid- and dashed-line plots show the amount of binding of Pt to DNA after a 2-h incubation with cisplatin as a function of extracellular [CDDP]. Asterisks and dashed lines are for sulphydryl initially available at a 2 mM concentration, filled circles and the solid line for sulphydryl blocked and unavailable. Triangles represent available sulphydryl concentration after 2 h as a function of extracellular [CDDP].

![Figure 5](image2.png)

**Fig. 5. Results of calculations using a simple phenomenological model (for values of parameters, see text).**

Extracellular [CDDP] is 7 μM. Solid- and dashed-line plots show the amount of binding of Pt to DNA after incubation with cisplatin as a function of incubation time. Asterisks and the dashed line are for sulphydryl initially available at a concentration of 2 mM, filled circles and the solid line for sulphydryl blocked and unavailable. Triangles represent available sulphydryl concentration as a function of extracellular [CDDP].
nuclear [CDDP] to come to a steady state). Afterward, the rate of cisplatin uptake is the same as for thiol-blocked cells. Since total cellular thiol concentration has not been measured experimentally, the model gives useful information about the contribution of cellular sulfhydryls.

Figure 4 also includes a plot of calculated thiol concentration remaining after 2 h versus [CDDP]. It appears that the concentration of cisplatin must exceed 8 μM to reduce the concentration of thiol groups by 90% during the 2-h incubation period. If the thiol groups are blocked by other reagents, so $S_0 = 0$, the entire cisplatin is effective in binding to DNA.

Figure 5 shows changes over time, from calculations assuming [CDDP] = 7 μM and the thiol concentration as either 0 or 2 mM. The same values for rate constants were used as in the calculations of Fig. 4. With no available thiol ($S_0 = 0$), the amount of Pt bound to DNA is close to a linear function of time, with a slope of 1.5 Pt atoms/10^6 nt/min. With $S_0 = 2$ mM, the slope starts out very small and rises with time. Even at $t = 2$ h, the slope is much smaller than the slope with $S_0 = 0$, even though three-quarters of the thiol groups have been saturated by Pt (as shown in Fig. 5). For larger times, the slope increases to approach 1.5 Pt atoms/10^6 nt/min.

The average slope with $S_0 = 2$ mM is about 0.2 Pt atoms/10^6 nt/min. The slope calculated from the experimental data (Fig. 2, open squares) is 0.46 ± 0.11, but the scatter in the data is too large to warrant fine tuning of the parameters in the calculation to get better agreement with this number. With $S_0 = 0$, the slope calculated from the experimental data is 1.3 ± 0.2 (Fig. 2, closed squares), fairly close to what we calculated (1.4). The scatter in the data for $S_0 = 0$ is less serious than for $S_0 = 2$ mM, largely because the quantities measured are bigger. Our model is too simple to account for the apparent rate changes found experimentally (Fig. 2).

The value of $k_0$, we used, $8 \times 10^3$ M$^{-1}$·h$^{-1}$, is several orders of magnitude larger than the rate constant reported (Bose et al., 1997) for reaction of CDDP with cysteine or glutathione (Dedon and Borch, 1987). It is likely, however, that the compound in the cell has been aquated to Pt(NH$_3$)$_2$(H$_2$O)Cl, and the rate constant for the reaction of Pt(NH$_3$)$_2$(H$_2$O)Cl with cysteine is given (Bose et al., 1997) only as “fast”. Recently, it has been shown that, for binding to DNA, the doubly aquated compound has a rate constant 2 orders of magnitude higher than Pt(NH$_3$)$_2$(H$_2$O)Cl, and this should also hold for binding to thiols (Legner et al., 2000). It should be emphasized that a large value of $k_0$ is demanded by the experimental results. The large increase in the slope of Fig. 1 (left panel) shows that the intracellular thiols are becoming nearly saturated when $P_e > ~5$ μM. If $|P|$ is fairly constant, the concentration of free thiols decreases according to:

$$\ln([S]/S_0) = -k_0(|P|)t.$$  

Assuming $|P| = 3$ μM (calculations show $|P|$ is somewhat more than half of $P_e$ at $t = 2$ h), $[S]/S_0 < 0.1$ for $t = 2$ h requires that $k_0$ be $4 \times 10^5$ M$^{-1}$·h$^{-1}$.

The effects of the cytoprotective agents can also be interpreted in the context of this model. WR-1065 or mesna enters the cells rapidly (Soud et al., 1999, 2001), thus effectively adding to the original concentration of thiols. Figure 6 shows the calculated amount of platination after a 2-h incubation as a function of the added thiol. The total thiol concentration at time 0 is equal to the added thiol concentration plus 2.0 mM, an approximate amount of endogenous cellular thiols (Reedijk and Teuben, 1999). Because of the scattered data in Table 3, we did not attempt to adjust parameters to get close agreement with experimental results. The effect of added thiols is small (probably undetectable) when the added concentration is <0.3 mM (first point), but the Pt-DNA adducts are reduced to a small fraction of its original value for added thiol concentrations exceeding 4 mM (Fig. 6).

In conclusion, it is well known that endogenous thiols intercept cellular cisplatin. Our data show that this phenomenon is less significant at a high cisplatin concentration. Moreover, therapeutic concentrations of WR-1065 and mesna do not decrease DNA platination by cisplatin. These two findings have important clinical implications.

References


