Platinum Incorporation and Differential Effects of cis- and trans-Diaminedichloroplatinum(II) on the Growth of Mouse Leukemia P388/D1

Gerald Just and Eggehard Holler

Institut für Biophysik und physikalische Biochemie der Universität Regensburg, Federal Republic of Germany

ABSTRACT

The parallel effects of cis-diaminedichloroplatinum(II) (1 μM) or trans-diaminedichloroplatinum(II) (20 μM) on the growth of mouse leukemia P388/D1 in culture, on the cellular content of protein/DNA, on the average cell volume, and on the distribution in different phases of the cell cycle were measured over a period of 70 h and compared with the amount of platinum residing in the cells.

Despite different concentrations in the culture medium, similar amounts of cis- and trans-diaminedichloroplatinum(II) were incorporated. The platinum content of cells passed through an early concentration maximum in the millimolar range and decreased during prolonged times of incubation. Effects of cis-diaminedichloroplatinum(II) were marked and irreversible whereas those of trans-diaminedichloroplatinum(II) were small and transient. Proliferation was inhibited, while synthesis of protein and DNA continued almost unaffected. Cells were arrested in G2-phase of the cell cycle, and their volume increased correspondingly. The results suggested an inhibition of mitotic events, rather than of DNA replication, that caused cell arrest.

INTRODUCTION

For several human tumors, treatment with cis-DDP provides an effective therapy while that with trans-DDP is not successful. DNA damage in tumor cells, especially by intrastrand cross-links of cis-DDP between adjacent guanine residues is generally accepted as one of the reasons of cytotoxicity (2–4). Only a marginal fraction of the total platinum incorporated into a cell, however, was found in DNA (5). There was no correlation between the amount of cis-DDP required for the inhibition of DNA synthesis in vitro and the concentration needed for inhibition of cell growth (6). The bulk of platinum seemed to be consumed by reactions with other constituents of the cell. Recent evidence suggests that resistance of cells against platinum treatment could be provided by reactions with certain avid components other than DNA (7), for instance with glutathione, which had increased levels (8). Another possibility considered was the inhibition of membrane transport activities, which are required for platinum compounds to enter cells (9–11). cis-DDP induced also a remarkable rise of many nucleotide levels (Refs. 12 and 13; Footnote 4) and the observed increase of intracellular cyclic AMP concentrations was found to be a direct result of cis-DDP interactions with the cytoplasmic membrane (14).

In the present investigation, we intended to establish the differential effects on properties of mouse leukemia P388/D1 cell cultures during treatment with cis-DDP and trans-DDP under conditions of equitoxic doses as defined by [3H]thymidine incorporation into DNA. The goal was to identify properties, which distinguish the therapeutically active cis-DDP from the inactive trans-DDP.

MATERIALS AND METHODS

Mouse leukemia P388/D1, cultures were obtained from Dr. Reubl (Munich). RPMI/glutamine (without carbonate) was purchased from Biochrom and Gibco, horse serum from Boehringer (Mannheim), [3H]thymidine from Amersham-Buchler (Braunschweig), scintillation cocktail from Roth (Munich), and all other chemicals of highest available grade from Sigma (Munich) or from Merck (Darmstadt). cis-DDP and trans-DDP were gifts of Degussa (Frankfurt).

Phosphate buffered saline, pH 7.0, contained 0.2 g KCl, 0.2 g KH2PO4, 8 g NaCl, 1 g Na2HPO4, and 0.15 g NaH2PO4·H2O in 1 liter distilled water.

Cell Culture. Mouse leukemia P388/D1 cultures were seeded at 80–100 × 106 cells/ml from a 2–3 × 105/ml stock in liquid nitrogen and grown in suspension cultures (200 ml, without shaking) in polystyrene bottles under humidified atmosphere with 5% CO2 at 37°C. Growth medium RPMI contained 10% heat-inactivated horse serum (30 min at 56°C), NaHCO3 (0.85 g/liter), HEPES buffer (10 mM, pH 7.4) and glutamine (2 mM). The doubling time of cells was 12–14 h (16, 17). Cultures were free of mycoplasma [standard assay from the distributor Boehringer (Mannheim)] (15). Freshly prepared solutions of cis-DDP or trans-DDP in dimethylformamide (0.01% by volume) were added at the end of the growth lag phase (9 h after seeding). The control culture (platinum absent) contained the same amount of dimethylformamide. Cell viability was assayed with trypan blue exclusion by the method of Phillips (15, 18).

Thymidine Incorporation. Incorporation of [3H]thymidine was measured 46 h after the addition of platinum complexes (15, 17). The incorporation was started by the addition of 0.3 nCi [3H]thymidine to 2-ml cell suspensions. After 2-h incubation, washed and counted cells were suspended in 1.5 ml water and sonified. Nucleic acids were precipitated over night at 4°C after addition of 4 ml of 10% (by volume) saturated trichloroacetic acid, collected on Millipore filter (0.45-μm pore) and counted for radioactivity. Incorporation of [3H]thymidine was measured in percentage of the control. Equally toxic doses of cis-DDP (1 μM) and trans-DDP (20 μM) were estimated for 90% inhibition.

DNA and Protein. The content of protein, DNA or platinum was measured after homogenization of 8 × 107 cells for each time point (twice washed with PBS, pelleted at 1000 × g in silane-treated vials and sonified; Branson Sonifier B15) in 0.1 vol PBS (with regard to the volume of the growth medium that had contained these cells). After centrifugation at 5000 × g, the supernatant or pellet was subjected to analysis as indicated.

DNA was measured in the supernatant according to Boer (19). Samples were incubated for 30 min at 37°C in the presence of 0.1 mg/ml of each pronase and ribonuclease. After addition of 40 μg/ml ethidium bromide to each sample, fluorescence intensity was measured at 590 nm wavelength (excitation wavelength, 360 nm). Intensities were calibrated against calf thymus DNA. Measurements were carried out with a Hitachi F-3000 fluorescence spectrophotometer.

Protein was measured in the supernatant according to the method of Bradford (20).

Platinum. The cell homogenates obtained as above were fractionated as follows. The supernatant after centrifugation was extracted with 1
were added at the lime (9 h) indicated by an arrow. Concentrations have been reported previously (26).

Reference volume of control cells (at the time of seeding) was 0.5± 0.1 x 10^6 M as measured with a Coulter Counter (average diameter, assuming proportionality between the scattering volume and intensities volume was calculated from the intensity of the forward light scatter of cells equipped with an Epics graphic display, a Tektronix 620 monitor and a OM2 camera body. Magnification was 300-fold in the phase contrast mode.

RESULTS

Effects on Cell Proliferation and Morphology. The growth of mouse leukemia P388/D, cells containing either 1 μM cis-DDP or 20 μM trans-DDP in the culture medium and of a control (platinum omitted) was followed in parallel over an incubation period of 70 h. In preliminary tests it had been established that cis- and trans-DDP at the given concentrations (46-h incubation) were equally toxic effecting the same degree of inhibition of [H]thymidine incorporation. Similar values for equitoxic concentrations have been reported previously (26).

The cell number per unit volume (Fig. 1) and the cell density

![Cell number per milliliter of culture medium. Platinum complexes were added at the time (9 h) indicated by an arrow.](image)

Effects on the Cell Content of DNA and Protein. The amounts of DNA or protein per unit volume of culture during the treatment of mouse leukemia P388/D, cells with the platinum compounds are shown in Fig. 4, A and C. While the dependence on the time of incubation with trans-DDP was of the kind that could be expected in relation to the growth curves in Fig. 1, the results for cis-DDP were surprising. The amounts of DNA (40% of the control, Fig. 4A, at 70 h) and of protein (65%; Fig. 4C, at 70 h) were considerably higher than expected from the cell number (20%; Fig. 1, at 70 h) at a constant cell volume. The results are also given in terms of an average content per cell for DNA (Fig. 4B) or protein (Fig. 4D). While these were very similar for trans-DDP and the control, they almost doubled for cis-DDP at the end of the observation period.

The observed doubling of DNA and protein content per average cell (Fig. 4) together with an increase in cell volume (Figs. 2 and 3) suggest that the effect of the incubation with cis-DDP was mainly to inhibit cell division rather than DNA and protein synthesis.

Growth Arrest in G2-Phase of the Cell Cycle. The number of cells in different phases of the cell cycle was measured by flow cytometry after staining of DNA with fluorescent acridin orange. The observed fluorescence intensity is a measure of the amount of DNA per cell. Because the DNA content per cell doubles during S-phase, cells sorted according to their intensities belong to G1- (low intensity), S- (medium intensity), or G2-phase (high intensity) of the cell cycle. Histograms are shown in Fig. 5A for the control and the platinum compounds. A major peak (see control) at low DNA content represents the portion of cells in G1-phase and a minor peak at high DNA content that in G2-phase. The area between the peaks refers to cells in the S-phase. Integration of each of these areas yields the particular fraction of the total number of cells counted (Fig. 5B).

The fraction of cells in G2-phase increased substantially during the first 10–20 h of incubation with cis-DDP and stayed constant from thereon (Fig. 5A). Interestingly, at that time an increase in the average cell volume was not yet visible (Fig. 3B). Treatment with trans-DDP at the equitoxic dose resulted in a similar effect; but the cell culture recovered after a period of approximately 20 h. At the end of the observation (after 70 h) these cells had nearly the same cell cycle distribution as those in the control. The accumulation in G2-phase occurred for both platinum compounds at the expense of the fraction cells in the G1-phase.

Incorporation of Platinum into Cells. The amounts of platinum

Fig. 1. Cell number per milliliter of culture medium. Platinum complexes were added at the time (9 h) indicated by an arrow.
were measured that resided in cell “compartments” which were defined by the fractionation protocol. More than 80% of total platinum from cells was found in the supernatant of cell homogenates after centrifugation containing soluble proteins, nucleic acids, low molecular weight compounds and free cis-/trans-DDP (Fig. 6A, cell supernatant).
EFFECTS OF cis-DDP AND trans-DDP ON P388/D1, MOUSE LEUKEMIA

Control
c/s-DDP trans-DDP

Control

1
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

n Control

20 /LI M transDDP

20 40 60 0 20 40 60
Growth time [h]

Fig. 3. Size (volume) distribution of mouse P388/D1 cells measured by flow cytometry (forward light scatter). A, histograms of each 0.3 x 10^6 cells of the control and platinum-containing cultures. Samples were taken at the times of growth indicated at the right of the figure. B, estimated average cell volume (left) and scattering range in terms of highest and lowest volume in the cell population (right) as a function of growth time. Averages were calculated as described in the text. Highest and lowest values were read from the histograms in A.

Fig. 4. Contents of DNA and protein in cultures of mouse P388/D1 cells. A and C, content of DNA and protein, respectively, per milliliter suspension culture. B and D, average content of DNA and protein, respectively, per million cells calculated on the basis of corresponding values in A, C and in Fig. 1. Cl, control; O, 1 μM cis-DDP; X, 20 μM trans-DDP.

Thus, the amount of platinum in cellular membranes and in other insoluble material in the pellet was low (Fig. 6A, cell pellet). In the soluble fraction the larger portion of platinum (30–70% of total cellular platinum) remained also soluble after ethanol precipitation of the aqueous phase that had been obtained after phenolic extraction (Fig. 6B). The pellet contained the nucleic acids and approximately 20% of total cellular platinum. The platinum content of the phenolic phase (containing proteins) were substantial for both trans-DDP and cis-DDP (Fig. 6B). The methanol/dichloroethane/water extract of the homogenate pellet was devoid of platinum, whereas the Triton/ HCl extract contained 5–14% platinum for cis-DDP and 3–7% platinum for trans-DDP. The amount resistant to extraction was 1–3% for both cis-DDP and trans-DDP.

Intracellular concentrations of platinum (Fig. 6A, solid lines) were calculated from the measured absolute amounts, the corresponding cell number (Fig. 1) and the mean cell volume (Fig. 3B). Maximum cellular concentrations of 1.6 mM for cis-DDP and 1.2 mM for trans-DDP were attained within 2 h (the time for the first sample) after the beginning of the treatment. Note that concentrations in the culture medium were 1 μM cis-DDP and 20 μM trans-DDP, respectively, and that the fractions of maximally incorporated platinum were 10 and 0.5%, respectively, of total platinum present in the cultures (Fig. 6, insets). During the course of the incubation, the level of platinum returned to 0.2 mM for cis-DDP after 20 h and to lower concentrations for trans-DDP. The concentration decay, which was faster for cis-DDP than for trans-DDP, could not be reconciled with simple dilution by cell growth (compare with Fig. 1).

DISCUSSION

Treatment of mouse leukemia P388/D1 cells with 1 μM cis-DDP in the culture medium resulted in an inhibition of proliferation accompanied by a 1.5- to 3-fold increase in cell volume that was detectable by microscopic inspection of cultures and by flow cytometry. Protein and DNA contents of cultures were within 40% of that of the control. Formation of enlarged cells was paralleled by increased cellular contents in DNA and protein. All these results indicated that DNA and protein synthesis were not severely inhibited despite a remarkable inhibition of proliferation. The existence of “giant” cells has been previously reported as a result of platinum treatment (6, 27).
has been suggested that inhibition of cell proliferation by cis-DDP might be caused by disturbing effects of cis-DDP in the context of cell mitosis rather than by inhibition of DNA replication (6). Our results show that in this sense arrest in G2-phase, almost undisturbed protein and DNA synthesis, and enlargement of cells fit together.

Arrest in G2-phase has been reported not only for cis-DDP (28, 29) but also for alkylating antitumor agents (30–32). Cells which cannot escape the arrest are bound to disintegrate (33). Thus, inhibition of DNA synthesis may not be the critical step which cannot escape the arrest are bound to disintegrate (33). Thus, inhibition of DNA synthesis may not be the critical step which cannot escape. If the uptake is considered the result of passive permeation of the cell's interior. If this were the case, the accumulation would have to follow a half-life of 3–4 h that is required for the hydrolysis of one of the chloro groups from the diffusible diamminedichloroplatinum(II) complex and that gives rise to the formation of the reactive monoaquaplatinum complex (35). Evidently, this explanation is unlikely.

On the basis of several observations we favor the assumption that the platinum complexes are taken up by particular active transport systems. An uptake associated with amino acid transport and an inhibition of the transport system by platinum has been reported (10, 36). Resistant cells incorporate much lower amounts of platinum probably due to mutational alteration in their amino acid transport systems (9, 11, 37). An active transport would explain the observed rapid rise of soluble platinum concentration in the tissue homogenate. An inhibition of this transport system and a slow passive diffusion of soluble, low molecular weight platinum complexes in the outward direction could explain the observed early concentration maximum followed by the slow decrease at later times. The plateau concentrations for cis- and trans-DDP in Fig. 6A could reflect amounts of platinum that had then indeed reacted with intracellular components. The amounts of incorporated platinum found in the various fractionation “compartments” in Fig. 6B did not reveal significant differences between cis-DDP and trans-DDP as far as experimental accuracy permitted.

Another observation is in support of the assumption that membrane transport systems could be affected by platinum complexes: at the employed “equitoxic” doses of 1 μM cis-DDP and of 20 μM trans-DDP, 90% of thymidine incorporation was depleted with regard to the control after a 48-h treatment. This is at variance with the finding of only 30–50% depletion in the DNA content per unit volume of treated cultures (Fig. 4A). A comparable situation has been reported for the treatment of Yoshida or Ehrlich ascites tumor cells with low doses of the alkylating agent triethyleneimino-cis-benzoquinone (Trenimmon) (32). Growth was arrested in G2 of the cell cycle, and DNA synthesis was normal. It was found that thymidine transport was inhibited by this drug. Their results together with those above suggest that the effect of platinum drugs on the activity of membrane-associated proteins may not be negligible towards an understanding of the mechanism(s) of action of platinum compounds.

Although trans-DDP in contrast to cis-DDP is inactive as a therapeutical antitumor agent (34), the effects by these drugs on the various aspects of mouse leukemia P388/D1 cells investigated here are qualitatively not much different during the first half period of the treatment. In fact, the intracellular concentration of platinum is almost indistinguishable for the isomers at “equitoxic” doses. What seems different is the quantity of the effects, being large and irreversible for cis-DDP but small and reversible for trans-DDP. These findings agree with the observation that, though the isomers react both with DNA, the frequent intrastrand cross-links by cis-platinum between neighboring guanine residues are more resistant to repair than the majority of DNA lesions by trans-platinum (5, 34).

ACKNOWLEDGMENTS

The authors wish to thank Dr. Rainer Laske, Universität Regensburg, for advice in performing cell cultivation.

REFERENCES

EFFECTS OF cis-DDP AND trans-DDP ON P388/D, MOUSE LEUKEMIA


Platinum Incorporation and Differential Effects of cis- and trans-Diamminedichloroplatinum(II) on the Growth of Mouse Leukemia P388/D

Gerald Just and Eggehard Holler