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Therapy-induced carboplatin–DNA adduct levels in human ovarian tumours in relation to assessment of adduct measurement in mouse tissues

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ABSTRACT

Despite an increasing understanding of the molecular mechanisms by which platinum drug DNA adducts interact with cellular processes, the relationship between adduct formation in tumours and clinical response remains unclear. We have determined carboplatin–DNA adduct levels in biopsies removed from ovarian cancer patients following treatment. Reliability of DNA adduct measurements in tissues samples were assessed using experimental animals. Platinum–DNA adduct levels were measured using inductively coupled plasma mass spectrometry (ICP-MS) and plasma drug concentrations determined by atomic absorption spectrometry (AAS). Adduct levels in tissues and plasma pharmacokinetics were determined in Balb/c mice exposed to platinum drugs. Comparisons of adduct levels in tumour and normal tissue were made in nu/nu mice carrying human neuroblastoma xenografts. At 30 min post-cisplatin administration, adduct levels in DNA from kidney and liver were approximately 10- and 6-fold higher than spleen or tumour. By 60 min, levels in liver and kidney, but not spleen or tumour, had fallen considerably. Carboplatin showed high adduct levels only in kidney. Adduct levels in tumour xenografts were comparable to those induced *in vitro* with similar drug exposures. In clinical samples removed 6 h after drug administration, adduct levels ranged from 1.9 to 4.3 and 0.2 to 3.6 nmol Pt/g DNA for tumour biopsies and peripheral blood mononuclear cells, respectively. No correlation was apparent between these two data sets. The present results demonstrate that reliable measurements of adducts in clinical tumours are feasible. Future results should provide insight into drug resistance.

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1. Introduction

Cisplatin and carboplatin are well-established agents used clinically against a broad range of malignancies, including testicular, ovarian and neuroblastoma [1]. The more recent introduction of oxaliplatin into clinical practice has extended the role of this class of drugs [2].

Platinum drugs exert their primary cytotoxic effects through covalent modification of cellular DNA [3,4]. However, only 5–10% of the total binding of platinum is to DNA [5], and additional effects related to the reaction with proteins and other non-DNA associated molecules seem plausible. Despite an increasing understanding of the molecular mechanisms by which platinum–DNA adducts interact with cellular processes [6–8] we still have a relatively poor understanding of the reasons for inter-patient variation in clinical response and toxicity. In particular, little is known about the levels of platinum–DNA adducts formed in tumour tissue and the relationship between this parameter and clinical outcome.

Measurement of drug–DNA adducts formed in tumours could provide an important biomarker in patients since it distinguishes variation in the extent of drug–DNA interaction from variation in cellular response to damage. Clinical studies investigating adduct levels (i.e. the ratio of drug–DNA adducts to total DNA) and relationships to response and/or pharmacokinetics have overwhelmingly relied on peripheral blood cells (PBCs) as a convenient source of DNA. Several investigations have indicated associations between drug–DNA adduct levels in non-tumour tissues and clinical response and toxicity [9–14]. *A priori*, correlations between drug–DNA adducts in normal cells and tumour response is likely to mainly reflect pharmacokinetic variation, since alternatives might imply correlation between normal blood cells and tumour tissue in factors such as drug uptake or DNA repair. In a dose-escalation

Abbreviations: AAS, atomic absorption spectrometry; AUC, area under the curve; C.V., coefficient of variation; *i.v.*, intravenous; *i.p.*, intraperitoneal; *i.u.*, international units; ICP-MS, inductively coupled plasma mass spectrometry; LC, liquid chromatography; PBCs, peripheral blood cells; PPB, parts per billion (by mass); PPT, (by mass) parts per trillion; *s.c.*, subcutaneous; S.D., standard deviation; S.E., standard error of the mean.

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setting, platinum–DNA adduct levels in PBCs were positively related to dose and area under curve (AUC) for carboplatin [15]. However, two later pharmacokinetic studies with cisplatin [16] or carboplatin [17] demonstrated a poor correlation between active drug AUC and platinum–DNA adduct levels in PBCs. These indicated that inter-individual variation in factors other than pharmacokinetics can significantly affect adduct levels in PBCs.

Of great relevance would be an understanding of the relationship between adducts levels achieved in blood cells and those achieved in solid tumours. However, measurements on tumour biopsies are challenging due to inherently low adduct levels and difficulties in obtaining biopsies at appropriate early times after drug administration, when data would be most informative. Recent important studies used a post-labelling assay to measure levels of drug–DNA adducts in biopsies of solid tumour and blood cells during cisplatin-based chemotherapy [18,19]. Drug–DNA adduct levels were found to be 2–5 times higher in tumour biopsies, but no correlation was observed between drug–DNA adduct levels in tumour tissue and PBCs. These findings further indicate that drug–DNA adduct levels in PBCs may not provide a useful biomarker for patient response to platinum-based chemotherapy. Factors other than the extent of initial DNA-adduct formation may have a significant impact on tumour response. However, many of the mechanisms that determine access of platinum-based drugs to their molecular target in patients are very poorly understood. Direct investigations of adduct formation should provide important insights into clinically relevant processes.

Immunological and post-labelling methods [6,15,16,20–23] are complex techniques but have the advantage of specifically quantifying platinum in the form of DNA adducts. Measurement of adducts by quantification of total platinum bound to DNA by atomic absorption spectrometry (AAS) has been used for several key investigations [24–26]. However, the lack of sensitivity of AAS has led to its replacement by inductively coupled plasma mass spectrometry (ICP-MS) for analysis of low levels of platinum present in small tissue samples and following clinical drug exposures [27–32]. ICP-MS offers a high degree of quality control of the data obtained through measurement of each of the four most abundant natural isotopes of platinum and internal standardisation [29,32]. We have applied this method to the analyses of total drug–DNA adduct levels in clinical tumour biopsies.

In the present report we initially describe analyses of platinum–DNA adduct formation in mouse tissues and human tumour xenografts. The aim of these initial studies was to establish the sensitivity and reliability of ICP-MS for measurement of DNA adduct levels in solid tissues and to determine the extent of variation in adduct level with sampling time after drug administration. The same methods were applied to a small number of accessible ovarian tumour biopsies obtained after administration of carboplatin, with plasma pharmacokinetics and blood cell adduct levels also determined. The numbers of samples for which we were granted ethical approval in this study was too small to permit reliable conclusions to be drawn about relationships between adduct levels in blood cells, tumours and therapeutic responses. A major aim of the present work was to demonstrate the reliability of DNA adduct measurements for solid tissue biopsies using the approaches chosen. This will justify future studies involving larger numbers of patients and possibly more intrusive sampling methods.

2. Materials and methods

2.1. Cell lines

The neuroblastoma cell lines IMR 32 [33] and SH-SY5Y [34] were cultured in RPMI 1640 culture medium with Dutch modification, supplemented with 10% (v/v) foetal bovine serum and antibiotics at 37 °C/5% CO_2. All cell culture reagents were obtained from Invitrogen, UK.

2.2. Reagents and drug preparations

Unless stated otherwise, all chemicals were of analytical grade or higher and were obtained from Sigma Aldrich, UK. Prior to *in vitro* experiments, cisplatin was diluted into culture medium immediately after dissolving in DMSO and carboplatin (Johnson Matthey, UK) was dissolved directly in culture medium. For *in vivo* experiments cisplatin (0.5 mg/ml) and carboplatin (10 mg/ml) were dissolved in water and sterile NaCl solution (4.5 M) was added to give a final NaCl concentration of 140 mM immediately prior to administration.

2.3. Patient details, treatment schedule and sampling times

Patients who had relapsed several years after adjuvant carboplatin treatment for ovarian cancer were included in this study (Table 1). Patients were selected for inclusion that had recurrent tumour which was accessible for biopsy; in three cases this was vaginal vault relapse and in one case this was tumour within the anterior abdominal wall, amenable to transcutaneous biopsy. During the present study, all patients received paclitaxel and carboplatin. All samples were collected according to a protocol approved by the local committee of the UK National Research Ethics Committee and were stored in accordance with the UK Human Tissue Act. At 45 min after administration of a 3 h *i.v.* paclitaxel infusion, carboplatin was infused *i.v.* over a 30 min period at a dose adjusted for renal function to target an AUC of 5.5 mg/ml [35]. For pharmacokinetic analyses, samples of peripheral blood were collected, from the opposite arm to that used for drug infusion, via a catheter which was flushed with saline after each use. Samples were collected pre-infusion and at 15, 30, 60, 90, 150 and 210 min after the start of carboplatin infusion. For isolation of PBCs, 20 ml blood samples were collected pre-infusion and at 210 min after the start of infusion. Biopsies were removed from accessible ovarian tumour at approximately 210 min after the start of carboplatin infusion and were immediately placed in vials in dry-ice for up to 18 h before transfer to −80 °C. Additional samples were processed in formalin for histology.

2.4. Tumour model and collection of experimental samples

Mice (Charles River, UK) were maintained and handled in isolators under specific pathogen-free conditions, according to the UKCCCR Guidelines for the welfare of animals in experimental

Table 1
Characteristics of patients/tumours at diagnosis and initial therapy.

	Age at diagnosis	Chemotherapy	Cancer type	Stage	Grade	Outcome of initial therapy	Age at relapse
Patient 1	47	Carboplatin	Serous cell	1C	1	Complete	52
Patient 2	74	Carboplatin	Endometrioid	1C	3	Complete	76
Patient 3	65	Paclitaxel/carboplatin	Clear cell	3C	3	Optimal	68
Patient 4	62	Carboplatin	Clear cell	1C	3	Optimal	68

neoplasia (second edition 1997). Balb/c mice were used to define plasma pharmacokinetics and adduct formation in normal tissues. CD1 *nu/nu* mice were used as hosts for xenografts of the human neuroblastoma tumour lines IMR 32 and SH-SY5Y. These cells were grown *in vitro* and suspensions (2×10^7 cells in 50 μl of PBS), were injected *s.c.* into the right flank. Approximately 80% of animals developed tumours which were visible within approximately 3 weeks. Mice were killed before the tumour reached more than 5% of the total body weight (approximately 6 weeks).

Mice (3 per group) were treated with single *i.p.* bolus doses of cisplatin or carboplatin when tumours reached an average volume of approximately 350 mm^3. At several time-points animals were bled under terminal anaesthesia (0.75 mg/kg fentanyl citrate, 25 mg/kg fluanisone and 12.5 mg/kg midazolam, 10 ml/kg *i.p.*), and the tumour, liver, kidney and spleen removed. Tissues samples were rinsed in 0.9% NaCl solution, frozen immediately in liquid nitrogen and stored at −80 °C until analysed. Blood was removed by cardiac puncture and collected into heparinised tubes (10 *i.u.*/ml).

2.5. Preparation of patient blood samples for determination of pharmacokinetic parameters

Blood samples, collected in lithium heparinised tubes, were immediately centrifuged (20 °C, 1200 × *g*, 10 min) to separate plasma. A 1 ml aliquot of plasma was transferred to an Amicon Centrifree micro-partition unit (MW cut-off 30 kDa, Millipore, UK) and centrifuged (20 °C, 1900 × *g*, 15 min) to obtain ultrafiltrate. Aliquots of whole plasma and ultrafiltrate were immediately frozen and stored at −80 °C.

2.6. Preparation of patient blood samples for measurement of adduct levels

Blood samples (20 ml) were collected into lithium heparinised tubes and immediately transferred to Leucosep tubes (Greiner Bio-One, UK) containing 15 ml sterile Lymphoprep (Axis Shield, Norway). Following centrifugation (20 °C, 1000 × *g*, 15 min), plasma was removed and mononuclear cells were transferred to a clean tube containing 20 ml of PBS at 4 °C. Following further centrifugation (4 °C, 1000 × *g*, 5 min) and washing twice with PBS, the final cell pellet was stored in dry-ice for up to 18 h before transfer to −80 °C prior to DNA extraction.

2.7. Clinical sample handling

As platinum levels in patient samples were predicted to be low, precautions were taken to ensure that external platinum was not introduced into the sample preparations. All sample handling was undertaken in a room in which the laminar flow cabinet and other equipment was kept free from use for handling platinum-based drugs. Water was from "Ultrapure" apparatus (Elga, UK) which was shown to give platinum levels below 0.1 parts per trillion (PPT). Nitric acid was ultrapure grade (UpA, Romil, UK) with certified < 0.1 PPT platinum.

2.8. DNA extraction

DNA was extracted from cell pellets or tissue samples using genomic DNA extraction kits ('for blood and cell culture', Qiagen, UK) according to the manufacturer's protocol, except for the addition of sonication prior to protease and RNAase digestion. Samples of solid tissues were part-homogenised using disposable plastic pestles (Anachem, UK) in buffer G2 (Qiagen, UK). Further homogenisation, without metallic contamination, was achieved by sonicating the vial in cooled water in a cup horn (VC-600 ultrasonic processor, Sonics and Materials, USA) at maximum power for 2 min. Samples were further processed according to the appropriate Qiagen protocol. After isopropanol precipitation, DNA was dissolved in water and aliquots removed and diluted where appropriate for measurement of concentration by Hoechst dye assay [36] for experimental samples or using an ND-1000 spectrophotometer (NanoDrop, USA) for clinical samples. The accuracy of the latter was verified by analysis of a quality control DNA sample (50 μg/ml) with every sample batch. With each batch of clinical blood cell and tumour samples, aliquots of two standard tissue homogenates prepared from pooled mouse kidneys were also processed; one from control CD-1 mice to check the background levels of Pt and the other from mice killed 60 min after *i.p.* injection of carboplatin (25 mg/kg) as a quality control test.

2.9. Analysis of platinum in plasma by AAS

Platinum in plasma ultrafiltrates was measured using a AAnalyst 600 graphite furnace spectrometer (PerkinElmer, UK) as described previously [16]. All samples were analysed in duplicate. Duplicate values were within 15% of each other in all cases. Intra- and inter-assay coefficients of variation for a quality assurance sample had to be <10% for an assay to be valid. The limit of detection for AAS was 0.1 μg/ml for cisplatin and carboplatin standards. AUC values of drug concentration in plasma ultrafiltrates versus time were calculated using the trapezoidal rule.

2.10. ICP-MS analysis of platinum in experimental DNA samples

DNA from experimental samples was prepared for ICP-MS by hydrolysis in 3.5% (w/v) nitric acid overnight at 70 °C and then analysed as described previously [28]. In brief, samples were delivered via a standard cross-flow nebuliser and Scott-type double-pass spray chamber into a Sciex Elan 6000 ICP-MS (PerkinElmer, UK). RF power was generally 1150 W. Nebuliser gas flow rates varied between 0.8 and 1.0 l/min and were optimised to keep the production of CeO^+ less than or equal to 3% of the total Ce^+ signal. Three isotopes of Pt were monitored, ^{194}Pt (32.97% abundance), ^{195}Pt (33.83% abundance) and ^{196}Pt (25.24% abundance) to evaluate possible isobaric interferences. Background Pt levels in 5% (w/v) nitric acid and Qiagen elution buffer were 0.1 PPT and 1.8 ± 1.3 (S.D.) PPT respectively. Solutions of DNA (50 μg/ml) extracted from cell lines and tissues which had not been exposed to platinum drugs contained Pt at levels corresponding to 0.16 ± 0.16 (S.D.) and 0.21 ± 0.21 (S.D.) nmol Pt/g DNA, respectively.

2.11. ICP-MS analysis of platinum in clinical DNA samples

DNA samples from clinical specimens were hydrolysed as for experimental samples. Thallium (Tl) was added (final concentration 1 part per billion (PPB)) to all samples and standards to act as an internal reference to enable monitoring of instrument performance and to correct for variations in matrix effects and in the rate of sample entry into the plasma torch. The instrument used was an Element2 Magnetic Sector Field ICP-MS (ThermoScientific, USA). Measurements were made of ^{194}Pt, ^{195}Pt, ^{196}Pt, ^{203}Tl, and ^{205}Tl. Possible mass interference by ^{143}Nd, ^{161}Dy, ^{178}Hf and ^{179}Hf was found to be less than 1% of the detected Pt signal. During analysis of each series of samples, repeat 5 PPT standards and blank nitric acid solutions were incorporated after every ten samples, to monitor instrument drift.

2.12. Analysis of ICP-MS data for clinical samples

The ratio of ^{194}Pt:^{205}Tl, ^{195}Pt:^{205}Tl and ^{196}Pt:^{205}Tl were calculated for all blanks, standards and samples. Standard curves

for these ratios versus platinum standard concentration were generated for each batch of samples and used to calculate the concentration of each platinum isotope in each sample. The average background level of platinum in a series of DNA samples extracted from samples of PBCs and solid tissue biopsies not previously exposed to platinum drugs were determined. The overall mean platinum concentration was 0.8 (±0.5 S.D.) PPT which equates to 4.4 (±2.4) fmoles/ml. The mean ratio of platinum to DNA in these samples was 0.08 (±0.05 S.D.) nmol Pt/g DNA.

3. Results

3.1. DNA adduct levels in cells exposed to cisplatin or carboplatin in vitro

As a foundation for subsequent *in vivo* analyses, the relationship between drug concentration and adduct formation under *in vitro* conditions was characterised for two human tumour cell lines. Cultures of IMR 32 and SH-SY5Y cells at 80–90% confluence were exposed to cisplatin or carboplatin for 1 h. DNA was extracted and platinum content measured by ICP-MS. Adduct levels were linearly related to drug concentration (see supplementary data, Fig. 1). Cisplatin treated IMR 32 cells accumulated more adducts than SH-SY5Y cells at the same exposures ($p = 0.0004$ by 2-way ANOVA and 1.8-fold difference in slope of linear regressions), but no difference was apparent for cells exposed to carboplatin. To achieve similar levels of DNA-adducts, concentrations of carboplatin approximately 50-fold higher than cisplatin were necessary.

3.2. Platinum plasma pharmacokinetics

Concentrations of free drug in plasma were measured at various time points in order to assess the availability of active drug in the circulation and to provide the basis for quantifying drug-exposure *in vivo*. Balb/c mice were used to minimise the numbers of nu/nu mice required. Cisplatin at 5 mg/kg and 10 mg/kg (Fig. 1) and carboplatin at 25 mg/kg and 100 mg/kg (Fig. 2) were administered to Balb/c mice. Blood was collected from mice killed at various times and platinum levels in ultrafiltrate samples determined by AAS. After administration of cisplatin at 10 mg/kg, the concentration of cisplatin in plasma ultrafiltrates was 19 ± 1 (S.E.) μg/ml at 5 min (Fig. 1b), decreasing to 5% of this value at 60 min. A similar pattern of results was observed after the lower dose (Fig. 1a). AUC values of unbound cisplatin measured in ultrafiltrate samples were 0.23 and 0.46 mg/ml min for the 5 and 10 mg/kg doses, respectively. The concentration of carboplatin in plasma ultrafiltrates was 125 ± 7 (S.E.) μg/ml at 15 min after administration of drug at 100 mg/kg (Fig. 2b), declining to 10% of this value by 60 min. AUC values for unbound carboplatin were 2.0 and 5.7 mg/ml min following doses of 25 and 100 mg/kg respectively. Tumour-bearing *nu/nu* mice showed similar pharmacokinetics to Balb/c mice for both unbound cisplatin and carboplatin (data not shown).

3.3. DNA adduct levels in normal mouse tissues exposed to cisplatin and carboplatin

Platinum–DNA adduct levels in normal tissues of Balb/c mice were determined to investigate the reliability of adduct measurements in solid tissue samples using ICP-MS and to investigate how these levels changed in relation to pharmacokinetic parameters determined in the same animals. As expected, adduct levels in kidneys, livers and spleens of the Balb/c mice following the 10 mg/kg cisplatin dose were approximately double those determined following the 5 mg/kg dose (Fig. 1). DNA from kidneys contained the highest levels of platinum. In both liver and kidney, DNA adduct levels were maximal 30 min post-treatment and fell to similarly low levels in both tissue types by 60 min. Adduct levels in spleen were more stable over this time period and were considerably lower than in kidney and liver.

Following administration of carboplatin at 100 mg/kg, adduct levels were approximately 4-fold higher than those following the 25 mg/kg dose (Fig. 2). Unlike the situation following cisplatin treatment, no transient increase in adduct levels was observed in DNA from liver after carboplatin administration. A transient peak in adduct levels was only observed in the kidney, where adducts had declined 50% by 60 min post treatment, relative to the 30 min time point. Even at 100 mg/kg carboplatin, maximum adduct levels were considerably lower (approximately 6, 4 and 2-fold for liver, kidney and spleen respectively) than at 5 mg/kg cisplatin, but were easily detectable at both dose levels.

3.4. DNA adduct levels in tissues from nu/nu mice and xenografts

In order to study adduct formation in human tumour tissue, the two tumour cell lines studied *in vitro* were grown as xenografts in nu/nu mice. DNA adduct levels in normal tissues were measured for comparison to the Balb/c mouse data. *Nu/nu* mice bearing neuroblastoma tumour xenografts were treated with cisplatin at 5 mg/kg or carboplatin at 100 mg/kg in the same manner as the Balb/c mice. Tumours were harvested together with samples of kidney, liver and spleen from each of three animals at each time point and platinum levels in DNA extracts were measured.

Levels of cisplatin adducts on DNA from xenografts of both cell lines changed only slightly between 1 and 6 h after drug

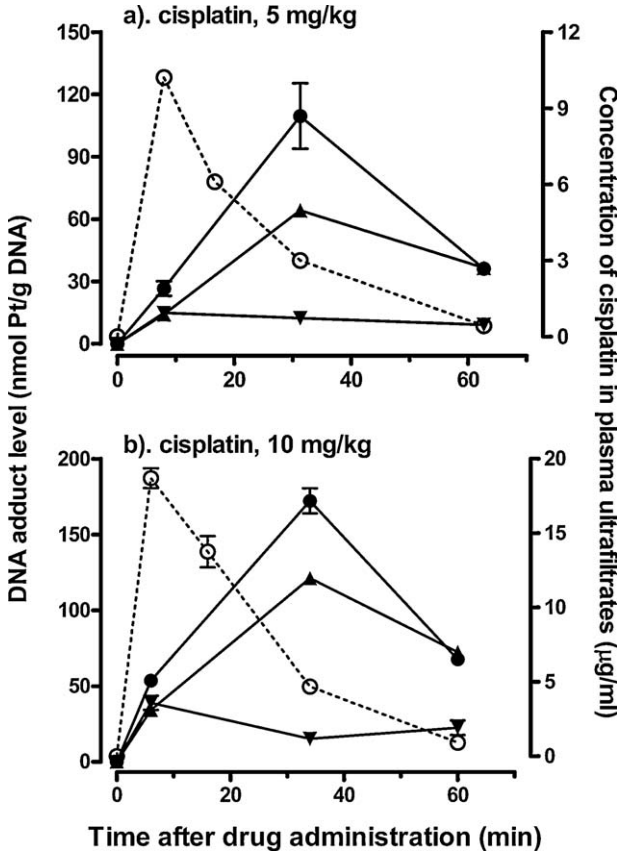

Fig. 1. Levels of DNA adducts in normal tissues and plasma ultra-filterable platinum, in Balb/c mice treated with cisplatin at 5 mg/kg (a) or 10 mg/kg (b). Mice were killed at various times after exposure and organs harvested. ICP-MS was used to measure Pt levels bound to DNA extracted from livers (▲), kidneys (●) and spleens (▼). Platinum in plasma ultrafiltrates was measured by AAS (○). Each point represents mean for 3 mice. S.E. lies within symbol unless shown by a bar.

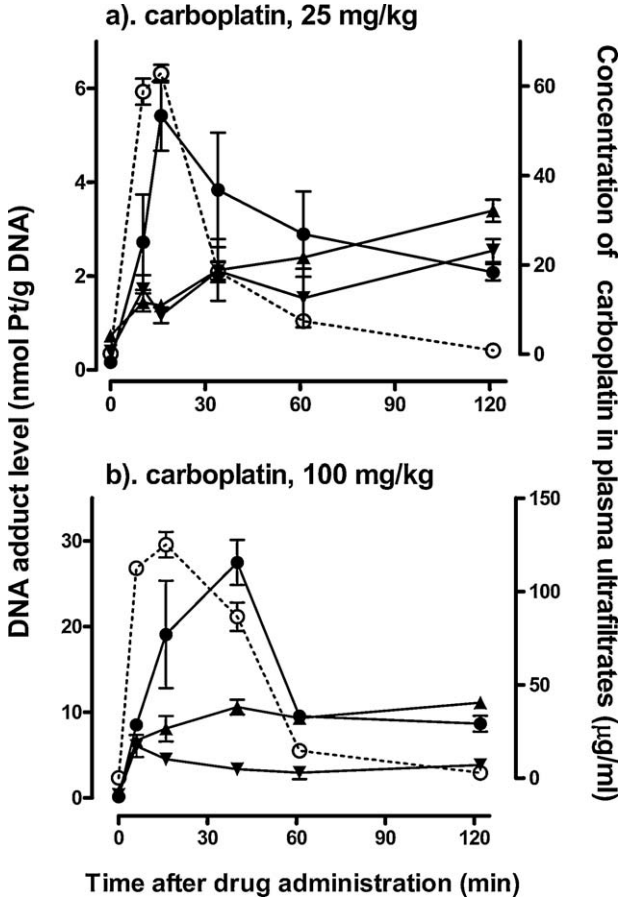

Fig. 2. Levels of DNA adducts in normal tissues and of plasma ultra-filterable platinum, in Balb/c mice treated with carboplatin at 25 mg/kg (a) or 100 mg/kg (b). Mice were killed at various times after exposure and organs harvested. ICP-MS was used to measure Pt levels bound to DNA extracted from livers (▲), kidneys (●) and spleens (▼). Platinum in plasma ultrafiltrates was measured by AAS (○). Each point represents mean for 3 mice. S.E. lies within symbol unless shown by a bar.

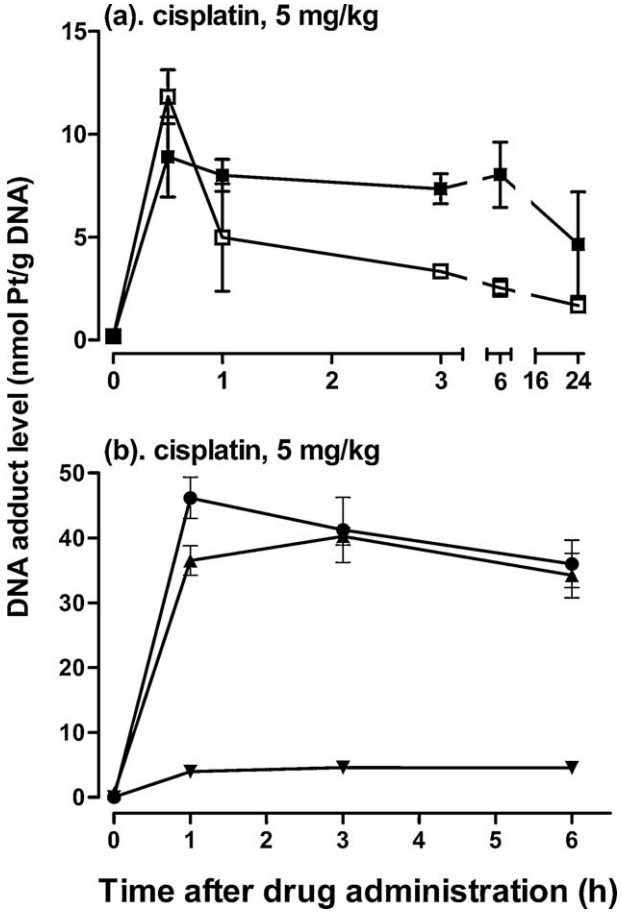

Fig. 3. DNA adduct levels in tumour (a) and normal (b) tissues following administration of cisplatin to *nu/nu* mice at 5 mg/kg. ICP-MS was used to measure Pt levels bound to DNA extracted from: IMR 32 tumours (■), SH-SY5Y tumours (□), livers (▲), kidneys (●) and spleens (▼). Each point represents mean for 3 mice. S.E. lies within symbol unless shown by a bar.

administration (Fig. 3a). Adduct levels in IMR 32 were higher than in SH-SY5Y at all time points after 30 min (2-way ANOVA, $p = 0.009$), comparable to the differential seen with cells treated in culture. Carboplatin–DNA adducts levels (Fig. 4a) were lower (approximately 3-fold) than those induced by cisplatin at 5 mg/kg in both normal and tumour tissues. Maximum carboplatin adduct levels in tumour DNA were approximately 2 nmol Pt/g DNA and no difference was observed between IMR 32 and SH-SY5Y tumours. In non-tumour tissues of animals treated with cisplatin or carboplatin, adduct levels present during the first few hours were similar to those seen in Balb/c mice (Figs. 3b and 4b). This indicates a general similarity between the two strains of mice regarding the pharmacodynamics of these drugs. Calculation of equivalent AUC values for active drug in cell cultures, using appropriate drug half-life values [37] together with the pharmacokinetic data for Balb/c mice, revealed close agreement between platinum–DNA adduct levels *in vitro* and *in vivo* for both IMR 32 and SH-SY5Y for both drugs (Table 2).

3.5. Analysis of patient blood samples

Measurements of ultrafilterable plasma platinum were made for determining concentrations of active drug (see supplementary data, Fig. 2), as a measure of *in vivo* drug exposure. Additional blood samples were taken for measurement of DNA adducts levels to directly compare with adducts levels measured at the same time point in the tumour biopsies. Platinum concentrations in DNA preparations from patient pre-treatment blood samples all fell

Table 2
Comparison of 1 hdrug exposures and platinum–DNA adduct levels *in vitro* and *in vivo*.

Drug		Drug dose or concentration	AUC (mg/ml/min)	Adduct level (nmol Pt/g DNA)	
				SH-SY5Y	IMR 32
Cisplatin	*In vitro*	16 μM	0.28[a]	4.1–7.0	9.1–10.1
	In vivo	5 mg/kg	0.23[b]	2.4–7.6	6.8–9.5
Carboplatin	*In vitro*	250 μM	5.5	1.0–2.0	1.0–2.0
	In vivo	100 mg/kg	5.7	1.0–2.0	1.0–2.0

[a] Calculated from drug half-life in culture medium [37].
[b] Measured in Balb/c mice.

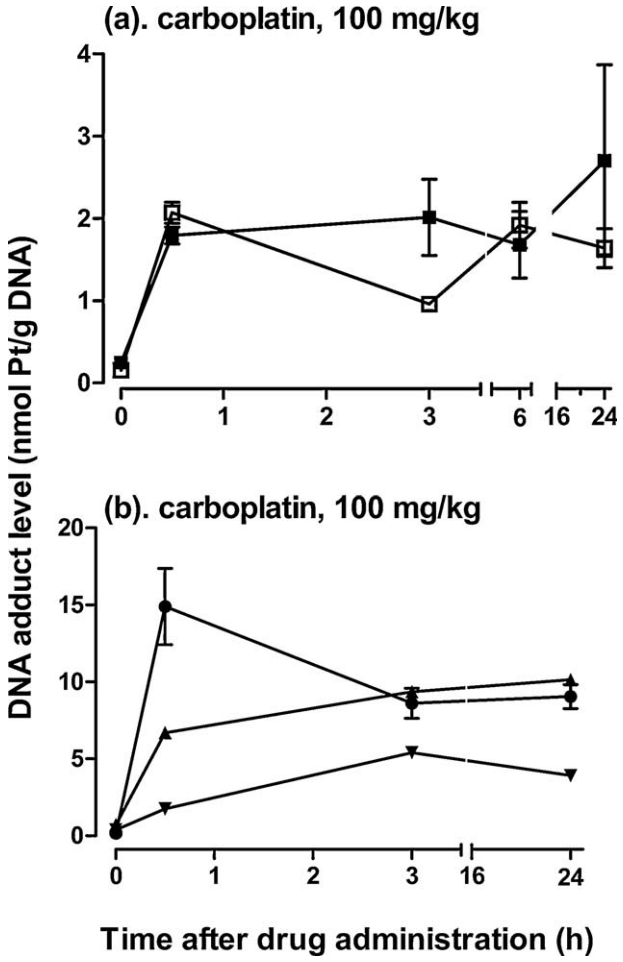

Fig. 4. DNA adduct levels in tumour (a) and normal (b) tissues following administration of carboplatin to *nu/nu* mice at 100 mg/kg. ICP-MS was used to measure Pt levels bound to DNA extracted from: IMR 32 tumours (■), SH-SY5Y tumours (□), livers (▲), kidneys (●) and spleens (▼). Each point represents mean for 3 mice. S.E. lies within symbol unless shown by a bar.

within 2 S.D. of the overall mean background Pt concentration (see Section 2). Pt concentrations in preparations of DNA from patient post-carboplatin blood samples were all more than 2 S.D. above the overall mean background Pt concentration (Table 3). Post-carboplatin blood platinum–DNA adducts levels ranged from 0.2 ± 0.01 (S.D.) to 3.6 ± 0.3 (S.D.) nmol Pt/g DNA (Table 3). Patients 2–4 had significantly higher platinum–DNA adducts levels in their post-carboplatin sample compared to their pre-carboplatin samples (Table 3).

3.6. DNA adduct levels in patient tumour biopsies

Tumour biopsies, obtained from patients 180 min after carboplatin infusion, were analysed for determination of therapy-induced platinum–DNA adduct levels. Biopsies were also taken from contiguous areas of the tumour and subjected to formalin fixation. Subsequent H&E stained sections confirmed the presence of viable recurrent tumour in keeping with the original histological subtype in all four cases. All sections showed high ratios of tumour to stromal tissue.

All of the 13 resulting DNA preparations were analysed by ICP-MS and 7 of these preparations contained sufficient DNA for ICP-MS analyses to be replicated on three separate occasions. The overall C.V. for these repeated ICP-MS analyses was 1.4%. The concentrations of platinum measured in the DNA preparations from the tumour samples exceeded the background level by 36–62 standard deviations (Table 4). Platinum–DNA adduct levels in different portions of the same tumour biopsies showed ranges of DNA adduct levels of 10–12% of the means, indicating good reproducibility. In the limited number of samples analysed, adduct levels in different biopsies from the same tumour also showed comparable adduct levels. The largest variation was seen in patient 3 where the adduct level in one biopsy appeared to be approximately 40% lower than the other two biopsies analysed. No significant correlations were seen between DNA adduct levels in PBCs and those in tumour ($r^2 = 0.196$, slope not significantly different to zero ($p = 0.56$)) or between free drug plasma AUC and tumour ($r^2 = 0.785$, slope not significantly different to zero ($p = 0.11$)) or blood cells ($r^2 = 0.572$, slope not significantly different to zero ($p = 0.24$). However, the small number of tumours studied limits the reliability of these results.

4. Discussion

Measurement of DNA adduct levels in tumours contributes to a better understanding of the causes of inter-patient variation in response to platinum-based anti-cancer drugs. However, as the collection of appropriate biopsies is clinically challenging, both for ethical and scientific reasons, it is essential that measurement of the low levels of adducts formed is based on reliable and validated techniques. The present studies assess the suitability of ICP-MS for analysis of DNA adducts in samples of solid tissue, investigate the extent of variation in adduct levels with sampling time after drug administration and finally apply the methods developed to a small exploratory set of clinical samples.

Quantification of drug–DNA adducts in blood cells has previously been performed by AAS [13], immunological methods [6,15,16,21,38] and post-labelling methods [22]. ICP-MS is a highly accurate method for measurement of very low levels of platinum and has been successfully applied to the analysis of adducts on DNA from nucleated blood cells and cell lines [27,30,39]. The accuracy of ICP-MS is inherently greater than other methods for adduct measurement and accuracy has recently been further improved by the use of plasma ionisation multicollector mass spectrometry (PIMMS) instrumentation with internal standardisation [29]. ICP-MS detects total platinum–DNA adducts as opposed to the specific adducts detected by immunoassay [31] or the combination of ICP-MS with LC [40].

Table 3
Platinum concentration and total DNA adduct level in patient post-carboplatin blood samples.

	Average Pt concentration in DNA sample			DNA adduct level		
	PPT (+/− range)	fmoles Pt/ml (+/− range)	Number S.D. above background	(nmol Pt/g DNA) (+/− range)	Significance vs. background	Significance vs. pre-sample
Patient 1	4.0 (0.5)	20.7 (2.6)	6.4	0.8 (0.1)	0.0008	ND[a]
Patient 2	35.0 (3.2)	178.9 (16.2)	68	3.6 (0.3)	<0.0001	<0.0001
Patient 3	2.2 (0.1)	11.2 (0.4)	2.8	0.2 (0.01)	<0.0001	0.009
Patient 4	2.2 (0.4)	11.3 (2.1)	2.8	0.2 (0.04)	0.0029	0.001

[a] ND = no data available for sample.

Table 4
Platinum concentration and total DNA adduct level in patient ovarian tumour biopsies.

Patient	Biopsy	Number of portions analysed	[Pt] measured in DNA preparations			Mean tumour adduct level for patient (nmol Pt/g DNA)	Mean blood cell adduct level for patient (nmol Pt/g DNA)
			PPT (±range)	fmol/ml	Number S.D. above background		
1	A	1	24.1	123.4	47	2.2	0.8
	B	2	20.7 (2.0)	106	36–44		
2	A	2	24.4 (2.8)	125	53–54	2.4	3.6
	B	4	21.9 (2.9)	112.1	36–48		
3	A	1	31.7	162.3	62	2.8	0.2
	B	2	31.7	162.3	62		
	C	3	19.9	102	38		
4	A	1	20.6	105.5	40	4.2	0.2

Plasma AUC values in Balb/c mice for unbound cisplatin (0.23 and 0.46 mg/ml min) and carboplatin (2.0 and 5.7 mg/ml min) were linearly related to dose and comparable to exposures typically achieved in patients [15,21]. At all time points, following both of the doses used for each drug, the levels of platinum–DNA adducts could be detected from all the tissues studied. Following cisplatin or carboplatin treatment the highest levels of DNA adducts were observed in the kidneys, consistent with the importance of renal clearance for both drugs [41]. Following cisplatin but not carboplatin, high DNA adduct levels were also observed in liver. This could reflect the much greater biliary clearance of cisplatin compared to carboplatin [41]. Splenic DNA contained considerably lower adduct levels, consistent with a previous study of rats [42]. For both cisplatin and carboplatin, the platinum–DNA adduct levels observed in tumours were similar to those observed in spleen samples. Similarity between adduct levels in spleen and tumour tissues is likely to reflect similar drug access and similar cellular pharmacology in these tissues. Furthermore, unlike liver and kidney, neither of these tissues was strongly involved in drug clearance. There is no evidence for preferential drug access to tumour cell DNA.

Following both doses of cisplatin in Balb/c mice, DNA adduct levels in the liver and kidney were maximal at the 30 min time point and had declined markedly by 60 min (Fig. 2). A similar transient peak in DNA adduct level was observed in kidney tissue from Balb/c mice exposed to carboplatin. If these results reflect levels of DNA adducts *in vivo,* they indicate an extremely rapid rate of DNA repair between 30 and 60 min. An alternative explanation is that high concentrations of active drug in these tissues at the time of freezing the tissue samples subsequently react with DNA during the tissue homogenisation and lysis procedures. High adduct levels in white blood cell DNA have previously been associated with an analogous artefact caused by inappropriate handling of blood samples [43]. Transiently high drug levels in kidney and liver would be expected because of the importance of these organs as routes of drug elimination. To avoid any risk of artefact, particularly in kidney and liver samples, biopsies should only be collected after free drug has cleared from the circulation.

The difference between IMR 32 and SH-SY5Y cells in DNA adduct formation by cisplatin (supplementary data, Fig. 1) may be due to differences in accumulation of active drug. The lack of a similar difference for carboplatin would reflect the different mechanisms that act on this compound. Comparison of adduct formation to available drug under *in vitro* and *in vivo* conditions showed that, for the two cell lines studied here, overall access of drug to cells grown as solid tumours in mice was as efficient as when the cells were grown as monolayers. Furthermore, the cellular factors governing access of drug to DNA in these two tumour lines showed a similar pattern of activity *in vitro* and *in vivo*. We are not aware of any previous direct comparison of drug–DNA adduct formation in relation to drug availability between cell lines grown both *in vitro* and as xenografts.

The adduct levels in mouse spleen and the experimental tumour changed very little between 1 and 6 h, which is consistent with available data on the rates of removal of platinum–DNA adducts by DNA repair processes [44–46]. This indicates that modest variation in timing of the removal of biopsies after drug administration is unlikely to have a marked effect on the DNA adduct levels observed.

Plasma AUC values for unbound carboplatin observed in the four patients studied (range 3.0–6.2 mg/ml min) were comparable to exposures typically achieved in patients [15]. A 2-fold range in unbound carboplatin AUC is typical for renal-based carboplatin dosing [16]. Platinum–DNA adducts levels in blood cells collected from patients post-carboplatin chemotherapy ranged from 0.2 to 3.6 nmol Pt/g DNA, significantly higher than background levels, and are comparable to previously reported adduct levels measured by ICP-MS in patients receiving carboplatin [17,30]. Platinum–DNA adduct levels measured in ovarian tumour biopsies by ICP-MS ranged from 1.9 to 4.3 nmol Pt/g DNA, all significantly higher than background levels. These values are in the same range as therapy-induced cisplatin–DNA adduct levels previously reported in head and neck cancer tissue [18,19].

It has been proposed that platinum–DNA adduct levels achieved in PBCs obtained from patients receiving platinum-based chemotherapy could be used as a biomarker for patient response. From the small numbers of patients in the present study, there is no obvious correlation between adduct levels in PBCs and in tumour biopsies. The lack of such a correlation is consistent with recently published data on this subject [18,19]. In three of the four patients studied, DNA adduct levels in blood cells were markedly lower than adduct levels detected in tumour biopsies collected at the same time point following carboplatin administration (Table 4). This may explain why carboplatin is an effective treatment despite the adduct levels detected in blood cells being lower than adduct levels detected following administration of cisplatin [16,21].

The current data confirm that measurement of total platinum associated with DNA by ICP-MS can be a reliable and sensitive method, providing appropriate precautions are taken with regard to sample collection and handling to minimise contamination with unreacted drug or extraneous platinum. The principle that any platinum that remains bound to DNA during the purification procedure is in the form of a DNA adduct has formed the basis of many previous studies. However, identification of specific adducts as described by Harrington et al. [40] would be a desirable refinement if sensitivity could be maintained. This is the first report of DNA adduct levels achieved during therapy with

carboplatin in a solid tumour patient setting. It indicates that adduct levels in tumour tissue can be several fold higher than levels achieved in mononuclear blood cells, consistent with recent data for cisplatin [18,19]. Platinum-based drugs are predominantly used to treat solid tumours and knowledge of variations in DNA adduct levels achieved in tumour tissue will improve understanding of resistance to treatment. The data presented here point to the possibility of a number of further experimental studies. However, extension of molecular pharmacological investigations to a fuller analysis of clinical tumours is clearly of particular relevance to understanding a number of fundamental principles underlying the differences in response of patients to treatment with these important drugs. The main challenge for wider studies will be the difficulty of obtaining suitable biopsies. Now that methodologies for measurement of platinum–DNA adduct levels in solid tumours have been established, it is hoped that it will be possible to exploit opportunities for such studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.10.005.

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