Ormaplatin Sensitivity/Resistance in Human Ovarian Cancer Cells Made Resistant to Cisplatin

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ABSTRACT

The human ovarian cancer cell lines A2780 and A2780/CP70 were studied to investigate the cellular basis for their relative sensitivities to tetraethyl(o-ntrans)-1,2-diaminocyclohexaneplatinum(IV) (ormaplatin). Cells were exposed to ormaplatin for 1 h in all experiments. As assessed by colony formation assays, the A2780/CP70 cell line [50% inhibitory dose (IC50) = 3.6 pM] was 9.5-fold more resistant to ormaplatin than the A2780 cell line [IC50 = 0.38 pM].

INTRODUCTION

Ormaplatin is a Pt(IV) complex containing DACH carrier ligand. Ormaplatin is less nephrotoxic in animals than cisplatin (1, 2) and is effective against a broad range of cisplatin-refractory tumors grown in vitro (2, 3). The efficacy of ormaplatin has been demonstrated in mice bearing L1210 leukemia cells (2–4), in primary human ovarian cancer cells (5), and in several cisplatin-resistant cell lines (6–8). The effective cytotoxicity and lack of cross-resistance against cells made resistant to cisplatin make it a promising agent for clinical development. The potential clinical utility of ormaplatin is currently under study, inasmuch as phase I clinical trials are currently ongoing (9, 10).

In culture medium or within the cell, ormaplatin is thought to undergo very rapid protein sulfhydryl-dependent reduction to form its reactive Pt(II) species Pt-DACH (2, 4, 11). It then behaves in a manner similar to that of other Pt(II) analogues. Inside the cell, the half-life of the rate of the displacement reaction of Pt-DACH ranges between 12 and 15 min, which is 6-10-fold faster than that observed extracellularly (2, 11). In tissue culture media, both ormaplatin and Pt-DACH produce similar biotransformation products (11), whereas in cells the uptake and metabolism of these complexes differ. Chaney’s group (12, 13), using high performance liquid chromatography monitored the intracellular uptake and metabolism of these complexes in murine leukemia L1210 cells. In these cells, there was preferential uptake of ormaplatin at early times at a rate 3–4-fold greater than that of Pt-DACH. Intracellular metabolism of ormaplatin yielded three biotransformation products; two were unique only to ormaplatin-treated cells; and small amounts of one product was seen in Pt-DACH-treated cells (12). In a similar fashion, Mauldin et al. (13) monitored the metabolism of Pt-DACH over 24 h in L1210 cells. At early times of drug exposure, most intracellular drug existed unchanged, after which it was gradually complexed to amino acids throughout the 24-h monitoring period. They also observed at early times an abundance of a platinum-glutathione complex, which increased with increasing platinum concentration; platinum-amino acid complex levels were unaffected. By using high performance liquid chromatography and DNA binding assays, these investigators identified the major DNA-reactive biotransformation product as an aquachloro complex which was gradually, but not completely, replaced over time mostly by stable amino acid complexes with negligible DNA-binding activity and several unknown DNA-reactive products. It remains unclear as to the characterization of the DNA-reactive biotransformation products and their relation to cytotoxicity.

Some studies suggest that ormaplatin may require intracellular activation by a sulfhydryl-dependent reduction to a Pt(II) analogue which elicits antitumor activity (11, 14). In tissue culture, ormaplatin undergoes rapid reduction activation with a 1/2 of 5–15 min, whereby protein sulfhydryl groups in serum-supplemented culture media are the major reducing agents (11). The binding kinetics of ormaplatin with human plasma proteins and bovine serum albumin, as reported by LeRoy and Thompson (15), indicates that its binding reaction with protein occurs quickly via a direct nucleophilic attack and that this reaction can occur prior to aquration. Eastman and Richon (16) observed that ormaplatin was cytotoxic to cisplatin-sensitive and -resistant murine leukemia L1210 cells and suggested that the cytotoxic effect of ormaplatin requires a non-rate-limiting reduction that can occur in the culture medium or intracellularly. In addition, by incubating DNA with ormaplatin and various concentrations of glutathione, Eastman and Richon showed that glutathione mediated the activation of ormaplatin, which then reacted with DNA. They suggested that the rate of reaction of reduced DNA with DNA is controlled by the dissociation of the two remaining chloride ligands of the Pt(II) species (14). The platinum-DNA adducts formed appear to be similar to those formed by cisplatin (16).

Cellular resistance to platinum drugs involves multiple mechanisms (17). Most evidence on the biological activities of platinum analogues indicates that their cytotoxicities result from interactions with DNA, producing platinum-DNA lesions which inhibit DNA replication (17–19). In human ovarian cancer cell lines A2780 and A2780/CP70, the major contributors to cisplatin resistance in vitro are reduced drug accumulation and enhanced DNA repair (20–22). The latter of which appears to be the prevalent mechanism in these cells. In this study, we sought to determine the effect of ormaplatin on human ovarian cancer cells that differ with respect to their levels of resistance to cisplatin.

MATERIALS AND METHODS

Cell Culture. The ovarian cancer cell lines A2780 and A2780/CP70 have been described previously (21) and were used in all experiments. Cells were
cultured in monolayer using RPMI 1640 supplemented with 10% fetal calf serum, 0.2 unit/ml of human insulin, and penicillin/streptomycin (GIBCO, Grand Island, NY). Cells were grown in a humidified 5% CO2 mixture with ambient air at 37°C. Sensitivity to ormaplatin was assessed by colony formation assays using 6-well plates, each well 35 mm in diameter. Cells were plated at 500 cells/well and drug treatments were performed as 1-h drug exposures on the day after plating. Ormaplatin was initially dissolved in phosphate-buffered saline at 1 mg/ml, and dilutions from this solution were made in media to obtain the desired drug treatment concentration. Cells were allowed to grow for 7–10 days, at which time colonies were stained with a methylene blue solution of 0.167 g % in absolute methanol. Visible colonies were counted by hand. Drug treatments were done in triplicate at each dose in each individual experiment. The value obtained in wells where no drug was added was assigned the value of 100% growth.

**Cellular Drug Accumulation.** Cisplatin-sensitive and -resistant cell lines were treated in monolayer with ormaplatin drug doses of 5, 10, 20, or 40 μM for 1 h for the purpose of measuring cellular accumulation of drug. After 1-h drug exposures, cells were immediately harvested and “wet-ashed” according to the method of McGahan and Tyczkowska (23), and total cellular drug accumulation was measured by AAS and assessed as pg of platinum/106 cells.

In separate experiments the volume consumed by 106 cells of each cell line was assessed in six replicates. This was determined by centrifugation of 6 × 106 cells into a pellet in a graduated centrifugation tube. Cells were spun at 2000 rpm for 20 min.

**Measurement of Rates of Cellular Drug Accumulation and Efflux.** A2780 and A2780/CP70 cell lines were treated at their respective IC50 doses for the purpose of measuring cellular drug accumulation and efflux. Cells were exposed to ormaplatin for 1 h in all experiments. To measure drug accumulation, cells were harvested at 15, 30, 45, and 60 min during the 1-h drug exposure. Total cellular accumulation was assessed as both pg platinum/106 cells and as the percentage of maximal drug accumulation (total drug accumulation at 60 min). To measure drug efflux, cells were treated for 1 h and aliquots were harvested immediately and at 1, 3, and 6 h after drug removal. Total cellular drug was assessed as pg platinum/106 cells. The percentage of total cellular drug accumulation and percentage of efflux in these two cell lines were compared by assigning the value of 100% to that drug level achieved at the end of the drug treatment and assessing all other values relative to the 100% value.

**Measurement of Platinum in Cellular DNA.** In one set of experiments, we sought to establish the relationship between ormaplatin dose and platinum-DNA adduct formation in the sensitive and resistant cell lines. Both cell lines were treated in monolayer with ormaplatin doses of 5, 10, 20, or 40 μM for 1 h, and cells were harvested immediately and frozen at −20°C until DNA isolation. DNA was isolated using cesium chloride density gradient centrifugation (24) and measured at 260 nm. Total platinum per unit DNA was measured by AAS (25).

In another set of experiments, we assessed the ability of these cells to remove platinum from cellular DNA. Cells were plated in T-150 flasks (Costar, Cambridge, MA) and allowed to grow in log phase with changes of fresh media twice weekly. Cells were labeled with a [3H]thymidine concentration of 250 μCi/ml of media for 24 h, after which fresh media were placed onto the cells, and further incubation was carried out overnight. At this time, cells were exposed to the specified concentrations of ormaplatin for 1 h. After 1 h labeling, an aliquot of cells was harvested before drug treatment (time zero control). Following a 1-h drug exposure, cells were harvested at 0 (immediately at the end of ormaplatin exposure) 1 h, 3 h, 6 h, and 24 h. Cells were frozen immediately at −20°C until DNA isolation. In experiments designed to compare adduct removal after equal levels of DNA damage, the A2780 cell line was treated with 10 μM ormaplatin and the A2780/CP70 cell line was treated at 20 μM.

Total cellular DNA was isolated using cesium chloride density gradients, yielding DNA which was 99.6% free of contamination (24). This DNA was dialyzed against four changes of distilled water over 36–48 h. DNA was then measured by absorbance at 260 nm. [3H]Thymidine content was assessed by liquid scintillation counting and platinum content was assessed by AAS. A decrease in the specific radioactivity of DNA (dpm/μg DNA) at each time point compared to that obtained at 1 h represents the amount of replication that occurred. This ratio was used to determine the platinum content of nonreplicated DNA.

**RESULTS**

**Differences in Survival between Cell Lines.** The relative sensitivities of the two cell lines to ormaplatin and to cisplatin, as assessed by colony formation, are shown in Fig. 1. Data obtained with cisplatin have been reported previously (20). In the A2780 cell line the IC50 for ormaplatin is 0.38 μM and the IC50 for cisplatin, is 3.0 μM (Fig. 1A). In the A2780/CP70 cell line the IC50 of ormaplatin is 3.6 μM and that of cisplatin is 40.0 μM (Fig. 1B). The IC50 drug doses for the two cell lines differ by 9.5-fold for ormaplatin (0.38 versus 3.6 μM) and by 13-fold for cisplatin (3.0 versus 40.0 μM). The A2780 cells are 7.9-fold more sensitive to ormaplatin than cisplatin, and in the A2780/CP70 cells the difference is 11-fold.

**Assessment of Total Cellular Accumulation of Drug.** We measured cellular accumulation of ormaplatin in the two cell lines after defined drug exposures. Fig. 2 shows these data with each data point representing the mean ± SD of four or six separate determinations. In A2780 cells, there is an increase in cellular platinum levels with increasing drug concentration which approaches linearity. Linear regression analysis up to the dose of 40 μM yields the equation \( y = 11.0x + 15.5, r = 0.96 \). In A2780/CP70 cells, there was a much greater increase in cellular platinum levels with increasing drug concentrations. The relationship between ormaplatin drug dose and total cellular accumulation also approaches linearity, with the linear regression...
A phosphate-buffered saline, trypsinized, and "wet-ashed," and total platinum content was measured using AAS with Zeeman background correction. Each data point is the mean ± SD (bars) from four or six separate determinations. Absolute values for drug uptake in A2780/CP70 cells during and following IC50 drug exposures for 1 h. The IC50s were: A2780, 0.38 nM; A2780/CP70, 3.60 nM. Cells were treated as described in the text. Each cell line accumulated more drug. We compared total cell volumes of 6 × 10^6 cells of A2780 and A2780/CP70 cells, using six replicates of each. For both cell lines, 0.033 ml was consumed by this number of cells.

Measurement of Rates of Cellular Drug Accumulation and Efflux. We measured the rates of total drug accumulation (Fig. 3) during 1-h exposures to IC50 drug doses in the two cell lines. Platinum concentrations are expressed in the figure as a percentage of maximal drug accumulation and each data point represents the mean of four determinations. After 1 h, the A2780 cells accumulated 50.0 ± 8.8 pg platinum/10^6 cells (9.1 ng platinum/ml) of total cellular drug and the A2780/CP70 cells accumulated 226.1 ± 64.0 pg platinum/10^6 cells (41.1 ng platinum/ml). As shown in Fig. 3A, the two cell lines accumulated drug at similar rates over the 1-h time period, although the cells differed in several respects. Cellular accumulation in the resistant cells was rapid, compared to the sensitive cells. At 15 min of the 1-h exposure, platinum levels did not reach detection threshold in A2780 cells, while the A2780/CP70 cells accumulated 19.7% of its maximal drug level. However, this difference in percentage of maximal drug accumulation was no longer present at 30 min into the drug treatment. Also, the resistant cells accumulated about 5-fold more drug than the sensitive cells when both are treated at their IC50 doses.

We also measured drug efflux in these cells over 6 h, after 1-h treatments with IC50 drug doses. Over the time period observed, absolute levels of drug changed more rapidly in the resistant cells. However, when expressed as the percentage of maximal drug accumulated, the sensitive cells effluxed drug more quickly (Fig. 3B). At 1, 3, and 6 h after treatment, the sensitive cells had removed 47.1, 72.6, and 78.7% of total accumulated cellular platinum at the respective time points. At the same time points the resistant cells removed 8.0, 50.2, and 65.3% of total accumulated cellular platinum. These data indicate that the sensitive cells had a rate of drug efflux over the first 3 h that was 2-fold greater than the resistant cells.

Measurement of Drug-DNA Binding after Defined Drug Exposures. Shown in Fig. 4 are the levels of platinum-DNA adduct formed following equivalent μM drug doses in the sensitive and resistant cell lines. For the resistant cell line, Pt-DNA adduct formation increases with increasing drug concentration, and the relation between drug dose and adduct level approaches linearity, with a linear regression equation of y = 0.24x + 0.65, r = 0.98. In the sensitive cell line, Pt-DNA adduct formation also increases with increasing drug concentration, and the relation between drug dose and adduct level also approaches linearity up to the dose of 40 μM (y = 0.64x + 1.26, r = 0.99). Based on the slopes of the linear regression equations, approximately 2.6-fold more ormaplatin is needed in the resistant cells to attain the same level of DNA damage as in the sensitive cells.
Comparative analyses of DNA adduct level with cellular accumulation of ormaplatin in A2780, wild type and A2780/CP70 cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug dose (µM)</th>
<th>Drug accumulation a (pg Pt/10⁶ cells)</th>
<th>DNA damage b (pg Pt/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>41.82 ± 11.06</td>
<td>1.73 ± 1.52</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>111.47 ± 27.36</td>
<td>4.24 ± 1.94</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>143.81 ± 18.75</td>
<td>10.60 ± 1.11</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>450.26 ± 141.37</td>
<td>24.92 ± 7.14</td>
</tr>
<tr>
<td>A2780/CP70</td>
<td>0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>338.27 ± 91.08</td>
<td>3.58 ± 1.18</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>432.60 ± 132.60</td>
<td>1.98 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1461.70 ± 223.78</td>
<td>9.36 ± 3.30</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3440.54 ± 895.56</td>
<td>9.36 ± 3.30</td>
</tr>
</tbody>
</table>

a After 1-h drug exposures, cells were treated as described in the text, and ormaplatin accumulation was assessed using AAS. Values represent the mean ± SD of four to six separate determinations.

b Following drug exposures, DNA was isolated as described in the text and platinum levels were measured by AAS. Values represent the mean ± SD of three or four separate determinations.

**DISCUSSION**

In this study, cisplatin-sensitive A2780 and resistant A2780/CP70 cell lines were assessed for their respective characteristics of ormaplatin drug accumulation and efflux, the relationship between drug dose and DNA damage level, and DNA repair. When assessed for ormaplatin cytotoxicity, the resistant cells (IC₅₀ 3.6 µM) were 9.5-fold more resistant to ormaplatin than the sensitive cells (IC₅₀ 0.38 µM). A2780/CP70 cells (IC₅₀ 40 µM) are 13-fold more resistant to cisplatin than A2780 cells (IC₅₀ 3 µM). For both cell lines, ormaplatin is more cytotoxic than cisplatin and the magnitude of increased sensitivity to ormaplatin was greater in the resistant cells (11.1-fold for A2780/CP70 and 7.9-fold for A2780). Resistant cells exhibited a moderate degree of cross-resistance to ormaplatin.

When these cell lines were treated with a range of doses of ormaplatin, there was a greater increase in drug uptake but reduced levels of platinum-DNA damage in the resistant cell line compared to the sensitive cell line. When treated at their respective biologically equivalent (IC₅₀) doses, the percentage of drug efflux was greater in the sensitive cells than in the resistant cells. When both cell lines were loaded to the same level of DNA-bound ormaplatin, both cell lines removed platinum from cellular DNA at relatively similar rates.

Cellular resistance to platinum compounds involves multiple mechanisms, which include: (a) altered cellular accumulation of platinum; (b) elevated levels of glutathione and/or metallothionein; (c) reduced removal of platinum from cellular DNA.

**Removal of Platinum from Cellular DNA.** The two cell lines were compared for their respective ability to remove Pt-DNA adducts from cellular DNA over a 24-h time period (Fig. 5). In this approach, we compared the sensitive cell line with the resistant cell line after both were treated with ormaplatin to achieve the same level of DNA-bound drug.

As shown in Fig. 5A, after a 10 µM dose in the A2780 cell line, the peak platinum level measured was 3.3 ± 1.7 pg/µg DNA. This was followed by a 6-h phase of rapid removal from cellular DNA to a level of 1.5 ± 1.2 pg/µg DNA. There was a slow rate of adduct removal during the remainder of the 24-h observation period, at which time levels measured 0.8 ± 0.6. For the A2780/CP70 cell line (Fig. 5A, closed diamonds), 20 µM ormaplatin was required to reach an equivalent level of DNA damage as observed in the A2780 cell line, 3.9 ± 0.7 pg/µg DNA. A rapid phase of adduct removal occurred over the next 6 h to a level of 1.2 ± 0.6 pg platinum/µg DNA. This was followed by a slow rate of removal during the remainder of the 24-h observation period, to the level of 0.6 ± 0.6 pg platinum/µg DNA.

When viewed as a percentage of the maximal value of platinum bound to DNA (Fig. 5B), the sensitive cell line removed 53% of its total platinum load in the first 6 h, whereas the resistant cell line removed 68%. Because of the error bars obtained at each time point, it appears that the amount of platinum removed from cellular DNA during the first 24 h after exposure is about the same for the two cell lines. In addition, a portion of the adduct load persists in both cell lines for at least 24 h after ormaplatin exposure.
DNA platination; (d) enhanced DNA repair; and (e) tolerance to unrepair DNA lesions (17–19). Altered drug transport and elevated glutathione levels appear to contribute to platinum resistance in some cell lines, whereas neither mechanism appears to correlate with the level of resistance (8, 18–22). In contrast to cisplatin, ormaplatin does not appear to influence cellular glutathione levels (26), which may be a contributing factor to the increased sensitivity to ormaplatin observed in cisplatin-resistant cells. Chaney (27) has reported that drug sensitivity may be carrier ligand specific, whereas changes in drug uptake are not. Changes in drug accumulation appear to result from general changes in membrane permeability (27). A number of mammalian cisplatin-resistant cell lines exhibit only partial or no cross-resistance to ormaplatin or other DACH complexes (1–4, 6–8, 11). In this study, A2780/C7070 cells exhibit moderate cross-resistance to ormaplatin.

The major factor in the cytotoxicity of platinum compounds is the binding to DNA. Studies with human ovarian cancer cell lines (28, 29), human small cell lung cancer cell lines (30), and cultured human glioma cells (31) indicate that the levels of platinum-DNA adducts formed is a good correlate for cytotoxicity. In a study of the comparative effects of ormaplatin and cisplatin in Escherichia coli, Razaka et al. (32) showed that alternative levels of drug exposure, the intracellular level of ormaplatin was 10-fold greater than that of cisplatin. In addition, at equal intracellular levels of drug, ormaplatin formed about one-half the number of DNA adducts compared to cisplatin. When both drugs platedig DNA to the same level, the cytotoxicity of ormaplatin was identical to that of cisplatin.

These two cell lines have been studied for cisplatin sensitivity and resistance. The A2780/C7070 cell line expresses a 2-fold reduction in cisplatin drug accumulation, enhanced drug efflux, and 2-fold reduced DNA platination compared to sensitive cells (20). In addition, resistant cells are 2-fold more efficient at repairing cisplatin-DNA lesions in cellular DNA than the parent sensitive cells (20–22). In this current study, ormaplatin had unexpected effects on cellular accumulation and DNA platination. At equal levels of drug exposure, resistant cells developed an 8-fold increased level of intracellular drug which did not correspond to an increased level of DNA-bound platinum in cellular DNA, as compared to sensitive cells. At equal intracellular levels of ormaplatin, sensitive cells formed over 12-fold more DNA adduct than resistant cells, which indicates that in resistant cells a large fraction of cellular drug was inactivated and/or made unavailable to react with DNA. Enhanced cellular efflux does not explain this observation, since sensitive cells effluxed drug more quickly than resistant cells.

In this study, one cannot easily distinguish between avid binding of ormaplatin to cellular components versus simple uptake and efflux of the drug. Increased cellular accumulation of ormaplatin was observed, and this phenomenon has been reported by Rahaman et al. (2), with murine leukemia L1210 cells, and by Chaney (27), with platinum-resistant variants of human carcinoma A2780 and HCT8 cell lines. While the usual route of ormaplatin activation is extracellular and rapid (11), the 2-fold elevated level of intracellular glutathione, a characteristic of the cisplatin-induced phenotype of this resistant cell line (21, 33), may have contributed to the differences in cellular drug uptake and efflux and Pt-DNA adduct formation compared to sensitive cells. Glutathione may modulate platinum cytotoxicity by inactivating platinum drugs through direct binding, quenching of monoadducts, and increasing DNA repair activity (11–13, 33). Whereas acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced DNA repair (20–22), ormaplatin-induced DNA damage is repaired with equal proficiency in the sensitive cells. Thus, in human ovarian cancer cells, cellular inactivation of drug and reduced ormaplatin-DNA adduct formation appear to be the primary determinants of increased resistance to ormaplatin.

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