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 (CBDCA) for cytotoxicity and myelotoxicity was studied in vitro. By incubating CDDP or CBDCA 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 $87^{e-0.1123t}$ ($r = -0.99$) and $101^{e-0.0087t}$ ($r = -0.99$) were determined for CDDP and CBDCA, respectively. Cytotoxicity in the human small cell lung carcinoma cell line GLC_4 and fPt fraction were closely related for CDDP ($r = 0.99$) and for CBDCA $r = 0.97$). However, at a similar fPt fraction the concentrations inhibiting cell survival by 50% (IC50) of CBDCA 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, CBDCA 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 CBDCA the 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 (CBDCA) is a cisplatin (CDDP) analogue, developed in an attempt to reduce the toxicity profile of CDDP. CBDCA has similar activity as CDDP but shows reduced nephrotoxicity. Leukopenia and anemia and especially thrombopenia are common in patients treated with CBDCA but less pronounced with CDDP (1–3). For CBDCA, 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 between 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 CBDCA once bound to DNA, are thought to share similar pharmacodynamics, their binding kinetics differ (6). Both CDDP and CBDCA are activated by loss of the leaving group(s), chloride and 1,1-cyclobutanedicarboxylic acid respectively. Both drugs have a similar reactive intermediate which binds to proteins and DNA. CBDCA 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 CBDCA (10) and 90% for CDDP (11, 12) during the elimination phase. Intact CDDP and CBDCA 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 myelotoxicity, whereas it is dose limiting for CBDCA. In the current study we investigated the effect of the fPt fraction of CDDP and CBDCA on cytotoxicity in a human lung carcinoma cell line GLC_4. Furthermore, we studied whether the occurrence of myelotoxicity of CBDCA is attributable to its high chemical stability and hence higher fPt fraction. Various fPt fractions of CDDP and CBDCA were compared and tested in-vitro on GLC_4 and human bone marrow.

MATERIALS AND METHODS

Drugs and chemicals

CDDP (Platinol; Molecular weight = 300.0) and CBDCA (Paraplatin; Molecular weight = 371.3) were purchased 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 μL 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°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 μM CDDP in RPMI 1640 medium plus 10% FCS.

For CBDCA, 50 μL of a solution containing 10 mg CBDCA/mL was added to 2.0 mL of FCS and incubated at 37°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 μM CBDCA in RPMI 1640 medium plus 10% FCS. Samples for total platinum (tPt) and fPt determinations were taken immediately after termination of drug incubation and stored frozen until analysis. The relation between % fPt fraction and incubation time (h) was estimated

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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 CBDCA 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 Centifree micropartition system provided with YMT membranes (Amicon, Oosterhout, The Netherlands) (MW> 30,000 Dalton) immediately following sampling. Samples were diluted with 4 M HNO_3. 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 HNO_3 and found linear (r= 0.99) 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

GLC_4 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% CO_2 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 GLC_4, the MTA has shown comparable survival curves for both CDDP and CBDCA when compared with the clonogenic assay. Per well 5,000 cells of the GLC_4 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 CBDCA 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 CBDCA 50%, 75% and 90% fPt fractions were tested. To achieve these ultrafilterable fractions, CDDP and CBDCA 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). 2×10^6 MNC were incubated for 4 h with various concentrations CDDP and CBDCA in 1.0 mL RPMI 1640 medium supplemented with 10% FCS. For CDDP incubations with 10%, 50% and 90% fPt fractions and for CBDCA 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 assessed 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 (1×10^5) 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 CBDCA 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= $87^{e-0.1123t}$ (r= -0.99) and % fPt fraction = $101^{e-0.0087t}$ (r= -0.99) for CDDP and CBDCA 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 CBDCA incubation times of 14, 35, and 82 h were required for a 90, 75 and 50% fPt fraction respectively.

Chemosensitivity

The chemosensitivity of GLC_4 cells was tested for the various fPt fractions of CDDP and CBDCA. The corresponding IC50s (total Pt concentration in FCS + RPMI 1640 medium) are summarized in Table I. At a fPt fraction of 50%, the respective IC50 values for GLC_4 at 4 h incubation with CDDP and CBDCA were 6.0 ± 2.1 μM and 63 ± 9 μM,

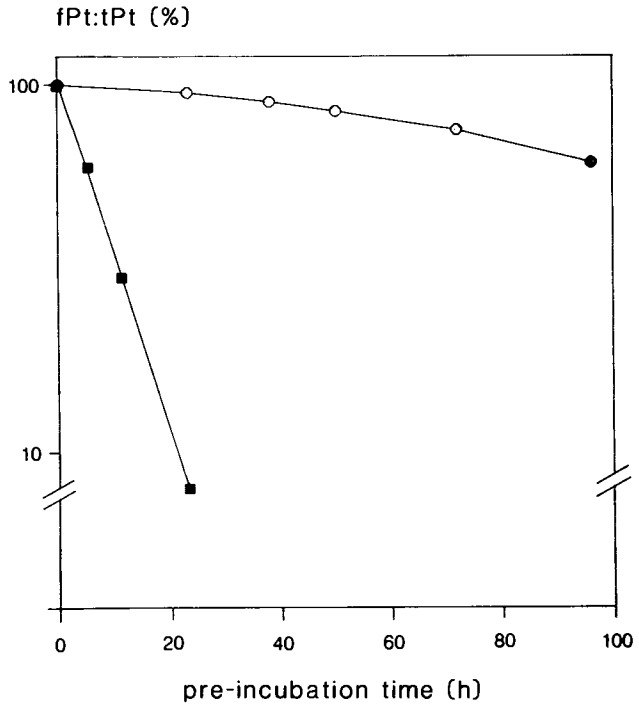

Figure 1. The correlation between mean ultrafilterable fraction (fPt-:tPt) of CDDP (■) and CBDCA (○) and incubation time (mean; n = 5).

whereas at a 90% fPt fraction they were 3.4 ± 0.4 μM and 62 ± 6 μM respectively. This means that at a similar fPt fraction and 4 h incubation, the IC50s of CBDCA exceed those of CDDP by a factor 10 and 18 respectively. With a 50% fPt fraction and continuous incubation (4 days), the IC50s for CDDP and CBDCA were 2.9 ± 0.1 μM and 15 ± 2 μM respectively. Under these circumstances CDDP is a factor 5.0 more cytotoxic than CBDCA.

The IC50 of CDDP at 4 h incubation decreased with increasing fPt fraction. This is also true for CBDCA at continuous incubation, except at a fPt fraction of 90%. The (linear) correlation coefficients between the IC50s and the reciprocal value of the fPt fraction for CDDP (4 h) and CBDCA (4 days) were 0.99 and 0.97 respectively.

Bone marrow toxicity was tested for the various fPt fractions of CDDP and CBDCA. MNC stimulated with FLF without drug supported 98 ± 6 (n = 12) and 295 ± 11 (n = 12) CFU-GMs/1x 10^5 cells for the respective aspirates. Corresponding IC50s (total Pt concentration in FCS + RPMI 1640 medium) are given in Table II. IC50 values decreased with increasing fPt fraction, for both DCCP and CBDCA. For CDDP, no IC50 value could be determined at a clinically relevant fPt fraction of 10%, because of low toxicity. A reduction in colony formation was noticed with CBDCA at all fPt fractions tested, including a clinically relevant fPt fraction of 50%. For CBDCA, IC50s were 233 ± 18 μM, 185 ± 9 μM an 155 ± 29 μM at fPt fractions of 50, 75 and 90% respectively. At similar fPt fractions the IC50 of CBDCA exceed those of CDDP by a factor 5.9 and 6.0 for 50 and 90% fPt fractions respectively. The linear correlation coefficient between the IC50s and the reciprocal value of the fPt fraction for CBDCA is 0.99.

DISCUSSION

In plasma or serum, protein binding of CDDP and CBDCA increase differently with time because of differences in chemical stability and pharmacokinetics. This results in 90% protein binding for CDDP and 40-50% for CBDCA during the elimination phase (10-12). Peak plasma levels after standard doses are between 10-40 μM for CDDP (11, 15) and 50-200 μM for CBDCA (16, 17). Gormley et al. (15) found that protein binding of CDDP destroys most of its cytotoxicity. Furthermore, studies of Egorin and Calvert revealed that the AUC of fPt of CBDCA is related to myelotoxicity (6-8, 10). Therefore, pharmacokinetic studies with platinum analogues preferably report on the kinetics of total platinum, fPt as well as on intact drug, instead of only total drug. In our study, designed to unravel the difference in myelotoxicity between CDDP and CBDCA, we found that in culture medium consisting of RPMI 1640 medium plus 10% FCS almost all CDDP and CBDCA is ultrafilterable. This situation does not correlate with the in vivo situation and might overestimate the effects of CDDP when compared with CBDCA in in-vitro assays. With the incubation procedure described, culture media can be prepared with fPt fractions corresponding to those in patients. As the intact drugs do not bind to proteins in contrast to their degradation products (18) and protein binding occurs instantaneous, the rate of protein binding reflects the rate of split off of the leaving groups. The estimated in-vitro degradation half lives in plasma or serum reported in the current study are in the same order as found by others (7-9, 18).

The chemosensitivity experiments of CDDP and CBDCA with the tumor cell line revealed, as expected, that cytotoxicity increases with increasing fraction fPt. A linear correlation was found between the reciprocal value of the fPt fraction and IC50 for both CDDP and CBDCA, suggesting that the fPt concentration determines drug cytotoxicity.

Table I. Cytotoxicity of various ultrafilterable fractions CDDP or CBDCA, expressed as IC50 (total Pt concentration (μM) in FCS + RPMI 1640 medium) tested in GLC_4 (mean ± SD, n = 3–4).

ultrafilterable Pt fraction	10%	50%	75%	90%
CDDP4 h	23 ± 4[a] μM	6.0 ± 2[c] μM	nd	3.4 ± 0.4 μM
CDDP continuous	nd	2.9 ± 0.1	nd	nd
CBDCA 4 h	nd	63 ± 9[d]	nd	62 ± 6
CBDCA continuous	nd	15 ± 2[b]	11 ± 2[d]	11 ± 2

p-value versus 90% ultrafilterable Pt fraction; a: <0.0005; b: <0.025; c: <0.05; d: not significant; nd = not determined.

Table II. IC50s (total Pt concentration (μM) in FCS + RPMI 1640 medium) for bone marrow cells measured as CFU-GM at various ultrafilterable fractions of CDDP and CBDCA (mean ± SD, n = 2 each in duplicate).

ultrafilterable Pt fraction	10%	50%	75%	90%
CDDP4 h	>100 μM	39 ± 1[b] μM	nd	26 ± 8 μM
CBDCA 4 h	nd	233 ± 18[a]	185 ± 9[c]	155 ± 29

p-Value versus 90% ultrafilterable Pt fraction; a: <0.0025; b: $p < 0.01$; c: not significant.

When the IC50s of CDDP and CBDCA (after 4 h incubaiton) with similar fPt fractions (50% and 90%) CDDP is a factor 10 and 18 more cytotoxic, respectively, than CBDCA. After continuous incubation (4 days) with an fPt fraction this factor is 5.

For bone marrow cells expressed as CFU-GMs at a 50% and 90% fPt fraction, CDDP was sixfold more toxic than CBDCA at 4 h incubation and at a similar fPt fraction. An explanation for this might be the lower rate of cellular accumulation and DNA platination of CBDCA. Hospers et al. (13) found in the same cell line that an eightfold higher drug concentration was needed with CBDCA to obtain similar cellular Pt contents after 4 h incubation. In order to obtain similar amounts of Pt-DNA cross links with CBDCA a fortyfold higher drug concentration was needed in comparison with CDDP after 4 h incubation.

For 4 h incubations with CBDCA, no differences in survival were found for fPt fractions of 50 and 90%. This might also be explained by the slow rate of cellular accumulation and DNA platination with CBDCA which makes it difficult to detect differences after short incubation periods. With a longer incubation period of 4 days significant differences were found between the IC50 of CBDCA at a 50% fPt fraction versus a 90% fPt fraction, but not between the IC50s of CBDCA 75% versus 90%. When IC50s of 4 days incubation with CDDP or CBDCA at a 50% fPt fraction are compared, the IC50 of CBDCA still exceeds that of CDDP (fivefold) but the decrease of the difference supports the role of intracellular phenomena. However, even when differences in cellular accumulation effects are minimized by using continuous incubations, CDDP appears to be more toxic than CBDCA and thus differences in cellular accumulation cannot fully explain their differences in cytotoxicity. In these cases the rate of DNA platination might play a determining role.

CDDP was shown to be more toxic to normal bone marrow cells when compared to CBDCA (19, 20), a finding not corresponding with the clinical experience, however, protein binding of the drugs was not taken into account. In the current study, CDDP with a fPt fraction of 10% and at a clinically relevant concentration did not show substantial toxicity in the in-vitro colony assay, whereas similar concentrations at the higher fractions fPt showed considerable toxicity. For CBDCA, all fPt fractions showed substantial toxicity in the FGU-GM assay at clinically relevant concentrations.

The mono- or diaqua intermediates, which are formed in solutions containing CDDP and CBDCA, might play an important role in both antitumor effect and toxicity. However, in the current study the influence of these metabolites was not evaluated.

It is concluded that, the use of different fPt fractions prepared by the technique described in this study, offers the opportunity to study platinum drugs in vitro in a more clinically relevant way. Furthermore, although CDDP shows potential toxicity to bone marrow cells in the CFU-GM assay at high fPt fractions, it is not pronounced at a 10% fPt fraction in concentrations usually found in patients. For CBDCA, in these circumstances considerable toxicity was found. The difference in protein binding of CDDP and CBDCA in patients is at least partly responsible for their difference in myelotoxity.

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