Effect of Ultrafilterable Platinum Concentration on Cisplatin and Carboplatin Cytotoxicity in Human Tumor and Bone Marrow Cells in Vitro

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The importance of the ultrafilterable platinum (fPt) fraction of cisplatin (CDDP) and carboplatin (CBDDA) for cytotoxicity and myelotoxicity was studied in vitro. By incubating CDDP or CBDDA with fetal calf serum (FCS) various fractions of fPt were prepared and determined by atomic absorption spectroscopy. A relation of % fPt fraction and incubation time (h) of $87^{0.124}$ (r = −0.99) and $101^{0.0087}$ (r = −0.99) were determined for CDDP and CBDDA, respectively. Cytotoxicity in the human small cell lung carcinoma cell line GLC4 and fPt fraction were closely related for CDDP (r = 0.99) and for CBDDA (r = 0.97). However, at a similar fPt fraction the concentrations inhibiting cell survival by 50% (IC50) of CBDDA exceeded that of CDDP by a factor of 10-18 with 4 h exposure and a factor of 5 with continuous exposure. Tested in the range of peak concentrations in plasma of patients and at a clinically relevant fPt fraction of 10%, CDDP was not toxic for human bone marrow cells in the CFU-GM assay, whereas it was toxic at fPt fractions of 50% and 90%. However, CBDDA was myelotoxic at a (clinically relevant) fPt fraction of 50%, and also at 75% and 90%. The use of different fPt fractions, produced by the incubation method described in this study, permits the study of platinum drugs in vitro while approximating in vivo conditions might be used to evaluate myelotoxicity of new platinum drugs prospectively. For CDDP and CBDDA the fPt fraction fPt determines cytotoxicity on tumor cells, and their different fPt fraction in patients account at least partly for their difference in myelotoxicity.

KEY WORDS: cisplatin; carboplatin; ultrafilterable platinum concentration; cytotoxicity; myelotoxicity; protein binding.

INTRODUCTION

Carboplatin (CBDDA) is a cisplatin (CDDP) analogue, developed in an attempt to reduce the toxicity profile of CDDP. CBDDA has similar activity as CDDP but shows reduced nephrotoxicity. Leukopenia and anemia and especially thrombocytopenia are common in patients treated with CBDDA but less pronounced with CDDP (1–3). For CBDDA, it was found that a decreased clearance of fPt, due to impaired renal function, was associated with higher reductions of platelets counts. Furthermore, correlations exist be-
tween the area under the curve (AUC) for fPt and the percentage change in the nadir platelet count (4, 5), indicating that fPt might be an important pharmacodynamic factor.

Although CDDP and CBDDA once bound to DNA, are thought to share similar pharmacodynamics, their binding kinetics differ (6). Both CDDP and CBDDA are activated by loss of the leaving group(s), chloride and 1,1-cyclo-
butanedicarboxylic acid respectively. Both drugs have a similar reactive intermediate which binds to proteins and DNA. CBDDA is much more stable than CDDP, with in-vitro degradation half lives in serum and plasma of 24-48 h and 2-3 h respectively (7-9). Protein binding increases with time to 40-
50% for CBDDA (10) and 90% for CDDP (11, 12) during the elimination phase. Intact CDDP and CBDDA do not bind to proteins in contrast to their degradation products. Therefore, the chemical reactivity is the rate-limiting step for plasma protein binding.

It is unknown why CDDP is essentially free of myelo-
toxicity, whereas it is dose limiting for CBDDA. In the current study we investigated the effect of the fPt fraction of CDDP and CBDDA on cytotoxicity in a human lung carcino-
ma cell line GLC4. Furthermore, we studied whether the occurrence of myelotoxicity of CBDDA is attributable to its high chemical stability and hence higher fPt fraction. Various fPt fractions of CDDP and CBDDA were compared and tested in-vitro on GLC4 and human bone marrow.

MATERIALS AND METHODS

Drugs and chemicals

CDDP (Platinol; Molecular weight = 300.0) and CB-
DDA (Paraplatin; Molecular weight = 371.3) were pur-
chased from Bristol Myers SAE, Spain and dissolved in sterile water.

Preparation of culture medium with different ultrafilterable functions

In initial experiments the relation between the percentage of protein binding in culture medium (without cells), at a concentration approximating the reported concentrations inhibiting cell survival by 50% (IC50) of the tumor cell line used (13), and incubation time was studied. For CDDP, 66 $\mu$L of a solution containing 0.5 mg CDDP/mL was added to 2.0 mL of fetal calf serum (FCS; Life Technologies, Paisley, Scotland) and incubated at $37^\circ$C for 0 h; 1 h; 4 h; 12 h; and 24 h. Incubation was terminated by adding 18.0 mL of RPMI 1640 medium (Life Technologies, Paisley, Scotland) resulting in a final concentration of 5.5 $\mu$M CDDP in RPMI 1640 me-
dium plus 10% FCS.

For CBDDA, 50 $\mu$L of a solution containing 10 mg CB-
DDA/mL was added to 2.0 mL of FCS and incubated at $37^\circ$C for 0; 24; 36; 48; 72 and 96 h. Incubation was terminated by adding 18.0 mL of RPMI 1640 medium resulting in a final concentration of 67 $\mu$M CBDDA in RPMI 1640 medium plus 10% FCS. Samples for total platinum (tPt) and fPt determi-
nations were taken immediately after termination of drug incubation and stored frozen until analysis. The relation be-
tween % fPt fraction and incubation time (h) was estimated.
by logarithmic regression analysis. The incubation time needed to achieve fPt fractions of 90%, 50% and 10% for CDDP and 90%, 75% and 50% for CBDCDA were calculated.

**Platinum (Pt) determinations**

Concentrations of tPt and fPt in the culture medium were determined by flameless atomic absorption spectrophotometry (FAAS) using a model AA1275 AAS with a GTA95 graphite furnace and an autosampler unit (Varian Techtron Pty. Ltd., Mulgrave, Victoria, Australia). Absorption was measured at 265.9 nm with a spectral band-width of 0.5 nm and deuterium background signal correction. The fPt samples were prepared by filtration over an Amicon Centrifer micropartition system provided with YMT membranes (Amicon, Oosterhout, The Netherlands) (MW > 30,000 Dalton) immediately following sampling. Samples were diluted with 4 M HNO₃. Samples of 10 μL were injected into the graphite furnace. Calibration curves were prepared with Pt chloride (BDH Chemicals Ltd., Poole, UK) in culture medium and diluted with 4 M HNO₃ and found linear (r = 0.999) from 0 to 3.0 mg Pt/L. The detection limit of this assay was 0.1 mg Pt/L. Each sample was injected in duplicate.

**Cell line**

GLC₄ is a human small cell lung carcinoma cell line (13) which was grown in RPMI 1640 medium supplemented with 10% FCS in a humidified atmosphere containing 5% CO₂ at 37°C.

**Chemosensitivity assay**

For cytotoxicity measurements on the tumor cell line, the Microculture Tetrazolium Assay (MTA) was used as described before (13). For GLC₄, the MTA has shown comparable survival curves for both CDDP and CBDCDA when compared with the clonogenic assay. Per well 5,000 cells of the GLC₄ cell line (doubling time 24 h) were incubated with CDDP concentrations ranging from 0.5-50 μM for either 4 h or 4 days, or with CBDCDA in concentrations ranging from 2.5-250 μM for 4 h or 4 days, in a total volume of 100 μL culture medium in microculture wells (96 well culture plates, Nunc, Gibco, Paisley, Scotland). For CDDP incubations 10%, 50% and 90% fPt fractions and for CBDCDA 50%, 75% and 90% fPt fractions were tested. To achieve these ultrafilterable fractions, CDDP and CBDCDA were incubated in FCS for periods as determined in the initial incubation experiment. For termination of the 4 h incubation period in the MTA, cells were washed three times with a mixture of equal amounts of HAM F12 medium and Dulbecco’s Modification of Eagle’s medium (HAM/DME: Flow Laboratories, Irvine, Scotland) plus 20% FCS and cultured in this medium. After a culture period of 4 days at 37°C in dark, the assay was performed as described previously (13). Controls consisted of media without cells (background extinction), and cells incubated in microculture wells with medium without the drug. Independent experiments were performed at least in threefold.

**Bone marrow assay**

Morphologically normal bone marrow cells were used and obtained after informed consent. Mononuclear cells (MNC) from human bone marrow aspirates were isolated by density-gradient centrifugation (Lymphoprep, Nycomed AS, Oslo, Norway). 2 × 10⁶ MNC were incubated for 4 h with various concentrations CDDP and CBDCDA in 1.0 mL RPMI 1640 medium supplemented with 10% FCS. For CDDP incubations with 10%, 50% and 90% fPt fractions and for CBDCDA 50%, 75% and 90% fPt fractions were tested. After incubation the cells were washed once by a tenfold excess of culture medium and centrifuged. After the supernatant was removed, cells were resuspended in fresh culture medium. The granulocyte-macrophage colony (CFU-GM) formation assay was chosen as a model for testing bone marrow toxicity. The in-vitro colony assay for the normal myeloid progenitor cell was assayed with 1.1% methylcellulose (Dow Chemical Co., Midland, MI), 20% fetal bovine serum (FBS; Hyclone, Logan, Utah), 1% deionized bovine serum albumin (BSA; Hyclone, Logan, Utah) and Iscoves medium (Flow, Rockville, MD), 2.5% supernatant of fetal lung fibroblast (FLF) activated with IL1β at 37°C and in dark and performed as described previously (14).

MNC (1 × 10⁶) were plated in methylcellulose, and on day 14 CFU-GM formation were counted by one individual with an inverted microscope. A CFU-GM contained more than 40 cells. Colonies were identified by their distinct morphologic appearance at 100× magnification. Two independent experiments were performed each in duplicate.

**Statistics**

The results were statistically analyzed using the two-sided Student’s t-test. Differences were considered significant at p-values <0.05.

**RESULTS**

**Preparation of culture medium with different ultrafilterable fractions**

The mean fraction ultrafilterable Pt (fPt:tPt) of CDDP and CBDCDA after various incubation times is given in Figure 1. After termination of the incubation by adding RPMI 1640 medium to the incubation mixture, the mean ultrafilterable fraction remains constant for at least 6 h (data not shown). Correlations between % fPt fraction and incubation time (h) were calculated by logarithmic regression analysis were % fPt fraction = 87× 0.1123t (r = 0.999) and % fPt fraction = 101× 0.9987t (r = 0.999) for CDDP and CBDCDA respectively. From these equations it was calculated that incubation times of 0, 4.9, and 19 h were needed for CDDP to have a 90, 50 and 10% fPt fraction respectively. For CBDCDA incubation times of 14, 35, and 82 h were required for a 90, 75 and 50% fPt fraction respectively.

**Chemosensitivity**

The chemosensitivity of GLC₄ cells was tested for the various fPt fractions of CDDP and CBDCDA. The corresponding IC₅₀ values (total Pt concentration in FCS + RPMI 1640 medium) are summarized in Table I. At a fPt fraction of 50%, the respective IC₅₀ values for GLC₄ at 4 h incubation with CDDP and CBDCDA were 6.0 ± 2.1 μM and 63 ± 9 μM,