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Fortification of blood plasma from cancer patients with human serum albumin decreases the concentration of cisplatin-derived toxic hydrolysis products *in vitro*†

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While cisplatin (CP) is still one of the world's bestselling anticancer drugs, its intravenous administration is inherently associated with severe, dose limiting toxic side-effects. Although the molecular basis of the latter are not well understood, biochemical transformations of CP in blood and the interaction of the generated platinum species with plasma proteins likely play a critical role since these processes will ultimately determine which platinum-species reach the intended tumor cells as well as non-target cells. Compared to healthy subjects, cancer patients often have decreased plasma human serum albumin (HSA) concentrations. Little, however, is known about how the plasma HSA concentration will affect the metabolism of CP. To gain insight, we obtained blood plasma from healthy adults (n = 20, 42 ± 4 g HSA per L) and pediatric cancer patients (n = 11, 26 ± 7 g HSA per L). After the incubation of plasma at 37 °C, a pharmacologically relevant dose of CP was added and the Pt-distribution therein was determined by size-exclusion chromatography coupled on-line to an inductively coupled plasma atomic emission spectrometer. At the 2 h time point, a 5.9% increase of toxic CP-derived hydrolysis products was detected in pediatric cancer patient plasma, while 9.8% less platinum was protein bound compared to plasma from healthy controls. These *in vitro* results suggest that the elevated concentration of highly reactive free CP-derived hydrolysis products in plasma may cause the toxic side-effects in cancer patients. More importantly, the deliberate increase of the plasma HSA concentration in cancer patients prior to CP treatment would represent a simple strategy to possibly alleviate the fraction of patients that suffer from drug induced toxic side-effects.

Introduction

Cisplatin (CP), which was serendipitously discovered by Rosenberg and co-workers in the mid-sixties remains one of the most widely used anti-cancer drugs worldwide.[1] Despite its remarkable anti-cancer properties, this intravenously administered platinum-based medicinal drug is associated with severe toxic side-effects, including nephrotoxicity, ototoxicity and neurotoxicity.[2] Although nephrotoxicity can be somewhat ameliorated by the administration of patients with hypertonic saline or mannitol,[3] no clinical procedures exist to completely eliminate ototoxicity or neurotoxicity.[4] Therefore, the inherent toxic side-effects constitute the primary dose limiting factor of this metal-based drug. One strategy to 'transform' CP into a better drug would be to mitigate its toxic side-effects in patients. To this end, its co-administration with small-molecular-weight compounds – so-called 'chemoprotective agents' – has been demonstrated to effectively reduce the toxic side-effects and recent *in vitro* studies have provided a first glimpse into the possible biomolecular mechanisms of action.[5] Seemingly unrelated to this, it has long been known that decreased human serum albumin (HSA) concentrations in blood plasma can significantly increase the fraction of patients that suffer from toxic side-effects following the intravenous administration of medicinal drugs.[6] The administration of patients (n = 1202) with the anti-anxiety drug diazepam (also known as Valium), for example, resulted in unwanted CNS depression in only 2.9% of patients with normal plasma HSA concentrations (>40 g HSA per L), while 9.3% of patients with severe hypoalbuminemia (<30 g HSA per L) displayed these symptoms.[6a] Similar observations were reported for the anticonvulsant drug phenytoin.[7] Considering that HSA is thought to play a critical role in the metabolism of CP,[8] systematic studies into the effect of the plasma HSA concentration on the metabolism of CP could provide guidance to develop strategies to

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mitigate the toxic side-effects of this otherwise very effective anticancer drug. To this end, an *in vitro* approach was chosen which involved the addition of a pharmacologically relevant dose of CP to human plasma from healthy adults (range 35–50 g HSA per L; 42 ± 4 g HSA per L) and pediatric cancer patients (range 8–34 g HSA per L; 26 ± 7 g HSA per L). The determination of all Pt-containing metabolites in plasma was achieved by using an established metallomic tool comprised of size exclusion chromatography (SEC) coupled on-line to an inductively coupled plasma atomic emission spectrometer (ICP-AES).[9] Monitoring dynamic changes of the Pt-distribution in plasma over a 2 h period was intended to reveal differences in the metabolism of CP in the respective groups. The obtained results were rationalized based on relevant blood plasma parameters, which included the HSA concentration and the concentrations of transferrin (Tf), creatinine and blood urea nitrogen (B.U.N.). Based on the differences that were observed for the metabolism of CP *in vitro*, it is recommended that boosting the plasma HSA concentration of cancer patients to levels that are prevalent in healthy individuals may represent a feasible strategy to decrease the fraction of cancer patients that suffer from severe drug related toxic side-effects by reducing the concentrations of highly reactive CP-derived hydrolysis products.

Experimental

Chemicals and solutions

Cisplatin (1 mg *cis*-$Pt(NH_3)_2Cl_2$ per mL; this sterile solution also contained 1 mg mannitol and 9 mg NaCl) was obtained from Hospira (Montreal, QC, Canada). An aqueous solution of highly pure HSA (12.5 g in 50 mL of buffered diluent (Alburex 25 - USP), stabilized with 0.02 M sodium caprylate and 0.02 M sodium acetyltryptophanate) was obtained from CSL Behring AG (Bern, Switzerland). Phosphate-buffered saline (PBS) tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the corresponding buffer (10 mM phosphate, 2.7 mM KCl, 137 mM NaCl) was prepared by dissolving PBS tablets in the appropriate volume of de-ionized water derived from a Simplicity water purification system (Millipore, Billerica, MA, USA) followed by pH adjustment to 7.4 with dilute HCl. The obtained solution was filtered through 0.45 μm nylon-filter membranes (Mandel Scientific, Guelph, ON, Canada) before use. A mixture of protein standards which contained thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa) was obtained from Bio-Rad Laboratories (Hercules, CA, USA) to calibrate the employed Superdex™ 200 SEC column.

SEC–ICP-AES system

This system was comprised of a Smartline 1000 HPLC pump (Knauer, Berlin, Germany) and a Rheodyne 9010 PEEK injection valve (Rheodyne, Rhonert Park, CA, USA) which was equipped with a 0.5 mL PEEK injection loop (0.5 mL). A pre-packed Superdex™ 200 10/300 GL Tricorn™ high performance size-exclusion chromatography column (30 × 1.0 cm I.D., fractionation range 600–10 kDa; GE Healthcare, Piscataway, NJ, USA) was used in conjunction with PBS-buffer mobile phase at a flow rate of 1.0 mL min^{-1} (column temperature 22 °C). Simultaneous multielement-specific detection of C (193.091 nm), Cu (324.754 nm), Fe (259.940 nm), Pt (214.423 nm), S (180.731 nm) and Zn (213.856 nm) in the column effluent was achieved with a Prodigy, high-dispersion, radial-view ICP-AES (Teledyne Leeman Labs, Hudson, NH, USA) at an Ar gas-flow rate of 19 L min^{-1}, an RF power of 1.3 kW and a nebulizer gas pressure of 35 psi. The nebulizer gas flow rate was 1.4 L of Ar per min. A 360 s delay was implemented between sample injection and data acquisition based on the void volume that was determined by the injection of blue dextran and monitoring of the C emission line. The data acquisition window was 1800 s. Raw data were imported into Sigmaplot 12 software, smoothed (bisquare algorithm) and the peak areas were determined using Origin 9 Data Analysis and Graphing software (OriginLab Corporation, Northampton, MA, USA). If two platinum peaks were not baseline separated, the point of lowest intensity between both peak maxima was used as the dividing line to obtain areas for each peak.

SEC–ICP-AES analysis of CP spiked human plasma

The collection of blood from humans was approved by the Calgary Conjoint Health Research Ethics Board (CHREB Approval No. E-25315). Over an 8 month period, a cross section of 11 pediatric cancer patients (age 3–18) were recruited for blood withdrawal based on pre-defined inclusion criteria, which stipulated that the HSA level in plasma had to be ≤30 g L^{-1} (ref. 6*a*) and that the patients must not have previously received treatment with Pt-based anticancer drugs. Owing to the ethical difficulty that is associated with the collection of blood from healthy children (which would constitute the proper control group), we instead recruited 20 healthy male and female adults (age 18–45). All subjects provided written informed consent for sample collection. Due to software related problems and isolated ICP-instrument failures, we obtained useful results only for 14 plasma samples (range 35–50 g L^{-1}) of the healthy control group. Approximately 12 mL of blood were collected from healthy controls into heparinized trace metal testing blood collection tubes (6 mL; Greiner-Bio-One Vacuette™, NC, USA) by a certified nurse from the Department of Kinesiology (University of Calgary). Blood was collected from pediatric cancer patients at the Alberta Children's Hospital (the cancer types are listed in S1, ESI†) in a similar manner using the same blood collection tubes. After centrifugation at 1000 rpm (4 °C) for 10 min, the buffy coat was removed and the supernatant plasma was withdrawn using a micropipette and pooled. If a sufficient amount of homogenous plasma stock was obtained, 1.6 mL were transferred to cyrovials and stored in liquid nitrogen. For analysis, plasma was thawed at room temperature for 45 minutes and incubated in a rotary shaker at 37 °C for 30 min. Then, a pharmacologically relevant dose of CP was added (0.04 mg CP per mL of plasma; ~0.13 mM).[10] This mixture was kept at 37 °C and samples were withdrawn for analysis (0.5 mL) after 5 min and 2 h. In order to corroborate the results that were obtained with plasma from pediatric cancer patients, aliquots of the latter were fortified with

pure HSA (Alburex 25) to achieve a total plasma concentration of 36 g HSA per L and 42 g HSA per L, respectively. This "HSA fortified" plasma was then spiked with the same dose of CP and analyzed in the same manner as outlined before. The recovery of Pt was determined by injecting a fresh solution of carboplatin in PBS-buffer (which had the same Pt concentration as that in the spiked plasma) before and after all plasma injections and expressing the total Pt peak area as a percentage of the carboplatin peak area. The Pt recovery was 97 ± 14%.

Statistical analysis

Due to an unequal variance between the two groups, a two-tailed Welch's *t*-test was used to compare the results for healthy *vs.* pediatric cancer patients. ANOVA was used to assess statistical significance among the results obtained for plasma from pediatric cancer patients and the HSA-fortified equivalents. With regard to both tests *P*-values of less than 0.05 indicate statistical significance.

Analysis of plasma for relevant analytes

Aliquots of all blood plasma samples were analyzed for HSA, Tf, creatinine, and blood urea nitrogen (BUN) by an accredited medical diagnostic laboratory (Calgary Laboratory Services, CLS) using validated analysis protocols. This involved colorimetric (HSA, creatinine) or immunoturbidimetric assays (Tf), while BUN was determined using a kinetic UV assay. A BUN/creatinine ratio of ≥ 20 in plasma is indicative of dehydration of a patient. This ratio was 9.1–24 in healthy adults and 5.6–121 in pediatric cancer patients. The plasma chloride concentration of all pediatric cancer patients was extracted from patient charts within ±1 day of the date of blood collection.

Results and discussion

Ever since CP was approved by the US Food and Drug Administration in 1978, numerous treatment regimens that include CP have had a major clinical impact in cancer patients, particularly with regard to testicular and ovarian cancer.[1c] After the intravenous administration of patients with CP, the parent drug as well as CP-derived Pt species will interact with blood constituents and eventually with cancer cells (intended) as well as healthy tissue cells (unintended). Conceptually, changes in the concentration of plasma proteins that are known to play a critical role in the metabolism of CP may contribute to determine the severity of the toxic side-effects at the organ level (*e.g.* nephrotoxicity). Cancer patients, for example, often display greatly decreased plasma HSA concentrations due to its decreased synthesis, its increased catabolism and/or the loss of HSA by kidney damage.[11] From a biochemical perspective three separate events occur after CP is injected into the bloodstream: (a) hydrolysis of CP, (b) binding of CP-derived hydrolysis products to plasma proteins (as well as erythrocytes and/or endothelial cells) and (c) the uptake of CP and/or CP-derived hydrolysis products (free or protein bound) into organ/tumor cells.[9] The hydrolysis of the neutral dichloro complex CP critically depends on the chloride concentration (~103 mM in plasma[12] *vs.* ~4–20 mM in the cell cytosol[13]) and results in the formation of the aqua-adducts $[PtClOH_2(NH_3)_2]^+$ and $[Pt(OH_2)_2(NH_3)_2]^{2+}$,[14] as well as other Pt-containing compounds, such as dimer and trimer complexes.[15] In blood plasma, CP-derived hydrolysis products can then bind to HSA (methionine and cysteine residues[8a]) and Tf (threonine, tyrosine, methionine and histidine residues[16]). This binding of Pt-species to plasma proteins can be directly visualized by analyzing plasma for Pt-species using metallomics methods, such as SEC–ICP-AES[9] and/or SEC–ICP-MS.[17] With regard to the transfer of Pt-species into cells, it is known that the uptake of CP is mediated by the copper transport uptake protein CTR-1,[18] but little is known about whether CP-derived hydrolysis products are transported into cells by this transport protein as efficiently as CP.[19]

Representative Pt-specific chromatograms that were obtained after the analysis of plasma from healthy adults are depicted in Fig. 1. At the 5 min time point 2.4 ± 0.3% of total platinum eluted in the form of a CP-derived hydrolysis product and 97.6% eluted in form of CP. At the 2 h time point, 53.0 ± 2.8% of Pt was protein bound, while 15.9 ± 1.7% corresponded to CP-derived hydrolysis products, and 31.1 ± 1.7% eluted as CP. The distribution of protein bound Pt peaks (referred to as PP-1, PP-2, PP-3), CP-derived hydrolysis products (HP-1, HP-2) and free CP are depicted in a representative chromatogram in Fig. 1 and the quantitative distribution is summarized in Table 1. The first protein bound Pt peak (PP-1) co-eluted with Zn (data not shown), which is in agreement with our previous observations[9] and can be explained by the formation of a Pt-containing α_2-macroglobulin complex.[20] Since PP-3 co-eluted with HSA (as evidenced by the co-elution of an intense sulfur-peak), this Pt-peak was assigned to a Pt–HSA complex.[8a] Based on the limited resolution of the utilized SEC column and considering that CP-derived hydrolysis products are known to bind to Tf,[16c] it is impossible to delineate whether PP-3 corresponds to a Pt–Tf (~79 kDa) and/or a Pt–HSA complex (~66.3 kDa). Owing to the >10 fold higher molar concentration of HSA in plasma and the comparatively higher affinity of CP hydrolysis products for HSA compared to Tf,[21] however, PP-3 most probably corresponds predominantly to Pt–HSA complexes. PP-2 was tentatively assigned to Pt–HSA-multimer complexes based on previous observations by others.[8a] Because Pt-peaks 4 & 5 eluted near the inclusion volume, they likely correspond to small molecular weight CP-derived hydrolysis products which will be referred to as HP-1 and HP-2. HP-2 was the more abundant Pt-species at the 5 min time point, which is in accord with previous studies.[9] Therefore, this Pt-species likely corresponds to the first hydrolysis product $[PtClOH_2(NH_3)_2]^+$. HP-1 – which was not detected at the 5 min time point – was present at the 2 h time point and likely corresponds to the second hydrolysis product $[Pt(OH_2)_2(NH_3)_2]^{2+}$. In accord with previous studies, the Pt-peak with the largest retention time was assigned to the parent drug CP and its elution past the inclusion volume can be rationalized in terms of an unknown interaction of CP with the stationary phase.[17b]

The results that were attained for pediatric cancer patient plasma yielded Pt-specific chromatograms, a representative of which is shown in Fig. 1. In sync with the results that were obtained for plasma from healthy adults 2.4 ± 0.4% of platinum

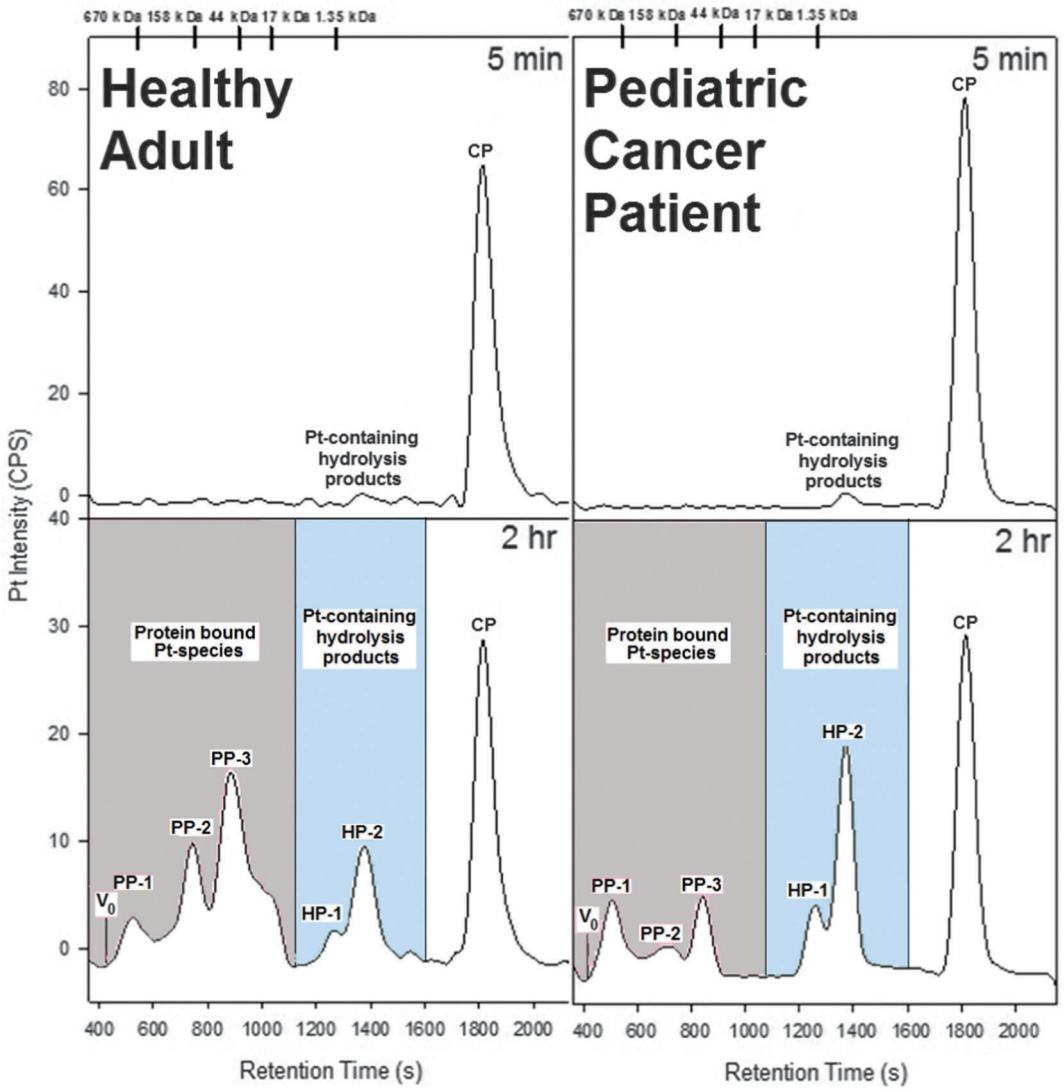

Fig. 1 Representative Pt-specific chromatograms obtained for the analysis of plasma from a healthy human adult (H-17) and a pediatric cancer patient (C-11) spiked with CP (0.04 mg mL^{-1}). The mixture was incubated at 37 °C and analyzed after 5 min and 2 h. Stationary phase: Superdex 200 10/300 GL column (30 × 1.0 cm I.D., 13 μm particle size) at 22 °C. Mobile phase: PBS buffer (pH 7.4). Flow rate: 1.0 mL min^{-1}. Injection volume: 500 μL. Detector: ICP-AES at 214.423 nm (Pt). Peaks 1–3: protein bound Pt-species, peaks 4 and 5: Pt-containing hydrolysis products, peak 6: CP. The retention times of the molecular weight markers are depicted on top.

eluted in form of HP-2 and 97.6% as the free drug. At the 2 h time point, 43.2 ± 9.5% of the Pt was protein bound, with the majority bound to PP-3 (22.3 ± 5.7%), while 21.2 ± 5.7% eluted as hydrolysis products, and 35.6 ± 4.4% as CP. Table 2 summarizes the quantitative distribution of protein bound Pt, CP-derived hydrolysis products and CP.

A comparison of the plasma Pt-distribution that was obtained for healthy adults (n = 14) with those of pediatric cancer patients (n = 11) are depicted in Table 3. The results that were obtained for the 5 min time point are virtually identical between the respective groups. At the 2 h time point, however, a different plasma Pt-distribution was observed between healthy adults and that of pediatric cancer patients with P-values <0.05 for PP-1, PP-3, HP-2 and CP. For the sake of clarity it is useful to discuss the results pertaining to CP first, followed by those corresponding to the hydrolysis products (HP-1 and HP-2), and finally the results that pertain to protein bound Pt (PP-1, PP-2 and PP-3). Overall, pediatric cancer patient plasma contained, on average, 4.5% more of the parent drug CP ($P < 0.05$) as well as 5.3% more hydrolysis products [+1.2% of HP-1 ($P > 0.05$) and 4.1% more of HP-2 ($P < 0.05$) compared to healthy adults]. The latter increase in hydrolysis products represents a net increase of 32% compared to the healthy controls. Related to this, the total protein bound Pt in pediatric cancer patient plasma was 9.8% less compared to that in plasma from healthy adults (Table 3). In contrast to this trend, the percentage of Pt bound to PP-1 was 1.9% higher in the pediatric cancer patients ($P < 0.05$). The increased percentage of Pt bound to PP-1 in pediatric cancer patient plasma can be rationalized by the apparently increased concentration of α_2-macroglobulin as

Table 1 Pt-peak areas expressed as % of total Pt obtained after SEC–ICP-AES analysis of human plasma from healthy adults spiked with CP after incubation at 37 °C for 2 h

ID #	PP-1 (t_r = 527 ± 9 s)	PP-2 (t_r = 751 ± 8 s)	PP-3 HSA (t_r = 890 ± 7 s)	HP-1 (t_r = 1273 ± 20 s)	HP-2 (t_r = 1388 ± 10 s)	CP (t_r = 1830 ± 12 s)
H-02[a]	8.1	13.5	32.1	3.7	13.3	29.2
H-04[a]	7.7	11.7	34.2	3.8	12.5	30.3
H-05[b]	6.8	11.2	31.1	4.0	13.9	33.0
H-06[b]	12.2	12.6	30.5	2.7	12.8	29.3
H-07[b]	9.2	13.1	29.9	4.3	12.8	30.6
H-08[b]	8.7	14.8	31.5	2.9	11.7	30.5
H-11[a]	8.3	11.9	33.2	3.3	12.3	30.9
H-12[b]	7.3	12.5	32.5	2.9	13.1	31.6
H-13[b]	9.3	9.7	28.6	2.5	14.4	35.5
H-14[a]	6.7	11.6	31.0	3.0	15.2	32.4
H-15[a]	8.5	12.6	32.0	1.6	13.6	31.7
H-16[a]	9.1	12.4	33.1	3.1	12.7	29.5
H-17[b]	6.9	13.3	33.6	2.6	12.3	31.3
H-19[a]	7.9	13.2	37.6	1.3	10.0	29.9
Avg ± STD	8.3 ± 1.4	12.4 ± 1.2	32.2 ± 2.2	3.0 ± 0.8	12.9 ± 1.2	31.1 ± 1.7

[a] Corresponds to one analysis. [b] Corresponds to the average of two analyses. The average difference between two consecutive analyses for PP-3 was 0.7%. PP = protein bound Pt-species, HP = Pt-containing hydrolysis product.

Table 2 Pt-peak areas expressed as % of total Pt obtained after SEC–ICP-AES analysis of human plasma from pediatric cancer patients spiked with CP after incubation at 37 °C for 2 h. PP = protein bound Pt-species, HP = Pt-containing hydrolysis product

ID #	PP-1 (t_r = 510 ± 9 s)	PP-2 (t_r = 724 ± 8 s)	PP-3 HSA (t_r = 868 ± 12 s)	HP-1 (t_r = 1259 ± 15 s)	HP-2 (t_r = 1377 ± 8 s)	CP (t_r = 1820 ± 8 s)
C-01	N/A	N/A	N/A	N/A	N/A	N/A
C-02	6.4	8.9	24.2	4.7	18.4	37.4
C-03	10.1	9.8	24.1	3.3	15.9	36.8
C-04	9.7	8.8	21.8	5.6	18.5	35.6
C-05	7.2	21.3	26.8	2.3	11.8	30.6
C-06	13.0	15.5	28.3	2.4	11.7	29.1
C-07	11.2	11.2	25.0	3.8	15.4	33.3
C-08	8.4	7.9	23.2	3.1	16.3	41.1
C-09	11.7	7.0	16.6	6.3	20.6	37.8
C-10	13.6	11.6	24.0	3.1	16.1	31.6
C-11	10.8	5.5	8.9	7.2	25.4	42.3
Avg ± STD	10.2 ± 2.4	10.8 ± 4.6	22.3 ± 5.7	4.2 ± 1.7	17.0 ± 4.1	35.6 ± 4.4

Table 3 Pt-peak areas expressed as % of total Pt obtained after SEC–ICP-AES analysis of plasma from healthy adults (average concentration 42 g HSA per L) and pediatric cancer patients (average concentration 26 g HSA per L) spiked with CP after incubation at 37 °C for 5 min and 2 h. PP = protein bound Pt-species, HP = Pt-containing hydrolysis product, H = healthy adults, C = pediatric cancer patients

Time	PP-1	PP-2	PP-3 (HSA)	Total PP	HP-1	HP-2	Total HP	CP
H – 5 min n = 13	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.4 ± 0.3	2.4 ± 0.3	97.6 ± 0.3
C – 5 min n = 11	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.4 ± 0.4	2.4 ± 0.4	97.6 ± 0.5
H – 2 h n = 14	8.3 ± 1.4	12.4 ± 1.2	32.2 ± 2.2	53.0 ± 2.8	3.0 ± 0.8	12.9 ± 1.2	15.9 ± 1.7	31.1 ± 1.7
C – 2 h n = 10	10.2 ± 2.4	10.8 ± 4.6	22.3 ± 5.7	43.2 ± 9.5	4.2 ± 1.7	17.0 ± 4.1	21.2 ± 5.7	35.6 ± 4.4
P value 2 h	0.042	0.287	<0.001		0.061	0.011		0.011

evidenced by the corresponding Zn-specific chromatograms (S2, ESI†). The increased concentration of α_2-macroglobulin (725 kDa) in plasma of pediatric cancer patients may be attributed to the relative loss of small molecular weight plasma proteins from the bloodstream by chemotherapy-induced kidney damage (http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/9270). Based on the assumption that PP-2 and PP-3 both likely correspond to HSA bound Pt, it is unsurprising that both Pt-peak areas were lower in the pediatric cancer patients [by 1.6% ($P > 0.05$) and 9.9% ($P < 0.05$)] compared to those observed for healthy adults.

To corroborate the different plasma Pt-distribution between healthy adults and pediatric cancer patients, aliquots of individual pediatric cancer patient plasma were fortified with highly pure HSA to achieve a final concentration of 42 g HSA per L and – if sufficient plasma was available – to 36 g HSA per L. At the 5 min time point, the plasma Pt-distribution was unaffected by the HSA concentration (Table 4 and Fig. 2). With regard to the 2 h time point, however, considerable differences were observed. It is useful to discuss the results pertaining to CP first, followed by those pertaining to the hydrolysis products, and lastly those for protein bound Pt. The plasma

Table 4 Pt-peak areas expressed as % of total Pt obtained after SEC–ICP-AES analysis of plasma from pediatric cancer patients and HSA fortified plasma spiked with CP after incubation at 37 °C for 5 min and 2 h. PP = protein bound Pt-species, HP = Pt-containing hydrolysis product, C = pediatric cancer patients

Time/[HSA]	PP-1	PP-2	PP-3 (HSA)	Total PP	HP-1	HP-2	Total HP	CP
C – 5 min (Avg 26 g HSA per L) n = 11	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.4 ± 0.4	2.4 ± 0.4	97.6 ± 0.4
C – 5 min (36 g HSA per L) n = 3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.3 ± 0.4	2.3 ± 0.4	97.7 ± 0.4
C – 5 min (42 g HSA per L) n = 9	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.1 ± 0.4	2.1 ± 0.4	98.0 ± 0.4
C – 2 h (Avg 26 g HSA per L) n = 10	10.2 ± 2.4	10.8 ± 4.6	22.3 ± 5.7	43.2 ± 9.5	4.2 ± 1.7	17.0 ± 4.1	21.2 ± 5.7	35.6 ± 4.4
C – 2 h (36 g HSA per L) n = 10	9.9 ± 3.0	9.1 ± 3.3	29.6 ± 2.5	48.5 ± 5.7	2.4 ± 0.9	13.2 ± 2.3	15.6 ± 3.0	35.9 ± 4.1
C – 2 h (42 g HSA per L) n = 10	10.4 ± 2.6	9.5 ± 2.8	32.8 ± 1.6	52.6 ± 4.3	1.8 ± 0.6	11.1 ± 2.1	12.9 ± 2.5	34.5 ± 3.0

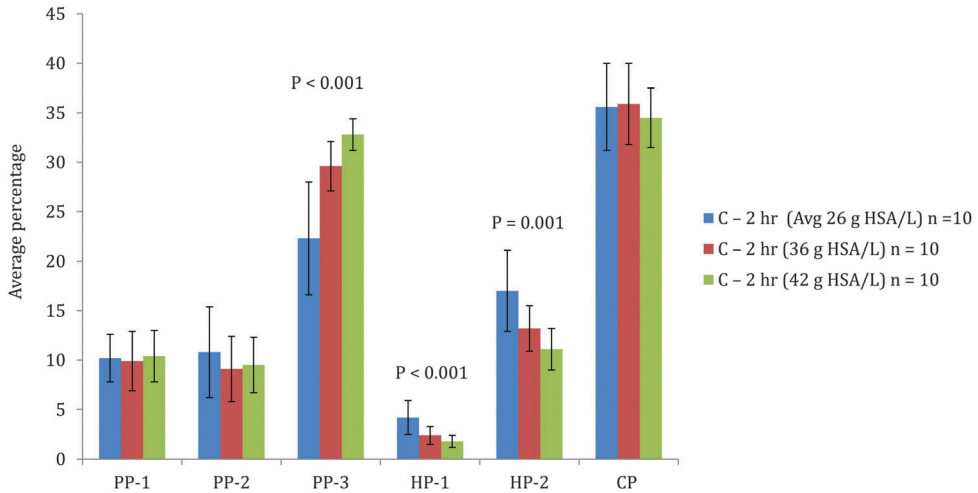

Fig. 2 Comparison of the Pt-peak areas expressed as % of total Pt for plasma from pediatric cancer patients (HSA = 26 g L^{-1}) and HSA fortified plasma (HSA = 36 and 42 g L^{-1}) spiked with CP after incubation at 37 °C for 2 h. The increase of Pt eluting as PP-3 and the decrease of Pt eluting as HP-1 and HP-2 were statistically significant.

concentration of CP was essentially independent of the HSA concentration, which can be rationalized in terms of its hydrolysis being mainly driven by water and the chloride concentration in plasma.[19] In contrast, the Pt-area of both hydrolysis products gradually decreased by 5.6% upon fortification to 36 g HSA per L, and by another 2.7% when the plasma concentration was increased to 42 g HSA per L. In the 42 g HSA per L fortified pediatric cancer patient plasma, the Pt-area of both hydrolysis products was 12.9%. Although this is lower than the 15.9% obtained for healthy adults (42 ± 4 g HSA per L), one needs to take into account that the hydrolysis of CP was faster in healthy adults. Related to this, the total protein bound Pt increased by 5.3% for a plasma HSA concentration of 36 g HSA per L and by another 4.1% for a plasma concentration of 42 g HSA per L, respectively. With regard to protein bound Pt, the percentage of Pt bound to PP-1 was essentially unaffected by the plasma HSA concentration, while the amount of Pt that eluted as PP-2 marginally decreased by 1.7% at 36 g HSA per L and remained essentially unchanged at 42 g HSA per L. The Pt-peak area that was obtained for PP-3 gradually increased by 10.5% over the investigated HSA concentration range and was expected since more binding sites were available for the *in situ* generated CP-derived hydrolysis products. As depicted in Fig. 2, the overall average change of PP-3 (increase), HP-1 (decrease) and HP-2 (decrease) were statistically significant ($P < 0.001$).

In order to rationalize the observed differences in the plasma Pt-distribution between pediatric cancer patients and healthy adults at the 2 h time point, it is instructive to discuss the percentages of Pt eluting in form of three major kinds of platinum species in human plasma, namely protein bound platinum (PP-1, PP-2 and PP-3), CP-derived hydrolysis products (HP-1 and HP-2) and parent CP. Pediatric cancer patient plasma contained on average 4.5% more of the parent CP which implies less hydrolysis compared to that observed in plasma from healthy adults. We hypothesized that this finding may be related to the increased dehydration of cancer patients (BUN/creatinine ratio 5.6–121 in pediatric cancer patients compared to 9.1–24 in healthy adults), which may in turn indicate an increased plasma chloride concentration. The consultation of patient charts for the plasma chloride concentration (±1 day around the time of blood collection), however, revealed an average chloride concentration of 101 ± 4 mM. Since this concentration is essentially identical to the 103 mM that has been reported for healthy adults[12] other factors must be responsible for the decreased hydrolysis of CP in pediatric cancer patient plasma.

Pediatric cancer patient plasma also contained on average 5.3% more CP-derived hydrolysis products. This apparent contradiction can only be rationalized if one also considers that pediatric cancer patient plasma contained 9.3% less protein

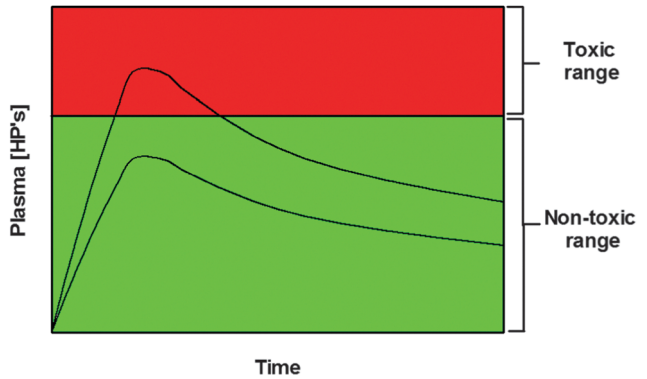

Fig. 3 Putative model which can explain the toxic side-effects of CP *in vivo* based on the obtained *in vitro* results. Lower curve: reduced concentration of hydrolysis products in plasma of healthy controls with >30 g HSA per L. Upper curve: increased concentration of hydrolysis products in patient plasma with <30 g HSA per L which exceeds the threshold for organ based toxicity.

bound platinum. It therefore appears that the decreased HSA concentration in pediatric cancer patient plasma (26 ± 7 g HSA per L) resulted in a reduced binding capacity for the *in situ* generated CP-derived hydrolysis products which increased the concentration of the unbound hydrolysis products HP-1 and HP-2 in plasma at the 2 h time point. The concentrations of free hydrolysis products may therefore exceed critical thresholds to result in toxic side effects as illustrated by the schematic depicted in Fig. 3. More importantly, the fortification of pediatric cancer patient plasma with pure HSA to 42 g L^{-1} decreased the fraction of free HP-1 and HP-2 in plasma by ~40%, while the hydrolysis of CP was essentially unaffected (Table 4). Although it is impossible to predict if the same would occur *in vivo*, our findings do suggest that the intravenous administration of cancer patients with exogenous HSA will increase the number of available binding sites for highly toxic CP-derived hydrolysis products, such as $[PtClOH_2(NH_3)_2]^+$.[22] The associated decrease of the concentration of highly toxic CP-derived hydrolysis products in the blood circulation (Fig. 3) could therefore mitigate the toxic side-effects of intravenously administered CP.

Conclusion

In the context of developing a clinical treatment protocol to reduce the toxic side-effects of CP in cancer patients, it is critical to better understand the role that the concentration of endogenous plasma proteins may play. To this end, we have conducted *in vitro* experiments in which human plasma from healthy controls and pediatric cancer patients was spiked with a pharmacological dose of CP and analyzed for the contained Pt-species by SEC–ICP-AES. At the 2 h time point, the decreased HSA concentration in pediatric cancer patient plasma (26 ± 7 g HSA per L) resulted in 9.8% less protein bound Pt (including HSA) and a 5.3% increase of the total Pt eluting in form of hydrolysis products. These results were corroborated by fortifying individual cancer patient plasma samples to 36 and 42 g HSA per L, which produced results that were somewhat similar to those observed for plasma from healthy adults. Given that CP-derived hydrolysis products are highly reactive and toxic,[22,23] our *in vitro* results – obtained with a relatively small number of plasma samples – suggest that HP-1 and HP-2 likely represent the species that cause the toxic side-effects *in vivo*. Although these findings need to be corroborated by *in vivo* studies using animal models,[24] a putative model suggests that increasing the plasma HSA concentration in cancer patients before CP is administered may alleviate some of its severe toxic side-effects.

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