Development and Application of a Simple Assay to Quantify Cellular Adducts of Platinum Complexes with DNA

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INTRODUCTION

Platinum(II) complexes play an important role in the treatment of various malignancies. Since the early eighties, cisplatin (*cis*-diamminedichloroplatinum(II)) has been widely used as a key drug in the treatment of solid tumors (1). Carboplatin (*cis*-diammine(1,1-cyclobutane-dicarboxylato)platinum(II)) was developed in the late eighties showing less severe kidney damage but cross resistance in cisplatin-resistant cell lines (1). Two very promising compounds overcoming cisplatin resistance, oxaliplatin ([*trans*-1R,2R-diaminocyclohexan]-oxalatoplatinum(II)) and lobaplatin ([*trans*-1,2-bis(aminomethyl)cyclobutane]-lactatoplatinum(II)), are currently being investigated in clinical trials (2).

The cytotoxic activity of the platinum (Pt) complexes is thought to be due to the substitution of the leaving groups and the formation of mono- or bifunctional adducts with DNA (3). Despite the same biological target for cytotoxicity, the extent of Pt-DNA adduct formation differs among complexes, patients and cell type. Since tumor tissue is not easily available, the amount of adducts in leukocytes has been considered as surrogate for the amount of adducts in tumor tissue (4). In addition, the adduct formation is independent from the relative amount of lymphocytes, granulocytes, and monocytes (5). A differentiation of white blood cells is hence not required. Clinical investigations in patients with ovarian or testicular cancer suggest that after platinum chemotherapy the amount of adducts formed correlates with tumor response (6). In order to isolate DNA from cells, several methods are used, e.g., CsCl gradient centrifugation (4), or ion-exchange chromatography after digestion (7). The Pt-DNA adducts can be quantified either by a ^{32}P-postlabeling method (7), an antibody/immunological assay (8), or an AAS method (5). The described procedures are either time consuming, laborious or not suitable for the determination of adducts from different platinum complexes. We therefore developed a simple, rapid, and versatile method that can be used in *in vitro* experiments as well as in the clinical situation. It was applied to investigate Pt-DNA adduct formation using buffy coat and leukocytes of tumor patients.

MATERIALS AND METHODS

Reagents

Cisplatin and carboplatin were purchased from Sigma (Deisenhofen, Germany), oxaliplatin was kindly donated by Debiopharm (Lausanne, Switzerland) and lobaplatin by ASTA Medica (Frankfurt/M., Germany). The solvent of DNA, Tris/EDTA-buffer, consisted of 10 mM Tris·HCl, titrated to pH 9.3, and 1.0 mM di-sodiumethylenediaminetetraacetate. PBS solution was a mixture of 0.14 M NaCl, 6.5 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4, and 2.7 mM KCl. All reagents were of analytical grade (Merck, Darmstadt, Germany).

Cell Isolation

Leukocytes from buffy coat (a leukocyte concentrate of healthy volunteers, German Red Cross, Germany) or whole blood samples of patients were separated from other blood components by density gradient centrifugation. 5 ml of the sample were added to 4 ml of separation medium (Ficoll-Paque™, Pharmacia Biotech, Uppsala, Sweden or Polymorphprep™, Life Technologies, Eggenstein, Germany). After centrifugation (30 min, 400 g) the cell layer formed was harvested and washed three times with PBS solution.

DNA Isolation and Purification

Leukocyte DNA was separated from Pt not bound to DNA and other components by solid phase extraction in 4 steps (Fig. 1). Approximately 10^7 cells of the cell pellet were digested by protease (QIAGEN, Hilden, Germany). The lysate was applied to a silica column (QiAamp™, QIAGEN) where the DNA was selectively bound using a caotrophic buffer with pH < 9. The residual cell components were removed in three wash spins (1–2 min; 6,000–10,000 g). After a 5 min incubation at 70°C, DNA was eluted with Tris/EDTA-buffer with pH > 9 and low salt concentration.

Quantification of Pt-DNA Adducts

Amount and purity of nucleotides were determined by UV spectrometry measuring the absorption at 260, 280, and 320 nm (A_{260}, A_{280}, A_{320}) using a UVIKON 930™ photometer (Kontron, Neufahrn, Germany) (9). The following purity requirements had to be met: $A_{320} < 0.010$ and A_{260}/A_{280} between 1.7–1.9. DNA concentration was calculated based on the Lambert-Beer law according to: $C_{DNA} = 50\ (\mu g/ml) \cdot A_{260}$.

The platinum content of the samples was assessed with a TJA Unicam PU9100X™ flameless atomic absorption spectrometer (TJA Unicam, Offenbach, Germany) using a matrix-adapted temperature program and deuterium background correction as described elsewhere (Kloft *et al.*, submitted for publication). Within-day and between-day precision were determined

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ABBREVATIONS: AAS, atomic absorption spectrometry; conc, concentration; Pt; platinum.

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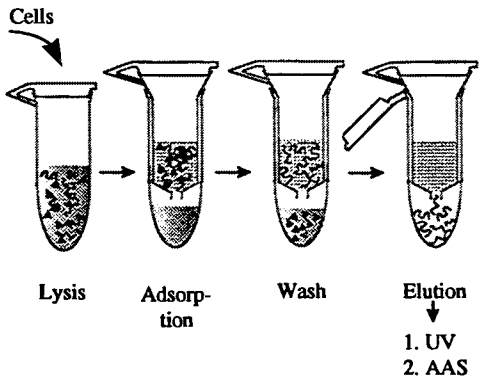

Fig. 1. Leukocyte DNA isolation and purification by solid phase extraction (ζ: DNA; ▲: other cell component).

by repeated injection (n = 10 or 6, respectively) of four different concentrations. Selectivity of platinum analysis was assessed by analyzing all buffers for potential interference with the platinum signal. Based on the results of the UV and AAS determination, the platinum-nucleotide ratio was calculated using the relative molar masses of platinum (195 g/mol) and nucleotides (mean: 330 g/mol).

Incubation with Leukocytes

For the *in vitro* incubation of therapeutically relevant Pt concentrations with leukocytes over a maximum of three days, a sufficient number with an appropriate life span was required. Among the leukocytes, mononuclear lymphocytes exhibit the longest one of at least seven days. These cells were yielded from buffy coat by Ficoll-Paque™ density centrifugation and incubated with cisplatin, carboplatin, lobaplatin, or oxaliplatin in a standardized experimental design with equimolar Pt concentrations. The Pt concentrations ranged from 12.8–102.6 μM and incubation periods were up to 72 h. In a 75 ml flask the appropriate volume of the stock solutions of the complexes (c = 1 mg Pt/ml), immediately prepared before incubation, was mixed with 30 ml of culture medium (RPMI 1640-Medium™ without phenolred, Biochrom, Berlin, Germany, fetal calf serum (10%), and a penicillin-streptomycin solution (0.2% both), Boehringer Mannheim, Germany). Leukocytes were added to obtain a final concentration of $2 \cdot 10^6$ cells/ml. The cell suspensions were incubated in a Cytoperm™ incubator (Heraeus, Hanau, Germany) at 37°C, approximately 100% humidity and 5.0% CO_2. Two sample aliquots were collected from each flask after the incubation periods, and the platinum-nucleotide ratios were determined.

Clinical Investigation

The method was also applied to determine the Pt-DNA adduct concentration in leukocytes of 5 germ cell cancer patients who had received either cisplatin (27–33 mg Pt corresponding to 20 mg/m² cisplatin) or high-dose carboplatin (462 mg Pt corresponding to 500 mg/m² carboplatin) as 1 h iv infusions. 4–7 hours after the infusion, a 5 ml (cisplatin) or 15 ml (carboplatin) peripheral blood sample was drawn. DNA containing cells were isolated with Polymorphprep™ to increase the number of cells. Apart from mononuclear lymphocytes, shorter living polymorphic nuclear granulocytes were also harvested. In contrast to the *in vitro* incubation experiments, the cells had not to remain viable for a longer period of time. After drawing the sample, the platinum-nucleotide ratio was quantified immediately.

In addition, heparinized plasma was obtained by immediate centrifugation of blood samples. Subsequently, 1 ml of plasma was centrifuged (20 min, 2000 g) through an ultrafiltration membrane (Centrifree™, Millipore GmbH, Eschborn, Germany) to determine the platinum concentration not bound to macromolecules.

RESULTS AND DISCUSSION

Platinum-DNA adduct formation was quantified after exposing cellular DNA to different platinum complexes *in vitro* or *in vivo* by a three step procedure: cell isolation, DNA isolation, and quantification of DNA and platinum.

Method Development

Solid phase extraction for isolation and purification of DNA-adducts has not been employed before. The yield of DNA with sufficient purity succeeded by fractional DNA elution from the column. Two elution steps were found to be sufficient. A third elution step increased the yield only marginally and this eluate did not meet the purity requirements: A_{260}/A_{280} was > 1.9. DNA could be rapidly and efficiently purified for direct quantification within only 20 min.

To obtain the platinum-nucleotide ratio, the amount of DNA was first quantified by non-destructive UV spectrometry, followed by the AAS determination of platinum. Using this sequence the total sample volume was still available for platinum analysis.

The AAS analysis method for platinum showed a linear range of 0.01–40 μg Pt/ml, a within-day precision of 1.1–8.6%, and a between-day precision of 2.6–7.3%. None of the buffers used for the isolation method interfered with the platinum signal. The spectrogram of a representative Pt-DNA adduct sample, analyzed within 5 min, is illustrated in Fig. 2.

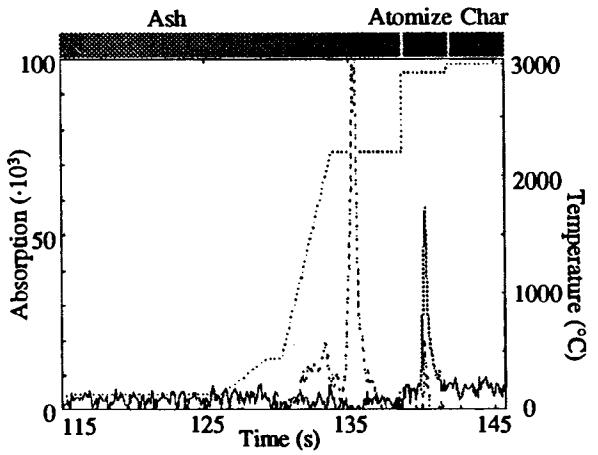

Fig. 2. Spectrogram of a platinum-DNA adduct sample (ash, atomize and char phase); dotted line: temperature program, dashed line: deuterium background signal, straight line: platinum signal.

Incubation with Leukocytes

Figure 3A shows the adduct formation in relation to the platinum concentration used after three days of incubation and Fig. 3B the adduct concentration depending on the incubation period. For all complexes, a time- and concentration-dependent Pt-DNA adduct formation was observed: the longer the incubation period or higher the platinum concentration incubated, the larger the platinum-nucleotide ratio. However, the extent and kinetics of adduct formation varied considerably among the four complexes. The 8-39fold lower adduct formation of carboplatin compared to cisplatin is most likely due to the higher stability of the dicarboxylate leaving group compared to the two rather weakly co-ordinated and readily substituted chloride ligands. Our result is in agreement with others obtained from isolated cell lines or animal investigations (10,11,12).

Oxaliplatin resulted in slightly lower adduct ratios than cisplatin, irrespectively of platinum concentration and incubation time. The highest adduct concentrations, 1.5-fold higher compared with cisplatin, were achieved by lobaplatin after a three day incubation of 102.6 μM Pt. After one day incubation, however, lobaplatin exhibited lower adduct ratios than cisplatin and oxaliplatin. Incubating non-cellular, histone-free calf thymus DNA with cisplatin, oxaliplatin, and lobaplatin over 48 h, Saris et al. found the following order in adduct formation: cisplatin > lobaplatin > oxaliplatin (11). Although the results cannot be directly compared with our results in leukocytes, these authors found a similar difference of adduct formation kinetics as we did using cellular DNA. These results suggest that by increasing the incubation period lobaplatin can bind more extensively to DNA than the other platinum complexes. This may be attributed to the different amine ligands of lobaplatin which may facilitate penetration to the target and/or inhibit cellular DNA repair processes. Further investigations regarding the mechanism of hydrolysis and binding will have to be performed to clarify this issue.

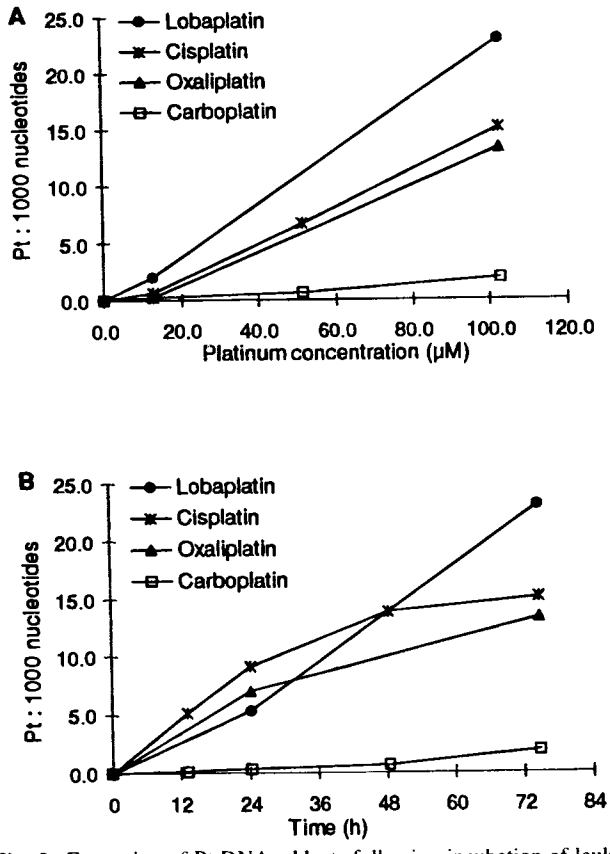

Fig. 3. Formation of Pt-DNA adducts following incubation of leukocytes with A) different concentrations of platinum complexes for 72 hours B) platinum complexes (102.6 μM) for different incubation periods.

The results obtained from the *in vitro* experiments may have important clinical implications. There is an inverse relationship between the platinum-nucleotide ratio in leukocytes after 72 h incubation and the doses used in chemotherapy. Related to lobaplatin, the platinum-nucleotide ratio decreased in the following order (in brackets a factor is given indicating how much lower the adduct formation is compared to lobaplatin): lobaplatin (1) > cisplatin (1.5) > oxaliplatin (1.8) ≫ carboplatin (11.5). From the clinically used doses of 50, 100, 130, and 400 mg/m^2 respectively, the following molar ratios indicate the increase in dose compared to lobaplatin: 1 : 2.5 : 2.6 : 8.7. *In vitro* experiments may hence provide valuable information for defining the dosage of new platinum complexes in an early phase of preclinical drug development.

Clinical Application of the Method

The developed method was applied to peripheral blood of germ cell tumor patients following cisplatin or carboplatin treatment. In Table I the corresponding platinum doses as well as plasma and ultrafiltrate platinum concentrations at the sampling times are summarized. Whereas the (platinum) dose of carboplatin was approximately 15fold higher compared to cisplatin, there was an about 100fold difference in ultrafiltrate Pt concentration due to the lower protein binding of carboplatin. The platinum-nucleotide ratios measured ranged from 1–12:10^5. Despite considerably lower ultrafiltrate concentrations, higher platinum-nucleotide ratios were found after cisplatin treatment demonstrating that the higher reactivity of cisplatin can be also measured *in vivo* in the leukocytes of tumor patients.

In contrast to an AAS method described by Reed *et al.* (4), our method needs only approximately 5 ml of blood after cisplatin and 15 ml after carboplatin treatment which is crucial for its feasibility in clinical studies. The method described by Welters *et al.* (7) using the more sensitive ^{32}P-postlabeling method requires laborious sample pretreatment (digestion to

Table I. Platinum-DNA Adduct Formation in Leukocytes of Patients After Platinum-Containing Chemotherapy

Platinum dose (mg)	Platinum complex	Sampling time after influsion (hours)	Platinum concentration (μg/ml) in		Platinum-nucleotide ratio
			plasma	ultrafiltrate	
30	Cisplatin	3.5	0.806	0.026	2:10^5
33	Cisplatin	3.5	0.507	<0.01[a]	6:10^5
28	Cisplatin	4	0.369	0.012	10:10^5
27	Cisplatin	5	0.827	0.026	12:10^5
462	Carboplatin	7	5.250	2.073	1:10^5

[a] Limit of quantification.

nucleosides, separation by cation-exchange chromatography, deplatination by CN^-, ^{32}P-postlabeling, separation and quantification) and has been applied to quantify adducts after cisplatin therapy only. Due to the very low adduct formation of carboplatin it is obvious why so little information is available from patients. Moreover, the determined *in vivo* platinum-nucleotide ratios of $1-12:10^5$ in leukocytes were markedly lower than the corresponding *in vitro* adduct ratios. This was also reported by Parker *et al.* in leukocytes after carboplatin administration (13). The *in vitro/in vivo* difference can be attributed to the fact that in the *in vivo* situation no steady-state was present. Other factors (e.g., distribution processes) may also contribute to this difference. In further clinical studies pharmacokinetics of (ultrafilterable) platinum must hence be taken into account.

In conclusion, our method allows the pharmacological screening of new platinum complexes at an early stage in the development of drugs with regard to extent and kinetics of Pt-DNA adduct formation. It also forms the basis for investigating the Pt-DNA adduct formation of different platinum complexes as a pharmacodynamic parameter in clinical studies. Adduct levels in tumor patients could be determined at different times after administration. Moreover, *ex vivo* incubations of platinum complexes with blood of tumor patients drawn prior to chemotherapy could be performed in order to predict *in vivo* adduct formation and tumor response. Future investigations should reveal the potential of this pharmacodynamic parameter to optimize platinum-based chemotherapy.

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