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, which modifies thiol groups, rendering them incapable of binding cisplatin. Analysis using high-pressure liquid chromatography showed that ~99% of cellular glutathione was modified by 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; Legenre 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; Demarqu 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., 1990), confirming the role of adduct removal by repair (O’Neill et al., 1999).

Thiol (sulfhydryl) groups, such as those on glutathione (GSH) 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 Pt, 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-2721, S-2-(3-aminopropylamino)ethanethiol (WR-1065), S-2-(3 aminopropylamino)ethanol: PBMC, peripheral blood mononuclear cells; [CDDP], molar concentration of cisplatin; NEM, N-ethylmaleimide; DA, diamide; m-BBr, monobromobimane; PBS, phosphate-buffered saline; HPLC, high-pressure liquid chromatography; RT, room temperature; nt, nucleotides; AAS, atomic absorption spectroscopy.
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 dH₂O 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 2HCI [mol. wt. 207.16] from U.S. Bioscience (West Conshohocken, PA); EDTA, N-ethylmaleimide (NEM); working solution, 0.1 M; made fresh in dH₂O, diamide [DA]; azodicarboxylic acid bis(dimethylamide); working solution, 0.1 M; made fresh in dH₂O, phenol (purchased as a saturated solution with 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, and stored at ~20°C), proteinase k (working solution, 20 mg/ml; made in dH₂O and stored at ~20°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°C], ribonuclease T1 (from aspergillus oryzae; working solution, 50 units/ml; diluted in ammonium sulfate and stored at ~20°C), SDS (working solution, 10% in dH₂O), and Tris from Sigma (St. Louis, MO); monobromobimane (mBBr) from Molecular Probes (Eugene, OR); methanesulfonic acid (containing 7 mol. wt., 207.16) from U.S. Bioscience (West Conshohocken, PA); EDTA, HCl; diluted in ammonium sulfate and stored at 4°C; for additional 2 h to allow for adduct repair before DNA extraction.

PBMC Collection. Blood was freshly drawn from healthy volunteers into heparin-containing Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ); ~10 ml of blood (containing ~1–2 × 10⁷ PBMC) was required per condition. PBMC were collected and counted as previously described (Souid et al., 2001). The yield was ~3 × 10⁶ cells/l.0 ml of blood. Cell morphology was evaluated on cytospin smears prepared with the Wright stain. Two-hour incubations with and without additions produced no noticeable morphologic changes.

Thiol Solutions. The WR-1065 and mesna solutions were prepared in dH₂O and stored at ~70°C. Their concentrations were determined by 5,5′-dithio-bis(2-nitrobenzoic acid) titration immediately before additions and during the course of incubation, as previously described (Souid et al., 1998).

Incubation with Drugs. In all experiments, ~1 to ~2 × 10⁷ cells/condition were incubated in RPMI medium in a final volume of 1.0 ml at 37°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 μl. Cells were incubated at 37°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°C for 5 min. Cisplatin was then added to a final concentration of 5 or 7 μM, and the incubation continued at 37°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 t½ 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, ~2.0 × 10⁷ cells/condition were incubated in RPMI in a final volume of 1.0 ml at 37°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°C for 15 min. Cisplatin was then added at various concentrations. The mixtures were incubated at 37°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 × 10⁷ cells/condition in RPMI) were incubated at 37°C for 15 min without or with 5 mM NEM. Cisplatin was then added to a final concentration of 7 μM to normal and thiol-blocked cells, and the incubation continued at 37°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°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 (Souid et al., 1999, 2001). Briefly, at the end of the 15-min incubation without or with 5 mM NEM, cells (2.1 × 10⁷ /condition; mean ± SD) were incubated in 20 mM Tris-methanesulfonic acid, pH 8.0, and 5 mM mBBr (final volume, 0.5 ml). After incubation in the dark at RT for 15 min, the cells were collected by centrifugation, and their acid-soluble supernatants were prepared by the addition of 300 μl of 2.5% perchloric acid/2 M sodium methanesulfonate. After vigorous vortexing, the supernatants were collected by centrifugation, and the amount of GS-histamine adducts was determined by HPLC (Souid et al., 1998).

Cellular Drug Thiol Determination. PBMC were collected from a healthy volunteer and purified with CD45 MicroBeads on a MiniMAC column attached to a magnet exactly as described (Souid et al., 2001). Purified PBMC (~10⁷ cells/condition) were incubated at 37°C for 15 min with 1.0 ml of PBS without or with 2 mM WR-1065 or 2 mM mesna. mBBr was then added to a final concentration of 30 mM, and the derivatization continued in the dark at RT for 20 min. The cells were collected by centrifugation and washed twice with PBS. The concentrations of WR-1065 and mesna in the acid-soluble supernatants were determined by HPLC.

Isolation of DNA. Cells were collected by centrifugation and resuspended in 10 mM Tris-Cl, 10 mM EDTA, pH 8, and 1% SDS (w/v) in the presence of 0.5 mg/ml proteinase k (final volume, 2.0 ml). The solutions were incubated overnight at 37°C and transferred to glass tubes. An equal volume of phenol was added, and the samples were mixed by vigorous vortexing and centrifuged.
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.

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 Beckman spectrophotometer (Model DU 640B; Beckman Coulter, Inc., Fullerton, CA) in a 1-cm cell, using the formula of 50 μg/ml double-stranded DNA corresponding to 1.0 absorbance unit at 260 nm (ε₂₆₀ = 12,000 M⁻¹cm⁻¹). The DNA concentration was 606 ± 42 (55) μg/ml [mean ± S.D. (n)], and A₂₆₀/A₂₈₀ was 1.73 ± 0.03.

Pt Analysis. Pt analysis was performed using the graphite furnace of a Shimadzu AAS (Model AA-6800; Kyoto, Japan), with an ist hollow cathode Pt lamp (Imaging and Sensing technology, Headsres, NY), deuterium arc background correction, and pyrolytically coated graphite tubes. The graphite tubes were changed after 100 ignitions. Argon gas and tap water flowed through the furnace hoses. The instrument operated at a lamp current of 14 mA, wavelength of 266.0 nm, and slit width of 0.5 nm. The Pt standard (H₂PtCl₆) was a 51.3 nM (0.01 mg/liter) solution, freshly prepared by serial dilutions of the Pt atomic standard stock in dH₂O plus 1% HNO₃ (v/v). A calibration curve was generated immediately before each measurement. It was linear from 0 to 1.0 pmol (r > 0.98); the lower limit of detection was ~20 pg of atomic Pt (~0.1 pmol). Each sample was measured in triplicate. The injection volume was 10 μl, containing 4 to 8 μg DNA. The furnace program used sequential drying (70°C for 10 s, 90°C for 10 s, and 120°C for 10 s), charring (250°C for 10 s and 800°C for 25 s), cooling (30°C for 20 s), and atomization (2600°C for 5 s) phases. The control samples (i.e., blood incubated without any addition) gave absorbance values that were the same as those of dH₂O. Calculations were based on 1 pg of Pt/μg of DNA = 5.13 fmol of Pt/μg of DNA (based on the molecular weight of Pt; 195.078), and 1 fmol of Pt/μg of DNA = 0.34 Pt molecules/10⁶ nucleotides (nt) (based on the average molecular weight of the nucleotides; ~343 g mol⁻¹) (McGahan and Tyczkowska, 1987; Reed et al., 1988).

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.

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 10⁶ 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/10⁶ 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).

Effects of WR-1065 and Mesna on DNA-Pt Adducts. The data in Table 2 show the number of Pt adducts in PBMC of healthy volunteers exposed to 5 μM cisplatin and 300 μM WR-1065 or mesna (volunteer 1) or 7 μM cisplatin and 150 μM WR-1065 or mesna (volunteer 2). These levels are routinely achieved in patients receiving cisplatin, WR-1065, and mesna (see Discussion). At these concentrations, neither agent affected DNA platination (Table 2).

Table 3 shows the effect of various concentrations of WR-1065 and mesna on Pt adducts in PBMC of two healthy volunteers exposed to...
thiols are indicated as cisplatin alone. In volunteer 1, the WR-1065 concentration was 300 μM, mesna 300 μM, and cisplatin 7 μM (patient 1) or 5 μM (patient 2), and the incubation continued at 37°C for 2 hr before DNA extraction. The number of adducts was determined on AAS. The values are mean ± S.D. of three separate measurements.

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., 2001). If these groups are blocked, so they cannot bind 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 10^5 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 unblocked, so they cannot bind Pt (as by treatment with NEM or DA).

Additions Pt Atoms/10^5 nt

<table>
<thead>
<tr>
<th>Additions</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin alone</td>
<td>241</td>
<td>260</td>
</tr>
<tr>
<td>Cisplatin plus WR-1065 (300 μM)</td>
<td>253</td>
<td>282</td>
</tr>
<tr>
<td>Cisplatin plus WR-1065 (5 mM)</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Cisplatin plus mesna (300 μM)</td>
<td>207</td>
<td>206</td>
</tr>
</tbody>
</table>

### TABLE 2

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

PBMC (~1.5 × 10^7 cells/condition) were incubated at 37°C for 5 min without or with WR-1065 or mesna. Cisplatin was then added, and the incubation continued at 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 a total of four individual experiments. Cells incubated without added thiols are indicated as cisplatin alone. In volunteer 1, the WR-1065 concentration was 300 μM, mesna 300 μM, and cisplatin 7 μM. In volunteer 2, the WR-1065 concentration was 150 μM, mesna 150 μM, and cisplatin 7 μM.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Pt Atoms per 10^6 nt</th>
</tr>
</thead>
<tbody>
<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>
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Additions Pt Atoms/10^5 nt

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<tbody>
<tr>
<td>Cisplatin (7 μM) alone</td>
<td>71</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + WR-1065 (0.15 mM)</td>
<td>92 ± 16</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + WR-1065 (0.20 mM)</td>
<td>88 ± 1</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + WR-1065 (0.25 mM)</td>
<td>80 ± 6</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + WR-1065 (0.30 mM)</td>
<td>87 ± 15</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + WR-1065 (0.50 mM)</td>
<td>47 ± 14</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + WR-1065 (0.75 mM)</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + WR-1065 (1.0 mM)</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + WR-1065 (1.25 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + mesna (0.3 mM)</td>
<td>106 ± 13</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + mesna (0.5 mM)</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + mesna (1.0 mM)</td>
<td>64 ± 7</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + mesna (1.5 mM)</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + mesna (5.0 mM)</td>
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Additions Pt Atoms/10^5 nt

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<td>Cisplatin (7 μM) + mesna (1.5 mM)</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>Cisplatin (7 μM) + mesna (5.0 mM)</td>
<td>0</td>
</tr>
</tbody>
</table>
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 thiols 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 therapeutically achievable 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 t½ 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 pKₐ 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 pKₐ 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 relations 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 sulfhydryl 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 (Pₓ) and intracellular [P] compartments (that is, Pₓ – [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 kₜ ([Pₓ – [P]]) to d[P]/dt. b) The reaction of cisplatin with cellular thiols (considered randomly distributed) is assumed second-order and equal to kₛ[P][S]. Here, [S] is the effective concentration of free thiols (equal to S₀ at t = 0). If all the thiols are blocked from binding cisplatin, S₀ = 0. c) The transfer of cisplatin through the nuclear envelope is like process a, with the rate assumed to be kₜ ([P] – [N]). d) The reaction of cisplatin with DNA is assumed second-order, equal to kₙ[N][D], where [D] is the effective concentration of free DNA sites capable of binding cisplatin, so the term −kₙ[N][D] appears in d[N]/dt and d[D]/dt. At t = 0, [D] is equal to D₀; at any time, the concentration of Pt-bound DNA sites is Dₙ – [D]. e) The repair of DNA, which reverses process c, has a rate kᵣ(D₀ – [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 $\text{Pt(NH}_3)_2\text{Cl}_2$ reacts slowly with thiols (Bose et al., 1997), and conversion into the active $\text{Pt(NH}_3)(\text{OH}_2)\text{Cl}$ 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.

$$
\frac{d[P]}{dt} = k_e(P_e - [P]) - k_s[P][S] - k_i([P] - [N])
$$

(1)

$$
\frac{d[N]}{dt} = k_e(P_e - [N]) - k_s[N][D]
$$

(2)

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

(3)

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

(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:

$$
\int_0^T k_s[N][D]dt = k_sD_0 \int_0^T [N]dt
$$

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 $S_0$, $k_a$, $k_s$, $k_c$, $k_b$, $k_d$, and $D_0$. If repair is neglected, $k_i$ does not enter, whereas $k_d$ and $D_0$ enter only as the product, $k_dD_0$, 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 $t \ll T$, $[D]$ 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,

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

$$
\frac{d[N]}{dt} = 0 = k_e[P_e - (k_c + k_0[D_0])[N]]
$$

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 $k_dD_0[N]^T$ and is also proportional to $P_e$ in this approximation. The constant of proportionality, or the slope, is smaller the larger 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 $k_a = 125\ h^{-1}$, $k_b = 8 \times 10^5\ M^{-1}\ h^{-1}$, $k_c = 150\ h^{-1}$, and $k_dD_0 = 15\ h^{-1}$; $S_0$ 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 approximation.

Figure 4 shows the expected behavior in the presence of free cellular thiols (assumed initial concentration, 2 mM). The plot of amount of DNA-bound Pt versus $[\text{CDDP}]$ is linear for small $[\text{CDDP}]$, with a slope of 1.8 Pt atoms/10^6 nt/μM. For larger $[\text{CDDP}]$, the slope is larger, about 10.5 Pt/10^6 nt/μM at $[\text{CDDP}] = 9\ μM$; the slope is still increasing at 9 μM. With thiol groups blocked, so $S_e$ = 0, the plot of amount of DNA-bound Pt versus $[\text{CDDP}]$ is close to linear for all $[\text{CDDP}]$. The slope is 25.8 Pt/10^6 nt/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
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 S0 = 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 (S0 = 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 S0 = 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 S0 = 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 S0 = 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 S0 = 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 S0 = 0 is less serious than for S0 = 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 k0 we used, 8 × 10^5 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(NH3)2(H2O)Cl, and the rate constant for the reaction of Pt(NH3)2(H2O)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 greater than Pt(NH3)2(H2O)Cl, and this should also hold for binding to thiols (Legenre et al., 2000). It should be emphasized that a large value of k0 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 > ~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 Pt at t = 2 h), [S]/S0 < 0.1 for t = 2 h requires that \( k_0 \) be 4 × 10^6 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 (Soudai 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 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.

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**References**


