The binding of cis-dichlorodiammineplatinum(II) to extracellular and intracellular compounds in relation to drug uptake and cytotoxicity in vitro

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Summary The biological consequence of the binding of cis-dichlorodiammineplatinum(II) (cis-DDP) to serum protein as well as to cellular components in general, was studied on human NHK 3025 cells in vitro. As expected, we found that the cytotoxicity of cis-DDP was lost by binding to serum protein, and that protein-bound platinum was impermeable to the cells. We have previously shown that electropermeabilisation may transiently increase the influx of cis-DDP, we applied this technique in an attempt to increase the efflux of cis-DDP or any other cytotoxic intermediates. Our data demonstrate that if cells are electropermeabilised shortly after treatment with cis-DDP, cell survival increased. This indicates that cis-DDP in an active form is released from the cells; furthermore, the plasma membrane represents a barrier against efflux, as it has also been shown to be against influx of active cis-DDP. Thus, our data are consistent with the idea that there must be an intracellular pool of either cis-DDP, or some biologically active intermediates, in cells treated with this drug. Additionally, our data indicate that the binding rate of cis-DDP to biological molecules is much quicker intracellularly than in the extracellular environment. We found the biological half-life at 37°C to be about 2.1 h in human serum and about 11 min inside our cells.

Materials and methods

Cells and cell culturing technique

Cells of the established cell line NHK 3025, derived from human uterine cervix carcinoma in situ (Nordbye & Oftebro, 1969; Oftebro & Nordbye, 1969) were used. The cells were routinely grown as a monolayer, at 37°C in medium E2a (Puck et al., 1957) containing 20% human serum prepared in the laboratory and 10% horse serum (Gibco). In order to maintain cells in continuous exponential growth, the cell cultures were trypsinised (0.25% trypsin, Difco 1:250) and recultured three times a week (Pettersen et al., 1977). Cells were routinely recultured the day before use in experiments.

Cell survival

The cell inactivating effect of cis-DDP was measured as loss of colony-forming ability of cells treated with cis-DDP. The cells were trypsinised and seeded as single cells into 6 cm Falcon plastic Petri dishes with 5 ml medium. The cell number was adjusted to give, after treatment, about 150 colonies per dish. The cells were incubated for a total of 12–14 days with a medium change on day 6, and thereafter fixed in ethanol and stained with methylene blue. Only colonies containing more than 40 cells were counted. Each observation was calculated as the mean of five replicate dishes.

Electropermeabilisation requires that cells be in suspension, therefore, for experiments involving electropermeabilisation, cells were treated in suspension. There was, however, no significant differences in cell survival for NHK 3025 cells treated with cis-DDP in suspension as compared to treatment of attached cells (Melvik et al., 1986). Treatment was stopped by removing cis-DDP by centrifugation (350 g in 5 min) with an immediate substitution to fresh medium. Treatment was performed either at room temperature (20–26°C) or at 37°C.

Atomic absorption spectroscopy

After treatment with cis-DDP the cells were centrifuged and washed once in fresh medium before they were dissolved in 60 µl 16 N HNO3. The day after, 60 µl H2O was added to each sample. The amount of cellular-bound platinum was measured using a Varian SpectraAA-30 atomic absorption spectrometer fitted with a GTA-96 graphite tube atomiser. Instrument control and data acquisition was by Varian DS-15 Data Station using Varian Atomic Absorption Software. The atomic absorption signal was measured in 30 µl aliquots with a platinum lamp at 265.9 nm. Automatic background
correction with a modulated deuterium lamp was utilised. The amount of Pt was calculated from a calibration curve run immediately before samples. Each experimental point represents the mean of three parallel measurements.

Electropermeabilisation
The apparatus and method for electropermeabilisation was essentially as previously described (Gordon & Seglen, 1982; Melvik et al., 1986). Briefly, 2 ml aliquots of cell suspensions were placed in a square-bottomed (1 x 1 cm) Perspex chamber; two of the opposing walls were flat stainless steel electrodes. The electrodes were connected to a 1.2 pF capacitor connected to a 2 kV power supply. The capacitor was charged by the power supply and discharged through the circuit containing the electrode chamber. Five consecutive discharges were given at intervals of about 3 sec, and the time constant of each discharge through the cell suspension was found to be about 45 µsec.

Ultrafiltration of protein-bound platinum
A suspension of 10 µM cis-DDP dissolved in E2a was stored at 37°C in the dark before 1 ml aliquots were placed in MPS-1 filters (Amicon, USA) and centrifuged at 10°C for 25 min at 1100 g. The concentration of Pt in the ultrafiltrate was measured by atomic absorption spectroscopy.

Drugs
Cis-dichlorodiammineplatinum(II) (cis-DDP) was from Farmitalia Carlo Erba (Barcelona, Spain). Cis-DDP was first dissolved in glycolze (Rofstad et al., 1980) as a stable stock solution with a concentration of 1000 µM before it was further diluted in the growth medium and added to the cells.

Results
Due to binding of cis-DDP to protein, the biological effectiveness of this drug may be of short duration when dissolved in serum or serum-containing medium. To characterise this parameter in our cell culture we performed the experiment shown in Figure 1. While panel a in Figure 1 shows the surviving fraction of cells treated with cis-DDP added immediately after the drug was dissolved in the medium, panel b shows the surviving fraction of cells which had medium with cis-DDP (10 µM) added to the cells at various times (abscissa) after the drug was dissolved (in either medium or serum). From panel b in Figure 1 the inactivating potential of cis-DDP decreases with the time it is dissolved in both serum and in medium E2a.

For cis-DDP concentrations above 2 µM in panel 1a, experimental points are well fitted by a straight line (slope: D(t) = 2.8 ± 0.2 µM (S.E.).

Figure 2 shows the relative effective concentration as determined for the experimental points in Figure 1b relative to the values obtained at time 0. The data are fitted with exponential functions indicating that the effective concentration of cis-DDP decreases with a half-life (t1/2) of 2.1 ± 0.03 (S.E.) h in serum and 6.0 ± 0.05 (S.E.) h in medium E2a. Thus, t1/2 is about three times longer in medium than in serum. Since the concentration of serum components are higher by a factor of three in serum as compared to medium this points to serum components as responsible for the reduced effective concentration of cis-DDP.

In Figure 2 the relative amount of Pt remaining in E2a medium after ultrafiltration, i.e. after removal of protein and protein-bound Pt (MW > 30,000 dalton) at different times after 10 µM cis-DDP was diluted in the growth medium (open circles) is also shown. The data show that the remaining amount of Pt in the ultrafiltrate decreases with a similar initial rate-constant as the effective concentration of cis-DDP in the E2a medium (6.8 ± 0.1 h).

Figure 1 Cell survival of exponentially growing NIHK 3025 cells after treatment with cis-DDP. a. Cells were treated for 2 h with cis-DDP immediately after the drug was dissolved in medium E2a. Different symbols show different experiments. The straight line was fitted by the method of least squares, b. cells were treated for 2 h (O, A) or 6 days (V, one point) with 10 µM cis-DDP. The abscissa shows the time from dilution of cis-DDP in medium E2a (O) or human serum (A) (i.e. time 0) until start of treatment. During this time E2a or serum was stored at 37°C. The dotted line shows how data in b was used in a to calculate the 'effective concentration' of cis-DDP still remaining at different times after the solutions were prepared. The standard error (S.E.) is indicated when exceeding the symbol size.

Figure 2 The relative effective concentration of cis-DDP, calculated from data in Figure 1 as explained in the text, still remaining in medium E2a (O) or serum (A) at different times after the solutions were prepared and stored at 37°C. The amount of Pt in the ultrafiltrate was also measured after dissolving cis-DDP in the E2a medium (V). The effective concentration of cis-DDP decreased exponentially and t1/2 in serum was calculated to 2.1 ± 0.03 h and in medium E2a to 6.0 ± 0.05 h (S.E.). The half-life of the amount of ultrafilterable Pt was calculated to 6.8 ± 0.1 h. Symbols for the relative effective concentration of cis-DDP are shown with S.E.
The temperature dependence of the loss of cytotoxic potential of cis-DDP in the presence or absence of serum components was studied in a separate experiment (Figure 3). A concentration of 1000 μM of cis-DDP was made up in either glycozole (an isotonic salt solution), medium E2a, or whole serum and stored at 4°C, 22°C or 37°C for 24 h before the solutions were diluted 1:100 with medium E2a and added to the cells for 2 h. In Figure 3 both the cell survival (panel a) and the amount of cell-associated Pt (panel b) are shown. When dissolved in glycozole, which contains no serum, cell survival was not affected by the duration of storage of cis-DDP before cell treatment started, irrespective of the storage temperature. In medium E2a as well as in serum, the effect on cell survival as well as the amount of cell-associated Pt, decreased with storage time at all temperatures tested, but the rate of decrease in effect was clearly temperature dependent.

To try to shed some light on the rate of cis-DDP binding to cellular macromolecules, we have made use of our previous observation that the use of electrical discharges to cells renders the cell membrane transiently permeable to cis-DDP without affecting normal cell growth (Melvik et al., 1986). In Figure 4 cell survival is shown for NHIK 3025 cells treated with a high concentration of cis-DDP (filled symbols) for 25 min before the drug was removed, and the cells were exposed to electrical discharges (electropermeabilisation).

What we will hereafter denote a 'standard discharge treatment', consisted of five consecutive, single discharges given over a period of 15 s. The cells received one such standard discharge treatment about 3 min after cis-DDP was removed (which was the quickest we could manage), and thereafter the procedure was repeated at intervals of 20 min. The data in Figure 4 show cell survival as a function of the number of standard discharge treatments received by the cells both at 37°C and at room temperature. Cell survival is also shown for cells treated with electrical discharges alone, i.e. without treatment with cis-DDP (open symbols). For these cells only a very slight effect on cell survival is seen, except for cells given a maximum of four standard discharge treatments at room temperature, where only 10% of the cells retained their colony-forming ability. For cells treated with cis-DDP, the data have been normalised relative to the effect of the electropermeabilisation procedure itself, such that the drug effect alone is shown. At both temperatures cell survival was higher when the cells were electropermeabilised 3 min after the end of cis-DDP treatment compared to cells not electropermeabilised. This effect is more clearly pronounced at room temperature than at 37°C where only a small increase in cell survival was seen. Repeating the electropermeabilisations at 20 min intervals resulted in no further increase in cell survival.

We have recently found that release of cell-associated Pt from NHIK 3025 cells after exposure to cis-DDP is relatively slow with time (Melvik et al., 1992); 2 h after a 25 min exposure the total amount of cell-associated Pt was lowered by only about 10%. The increase in cell survival observed after electropermeabilisation can therefore be explained by the loss of either cis-DDP or some cytotoxic intermediates from the cells due to the increased permeability of the plasma membrane caused by the electropermeabilisation. With this in mind we performed a similar experiment where we subjected the cells to only one standard discharge treatment but varied the time between the end of the 25 min cis-DDP treatment and the start of electropermeabilisation. From Figure 5 one can see that survival of cells subjected to electropermeabilisation at room temperature decreased with increasing time interval from the end of the cis-DDP treatment to the start of the electropermeabilisation. When cells were subjected to electropermeabilisation later than 80 min after cis-DDP was removed, the cell survival was similar to that of cells treated with cis-DDP without electropermeabilisation. Thus, for treatment occurring at room temperature it seems to take up to 80 min before cis-DDP, i.e. in an active form, can no longer be released from the cells by the electropermeabilisation procedure.

In order to compare the time course of the intracellular binding of cis-DDP versus the extracellular loss of drug
activity, survival curves were used to calculate effective cis-DDP concentrations from data like those shown in Figure 5 in the same way as was described for the data presented in Figures 1 and 2. In this case, however, we used survival curves obtained after 25 min treatment with cis-DDP (not shown) since this was the treatment duration used in the corresponding electropereamobilisation experiments. In Figure 6 the effective concentration of cis-DDP is presented as relative units, i.e. relative to the concentration of cis-DDP which, if added to the cells for 25 min without electropereamobilisation, would give a similar cell survival. The data presented in Figure 6 are from experiments performed at room temperature and at 37°C.

Clearly the relative effective concentration of cis-DDP is considerably lower than 1 if cells are electropereamobilised immediately after the 25 min cis-DDP pulse (time 0), but increases up to 1 if cells are electropereamobilised more than 0 (37°C) or 80 (room temperature) min after the cis-DDP pulse. This finding supports our suggestion that subjecting cells to electropereamobilisation immediately after the cis-DDP pulse permits leakage of active cis-DDP out of the cells, thus reducing the degree of cell damage. However, within half an hour at 37°C and about 2–3 times that period at room temperature, all intracellular cis-DDP may have reacted with cellular constituents. The data therefore suggest that the reaction rate of cis-DDP in the intracellular environment is about 2–3 times slower at room temperature as compared to 37°C.

If this interpretation of the data in Figure 6 is correct, one would also expect that analysis of cellular-bound Pt would yield data similar to those shown in Figure 6. This was investigated by measuring the amount of cell-associated Pt in cells treated similarly as those shown in Figure 6. These results are shown in Figure 7. A direct comparison shows that the time course of the curves in Figure 7 is similar to that of the curves in Figure 6.

Discussion

From previous studies it is known that binding of cis-DDP to protein involves loss of cytotoxic activity (Takahashi et al., 1985; Uchida et al., 1986; Cole & Wolf, 1980; Repta & Long, 1980; Gormley et al., 1979; Holdener et al., 1982; Hegedus et al., 1987). In the present studies we performed

**Figure 5** Cell survival of NHK 3025 cells after treatment with 400 μM cis-DDP for 25 min at room temperature and one standard discharge treatment (consisting of five consecutive discharges) given at different times after the end of drug treatment. The dashed line (with S.E.) indicates the level of cell survival for cells treated with cis-DDP alone (i.e. without electrical discharges). The data were also used to calculate the 'effective concentration' of cis-DDP (shown in Figure 6) for each observed surviving fraction by the same procedure as described for Figures 1 and 2. In this case, however, survival curves obtained after 25 min exposure to increasing concentration of cis-DDP were used.

**Figure 6** The relative concentration of biologically effective cis-DDP as a function of the time between the end of a cis-DDP pulse and electropereamobilisation of NHK 3025 cells. The effective concentration of cis-DDP was determined by the same procedure as described for Figures 1 and 2, but with the use of survival curves obtained after 25 min exposure to increasing concentrations of cis-DDP using survival data from experiments of the type shown in Figure 5. The cells were treated with cis-DDP for 25 min either at room temperature (400 μM) or at 37°C (130 μM). Different symbols (with S.E.) represent different experiments. The dashed line (with S.E.) shows the effect of treatment with cis-DDP alone (without any electrical discharges).

**Figure 7** The relative amount of cell-associated Pt with NHK 3025 cells treated for 25 min with 400 μM cis-DDP at room temperature or with 130 μM cis-DDP at 37°C, and thereafter subjected to one standard discharge treatment at the indicated times after ended drug treatment. Different symbols (with S.E.) show different experiments. The dashed line (with S.E.) shows the effect of treatment with cis-DDP alone (without any electrical discharges).
comparative measurements of the half-life of cis-DDP binding to extracellular as well as to intracellular macromolecules by means of loss of cytotoxic activity.

In the extracellular environment binding to serum protein is the major factor of importance concerning loss of cis-DDP activity. This is strongly supported by the fact that the total amount of ultrafilterable Pt decreases with a similar half-life in E2a as the effective concentration of cis-DDP. Furthermore, there is no observed loss of cytotoxicity of cis-DDP with time when the drug is dissolved in the absence of protein. The reaction rates obtained here support earlier findings reported earlier (Takahashi et al., 1985; Repta & Long, 1980; LeRoy et al., 1979; van der Vijgh & Klein, 1986).

The binding of cis-DDP to serum proteins is also, as expected, strongly temperature-dependent (Figure 3). Furthermore, there is a clear correlation between the amount of cell-associated Pt and drug cytotoxicity, indicating that the protein-bound fraction of cis-DDP is impermeable to the cells.

We have recently demonstrated only a marginal release of Pt from these cells with time after a cis-DDP pulse (Melvik et al., 1992), thus demonstrating only a slow drug efflux. It was thus of interest to study if there could be an increased efflux of drug when the cells were electroporabilised after the cis-DDP treatment. The reduced cell survival observed in these experiments, as measured by a decrease in the number of viable cells per dish, thus may indicate a more rapid drug loss, but the efflux is probably no greater than that when the Pt was released by the cell membrane damage.

Electroporation may significantly protect cells against the cytotoxicity of cis-DDP if cells are permeabilised shortly after drug treatment (Figures 4 and 5). Thus, electroporabilisation may increase the efflux of cytotoxic Pt molecules, as it was shown earlier to increase the influx of such molecules (Melvik et al., 1986; Dornish et al., 1986). Our data, therefore, indicate that there is an intracellular pool of active drug shortly after drug treatment. Furthermore, as there is a reduction in the cellular amount of Pt by the electroporabilisation procedure (Figures 6 and 7) our data therefore suggest that drug efflux is inhibited by the cell membrane. It is therefore also likely that cis-DDP does not induce all damage immediately after entrance through the cell membrane.

The use of reversible electroporabilisation renders the cell membrane permeable to small molecular weight compounds only (Zimmermann et al., 1980; Gordon & Seglen, 1982; Riemann et al., 1975). Thus, cis-DDP bound to protein and other large molecules will not be lost from the cells after the electroporabilisation procedure. This explains why no further loss of Pt is seen when cells are electroporabilised after all drug has been bound intracellularly (Figure 6 and 7). Furthermore, this finding is in accordance with the observation that no further increase in cell survival is seen when repetitive treatments with electrical discharges are given (Figure 4).

A comparison between the time course of the curves shown in Figures 2 and 6 suggests that cis-DDP is bound more rapidly in the intracellular than in the extracellular environment. While from Figure 2 the t1/2 in serum at 37°C is more than 2 h, Figure 6 shows that electroporabilisation of cells later than 30 min after drug treatment had no influence on cell survival. This observation may be explained if one assumes that the reaction stage is disposed to t1/2 ≈ 11 min. Thus, the binding rate of cis-DDP is more than ten times higher in the intracellular environment as compared to human serum. Two factors may explain this difference: Firstly, more reactive Pt-metabolites are expected to appear in an environment having a low chloride concentration, as inside of the cells, rather than a high one, as in the medium (Chadwick et al., 1976; Szumiel & Nias, 1976; Segal & Le Pecq, 1985; Dedom & Borch, 1987). Secondly, it is reasonable to assume that the concentration of nucleophilic target molecules is higher in the intracellular than in the extracellular environment. The intracellular binding of cis-DDP may include binding to particular nucleophilic proteins like metallothioneins (Basu & Lazo, 1990), as well as to other compounds like glutathione and methionine (Melvik & Pettersen, 1987; Dedom & Borch, 1987; Andrews et al., 1986; Sekiya et al., 1989; Melvik et al., 1992; Anderson et al., 1990; Newman et al., 1979).

The data of Figures 6 and 7 suggest that cis-DDP is bound 2–3 times more slowly at room temperature that at 37°C in the intracellular environment (Figure 2). This is in accordance with the respective reaction rates of cis-DDP binding to serum components at the two temperatures as suggested from the data in Figure 3. We have, however, previously found a correlation between drug cytotoxicity and cellular drug accumulation when comparing experiments performed at room temperature and at 37°C (Melvik & Pettersen, 1988), and a reduced uptake of cis-DDP through the cell membrane at the former as compared to the latter temperature is therefore probably responsible for this difference. Thus, it is reasonable to believe that the reaction rate of cis-DDP towards intracellular macromolecules is not the critical factor here, but that the cell membrane is more rapidly lost at 37°C than at 30°C, as it is unpractical to compare the rate of binding to critical macromolecules with a reduced activity observed in an environment that is hypo- as compared to normothermic temperatures (Melvik & Pettersen, 1988; Herman, 1983). The cell membrane may therefore be the critical factor for the cytotoxicity of cis-DDP as the influx (Melvik et al., 1986) as well as the efflux (Melvik et al., 1992) of cis-DDP, and other cytotoxic intermediates through the plasma membrane is relatively slow compared to the rate of binding to critical molecules (Figure 6). cis-DDP molecules that are accumulated by the cells will therefore be trapped and ultimately exert its cytotoxic damage independent of the lowered temperature (room temperature). However, it is possible that the reduced reactivity of cis-DDP at lower temperatures could reflect a reduced transport of cis-DDP into the cells.

It is known that cis-DDP may act as a hypoxic radiosensitizer (Melvik & Pettersen, 1988; Overgaard & Khan, 1981; Stratford et al., 1980; Chibber et al., 1985; Douple & Richmond, 1979). This effect is strongly temperature dependent as we found a clear radiosensitising effect of cis-DDP for NHK 3025 cells irradiated under extremely hypoxic conditions at 37°C, but not at room temperature (Melvik & Pettersen, 1988). The data presented here therefore support the idea discussed in our previous paper (Melvik & Pettersen, 1992), that it is the lowered reactivity of drug macromolecules that is critical for the temperature-dependent hypoxic sensitisation, while it is the cell membrane that is critical for the temperature-dependent cytotoxicity of cis-DDP under hypothermic conditions. The underlying mechanisms for these two temperature-dependent effects are, therefore, fundamentally different.

The data presented here show that electroporabilisation may be used to demonstrate the significance of the cell membrane as a barrier against the efflux as well as the influx of cis-DDP, as presented earlier (Melvik et al., 1986; Dornish et al., 1986). Our data are thus consistent with the presence of a pool of active drug under and shortly after exposure to cis-DDP. Furthermore, the electroporabilisation method has been used to establish data on the binding rate between cis-DDP and cellular macromolecules, and confirms a much faster binding rate between cis-DDP and macromolecules in the intracellular as compared to the extracellular environment.

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