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Modulation of the metabolism of *cis*-platin in blood plasma by glutathione

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Abstract

The anticancer drug *cis*-platin (CP) is in worldwide clinical use to treat a variety of cancers, but is inherently associated with severe toxic side-effects. Previous animal studies revealed that its neurotoxicity can be significantly reduced by the co-administration of L-glutathione (GSH) without affecting the anti-cancer effect. The underlying molecular mechanism, however, has remained elusive. Since the bloodstream is a likely biological compartment where CP-derived hydrolysis products may react with GSH, we have employed a recently developed metallomics tool to gain insight into the interaction of CP and GSH in rabbit plasma *in vitro*. After the addition of increasing GSH:CP molar ratios to plasma (25:1, 50:1 and 100:1), the determination of the Pt-distribution 5 min and 2 h later revealed the formation of a Pt-GSH complex which did not bind to plasma proteins. The simultaneously obtained Zn-distribution in plasma revealed a progressively more pronounced perturbation of the Zn metalloproteome with increasing GSH:CP molar ratios at the 5 min time point, which partially reversed at the 2 h time point. The formation of Pt-GSH species in plasma is therefore likely to be directly involved in the process by which GSH protects mammalian organisms from CP-induced neurotoxicity, nephrotoxicity and possibly other organ-based toxicities.

Key words

Blood plasma, *cis*-platin, chemoprotection, glutathione, metallomics

70 Introduction

71 The serendipitously discovered anticancer drug *cis*-platin (CP)^{1,2} heralded the dawn of metallodrugs
72 and remains one of the most effective chemotherapeutic agents more than five decades after its
73 discovery³. In fact, 50-70% of all cancer patients are treated with CP either alone or in combination
74 with other anticancer drugs.⁴ Despite its broad spectrum of activity against a large variety of cancers,⁵
75 the intravenous administration of patients with CP is associated with severe toxic side-effects,
76 including nephrotoxicity, ototoxicity and neurotoxicity^{6,7}. Although the nephrotoxicity of CP can be
77 somewhat ameliorated by the administration of patients with hypertonic saline or mannitol,⁸ no
78 clinical procedures exist to completely eliminate its ototoxicity and neurotoxicity which therefore
79 constitute the primary dose limiting factors.⁹

80 The aforementioned toxic side-effects of CP, however, have been shown to be significantly reduced in
81 animal studies when small-molecular-weight (SMW) compounds – also referred to as
82 ‘chemoprotective agents’ – were co-administered.^{9,10} Although this principle approach can adversely
83 affect the anti-tumor effect of CP,⁹ it has been demonstrated that this can be sometimes altogether
84 avoided if the timing between the administration of CP and the chemoprotective agent as well as their
85 route of administration is carefully optimized.¹¹ More recent investigations aimed at better
86 understanding the biomolecular mechanisms by which chemoprotective agents ameliorate the toxicity
87 of CP revealed that sodium thiosulfate, N-acetyl-L-cysteine and D-methionine react with CP-derived
88 hydrolysis products in human blood plasma *in vitro*.¹²⁻¹⁴ Since some of the CP-derived hydrolysis
89 products that are formed in blood plasma are highly toxic,⁹ their ‘neutralization’ by chemoprotective
90 agents likely constitutes one important mechanism of action by which the latter mitigate the toxic
91 side-effects of CP.¹⁵

92 L-Glutathione (GSH) represents another SMW sulfur compound that has been demonstrated to be
93 effective in terms of reducing the toxic side-effects of CP in animals^{16,17} and humans¹⁸. In contrast to

the aforementioned chemoprotective agents, however, GSH is naturally present in human blood plasma at concentrations of 1-5 μM ¹⁹ and the plasma concentration of oxidized glutathione (GSSG) is $\sim 2 \mu\text{M}$ in rats.²⁰ The administration of exogenous GSH to animals to decrease the neurotoxicity of CP without diminishing the anti-tumor effect of the latter.¹⁶ Given that CP (1.0 mg/kg) was administered intraperitoneally and GSH (200 mg/kg) intravenously to rats in one particular study,¹⁶ it is possible that the underlying toxicologically relevant processes occurred in blood plasma.¹²⁻¹⁴ Since no direct experimental evidence in support of this possibility has been obtained, we have conducted systematic *in vitro* experiments with rabbit plasma to which pharmacologically relevant GSH:CP molar ratios – defined as those close to those where the desired amelioration of the toxicity of CP was achieved *in vivo* – were added. The analysis of the obtained mixtures with a metallomics tool – a size-exclusion chromatography (SEC) system coupled on-line to an inductively coupled plasma atomic emission spectrometer (ICP-AES)²¹ – allowed us to observe changes in the Pt-distribution in plasma between the 5 min and 2 h time point. Since ICP-AES allows to simultaneously monitor the emission lines of the endogenous Fe, Cu and Zn-metalloproteins in addition to those of Pt, changes in the plasma distribution of these metals (e.g. caused by GSH) can also be observed. The results revealed a rapid formation of Pt-GSH adduct(s) in plasma, which strongly suggests their formation to be directly implicated in the mechanism by which GSH can ameliorate the toxic side-effects of CP *in vivo*.^{16,17,22}

Experimental

Chemicals and Solutions

Cis-platin (1 mg cis-Pt(NH₃)₂Cl₂/mL; this solution also contained 1 mg mannitol and 9 mg NaCl; sterile) was obtained from Hospira (Montreal, QC, Canada). Phosphate-buffered saline buffer (PBS) tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the corresponding PBS-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 (Simplicity water purification system; Millipore, Billerica, MA, USA) followed by pH adjustment to 7.4. 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 SuperdexTM 200 Increase SEC column. GSH ($\geq 98\%$) was obtained from Sigma-Aldrich and an aqueous stock solution with a concentration of 142 mg/mL (0.46 M) was prepared by dissolving it in PBS-buffer followed by pH adjustment to 7.4 using 4 M NaOH. This solution was prepared daily and was used within 6 hours during which it was stored at 5 °C in a vial with minimal head space.

SEC-ICP-AES system

The SEC-ICP-AES system that was employed 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 SuperdexTM 200 Increase 10/300 GL high performance size-exclusion chromatography (SEC) column (30 x 1.0 cm I.D., particle size 8.6 μm , fractionation range 600 - 10 kDa; GE Healthcare, Piscataway, NJ, USA) was used in conjunction with a PBS-buffer mobile phase (as described in ‘chemicals and solutions’) and a flow rate of 1.0 mL/min (column temperature 22° C). We note that the employed SEC column contains smaller particles than the columns that we used in our previously studies,^{12-14,23} but that the backbone is identical and resulted in a similar separation of plasma proteins (data not shown). Simultaneous multielement-specific detection of Ca (317.933 nm), C (193.091 nm), Cu (324.754 nm), Fe (259.940 nm), Mg (280.271 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, 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/min. A 400 s delay was implemented between injection and data acquisition and data acquisition window was 2000 s. The raw data (Salsa Software) were imported into Sigmaplot 13.0, smoothed using the bi-square algorithm and then the peak areas were determined.

Preparation of blank plasma, CP-spiked plasma and GSH-spiked plasma

Rabbit plasma (Lot 0165) was obtained from Cedarlane Corporation (Burlington, ON, Canada) and had been prepared by pooling plasma from 3.5 months old male New Zealand white rabbits using sodium heparin (158 USP units/10 mL of whole blood) as the anticoagulant. Plasma (2.2 mL aliquots) was stored at -60 °C and received frozen on dry ice. Individual plasma vials were thawed at room temperature for 45 minutes and incubated at 37° C for 30 min before pharmacologically relevant doses of CP and/or GSH were added. “Blank” plasma was prepared by spiking 1.8 mL of rabbit plasma with 130 µL of PBS-buffer. The solution that served as the CP control plasma was prepared by adding 75 µL of the CP stock solution (1 mg/mL) and 54 µL of PBS-buffer to 1.8 mL of plasma. The final concentration was ~0.04 mg CP/mL of plasma (~0.13 mM), which is pharmacologically/therapeutically relevant²⁴. This mixture was incubated at 37° C and samples were withdrawn for analysis after 5 min and 2 h.

Analysis of GSH and CP-spiked plasma (GSH:CP molar ratios 25:1, 50:1 and 100:1)

Plasma (1.8 mL) was incubated at 37° C, spiked with 54 µL of the GSH stock solution and 1 min later 75 µL of the CP stock solution (1 mg/mL) was added (GSH:CP molar ratio of 100:1). The obtained mixture was incubated at 37° C and analyzed after 5 min and 2 h. The solution that served as the GSH

control (corresponding to the GSH:CP=100:1 molar ratio) was prepared by adding 54 μ L of GSH stock solution and 75 μ L of PBS-buffer to 1.8 mL of plasma. The obtained mixture was incubated at 37° C for 6 min and 2 h + 1 min and was subsequently analyzed.

The solution corresponding to the GSH:CP molar ratio of 50:1 was prepared by spiking 1.8 mL of plasma (that had been incubated at 37° C) with 27 μ L of the GSH stock solution and 27 μ L of PBS-buffer. After mixing 75 μ L of the CP stock solution (1 mg/mL) were added 1 min later. The solution corresponding to a GSH:CP molar ratio of 25:1 was prepared in an analogous manner by spiking 1.8 mL of plasma with 13.5 μ L of the GSH stock solution and 41 μ L of PBS-buffer. After incubation at 37° C for 1 min, 75 μ L of the CP stock solution (1 mg/mL) was added and the obtained mixture was incubated at 37° C and analyzed 5 min and 2 h later. All experiments were carried out in triplicate and representative chromatograms are presented.

Analysis of CP-spiked PBS-buffer and GSH+CP-spiked PBS-buffer (GSH:CP molar ratio 100:1)

The CP control was prepared by spiking PBS buffer (pH 7.4, 1.8 mL) that had been incubated at 37° C with 75 μ L of the CP stock solution (1 mg/mL) and 54 μ L of PBS-buffer. After incubation of this mixture at 37° C, the latter was analyzed after 5 min and 2 h. Then, PBS buffer (pH 7.4, 1.8 mL) was spiked with 54 μ L of GSH stock solution, incubated at 37° C for 1 min and then spiked with 75 μ L of the CP stock solution (1 mg/mL). The obtained mixture was maintained at 37° C and samples were withdrawn for duplicate analysis after 5 min and 2 h. All attempts to separate excess GSH from the co-eluting Pt-S peak on a PRP X-100 anion-exchange HPLC column (30 x 0.46 cm I.D.) using a variety of different PBS-buffer concentrations were unsuccessful.

Results and Discussion

189 After the intravenous administration of cancer patients with CP, all CP-derived metabolites will
190 interact with blood constituents, tumor cells (intended) and healthy tissue cells (unintended). Since the
191 latter interaction is associated with organ-based toxicities²⁵⁻²⁸, various approaches are currently being
192 explored to increase the tumor selectivity of metal-based drugs⁷. In principle, this can be achieved by
193 optimizing the tumor selectivity of novel (Pt-based) drug structures^{3,29,30} or by improving the selective
194 delivery of established anticancer drugs to tumor cells (e.g. using substituted β -cyclodextrins and calix
195 [4] arenes)³¹⁻³³.

196 Another feasible strategy to improve the safety of clinically used anticancer metallodrugs aims to
197 selectively reduce their toxic side-effects by the co-administration of chemoprotective agents.
198 Unfortunately, the only chemoprotective agent that has been clinically approved to date, amifostine,
199 did not result in significantly reduced ototoxicity compared to the cisplatin only group.⁹ Three other
200 chemoprotective agents, sodium thiosulfate, N-acetylcysteine and D-methionine, however, were
201 recently demonstrated to modulate the metabolism of CP in human plasma *in vitro*.¹²⁻¹⁴ The common
202 underlying biomolecular mechanism appears to involve the reaction of CP-derived hydrolysis products
203 with each chemoprotective agent to form Pt-chemoprotective agent complexes, some of which could
204 be structurally characterized.¹⁴ The fact that the parent anticancer drug CP remained in plasma for up
205 to 50 min – at least these *in vitro* studies – demonstrates that the co-administration of CP with
206 chemoprotective agents will not entirely inactivate the parent anticancer drug. Since this principle
207 approach has potential to improve the quality of life of patients that are being treated with CP, we
208 wanted to gain insight into the mechanism by which GSH reduced the toxic side-effects of CP in
209 previously conducted animal studies^{16,17} and one study involving cancer patients.¹⁸ To elucidate if and
210 in what way GSH will affect the metabolism of CP in rabbit plasma, we have conducted *in vitro*
211 experiments which involved the addition of increasing GSH:CP molar ratios to plasma followed by its
212 analysis with a recently developed metallomics method. The latter allows to simultaneously determine

213 essentially all CP-derived Pt-metabolites in plasma²³ and all major Cu, Fe and Zn-containing
214 endogenous metalloproteins.²¹

215

216 **Metabolism of CP in plasma**

217 After the addition of a pharmacologically relevant dose of CP to plasma, the obtained mixture was
218 analyzed after 5 min and 2 h (Fig. 1A). At the 5 min time point 1.5% of total Pt eluted bound to plasma
219 proteins (Pt-PP), ~4% in form of CP-derived hydrolysis products (CPHP) and almost 95% as CP
220 (Table 1). The results that were obtained for the 2 h time point revealed a significant increase of Pt-PP
221 (26.6%), that 22.6% of total Pt eluted in form of CPHP's and that ~51% of free CP was present in
222 plasma (Table 1). In general, these results are in good accord with previous results that were obtained
223 after the addition of the same CP-dose to human plasma.²³

224

225 **Effect of increasing GSH:CP molar ratios on the metabolism of CP in plasma**

226 In order to observe the effect of GSH on the metabolism of CP, increasing amounts of GSH were
227 added to plasma first followed by a pharmacologically relevant dose of CP 1 min later to achieve
228 GSH:CP molar ratios of 25:1, 50:1 and 100:1. The obtained mixtures were analyzed after 5 min and 2
229 h (Fig. 1B-D). The average peak area counts for each detected Pt peak (as percent of the total Pt area)
230 are depicted in Table 1. For the sake of clarity, the results will be discussed first for the 5 min time
231 point, followed by those obtained for the 2 h time point.

232 At the 25:1 GSH:CP molar ratio (Fig. 1B) and compared to the control experiment (Fig. 1A), no
233 statistically significant increase was observed for Pt-PP, but 2.4% of Pt eluted in form of a Pt-peak that
234 was not detected in the control experiment and corresponds to a putative *in situ* formed Pt-GSH
235 complex. The retention time of this Pt-GSH complex was comparable to that of related Pt-complexes
236 that were detected in human plasma after incubation with CP and sodium thiosulfate,¹² N-acetyl-L-

cysteine¹³ or D-methionine.¹⁴ Furthermore, 0.6% less Pt eluted in form of CPHP's and 2.2% less Pt eluted in form of CP (compared to the control group). An increase of the GSH:CP molar ratio from 25:1 to 100:1 marginally affected the amount of Pt that eluted as Pt-PP, but significantly increased the amount of Pt that eluted in form of a Pt-GSH complex by 5.2% (Fig 1B-D, red lines). In accord with these results, the amount of Pt that eluted in form of CPHP's decreased by 0.4% and 7.2% less Pt eluted in form of CP at the 100:1 GSH:CP molar ratio (compared to the control group).

As expected, the results that were obtained for increasing GSH:CP molar ratios on the Pt-distribution in plasma at the 2 h time point were progressively more pronounced (Fig. 1B-D, blue lines) compared to the control group (Fig. 1A, blue line). Increasing the GSH:CP molar ratio from 25:1 to 100:1 dramatically decreased the amount of Pt that eluted in form of Pt-PP by 14.4% and increased that of a putative Pt-GSH complex by ~77%. The amount of Pt that eluted in form of CPHP's decreased by ~21% and that of free CP in plasma decreased by 41.2%. Overall, these results demonstrate that GSH can – depending on the employed GSH:CP molar ratio – profoundly affect the metabolism of CP in plasma by accelerating its hydrolysis and decreasing the amount of CPHP's. This apparent discrepancy can be rationalized in terms of the rapid formation of Pt-GSH complexes, either based on the reaction of GSH with CP-derived hydrolysis products or by the direct replacement of a chloro ligand from CP by GSH.³⁴ These Pt-GSH complexes did not bind to plasma proteins and therefore resulted in a decrease of the amount of total Pt that eluted in form of Pt-PP. Since the endogenous plasma GSH concentration in rabbit plasma is negligible compared to the concentrations that were obtained after the addition of GSH to plasma in our experiments, it is unlikely to have affected the obtained results.

Effect of increasing GSH:CP molar ratios on the Zn-metalloproteome in plasma

Since the analysis of plasma with the employed metallomics method allows to simultaneously determine all major endogenous Cu, Fe and Zn metalloproteins, we noted an effect of increasing GSH:CP molar ratios on the Zn-metalloproteome, but not on the Cu and Fe-metalloproteome (data not

shown). The average peak area counts for PP-bound Zn and SMW Zn (as percent of the total Pt area) are depicted in Table 2. The addition of a pharmacologically relevant dose of CP to plasma followed by its analysis 5 min and 2 h later did not reveal any significant changes to the Zn-metalloproteome (Fig. 2A, red and blue line). The fact that the obtained Zn-metalloproteome pattern for plasma spiked with CP was very similar to that observed for untreated plasma (Fig. 2A, green line) indicates that CP did not perturb the endogenous Zn-metalloproteome. These results are in accord with previous studies that were conducted with human plasma.²³

Strikingly different results, however, were observed for the Zn-metalloproteome in plasma with increasing GSH:CP molar ratios (Fig. 2B-D, Table 2). Correspondingly, all of these detected changes must be attributed to the presence of GSH. The results that were obtained for the 25:1 GSH:CP molar ratio revealed a distinct perturbation of the Zn-metalloproteome at the 5 min time point (Fig. 2B, orange line) and can be rationalized in terms of a GSH-mediated re-distribution of Zn from what appears to be human serum albumin (HSA) to higher molecular weight (HMW) PP's. In addition, a Zn-peak eluted in the inclusion volume (~1200 s), which is indicative of a SMW Zn-species in plasma, possibly a (GS)₂-Zn complex³⁵. Interestingly, the Zn-metalloproteome pattern that was obtained for the 2 h time point (Fig. 2B, blue line) more closely resembled the pattern of unperturbed (native) rabbit plasma (Fig. 2A, green line). This finding can be rationalized in terms of a partial reversal of the binding of Zn from HMW-PP and SMW moieties to HSA. A molecular explanation for this redistribution of Zn is unavailable at present, but may involve exposure to air which was not precluded in our experiments. The results that were obtained for the 50:1 GSH:CP molar ratio and the 5 time point (Fig. 2C, orange line) revealed an even more pronounced re-distribution of Zn from HSA to two HMW-PP and the elution of a more intense Zn-peak in the inclusion volume. The Zn-metalloproteome that was observed at the 2 h time point (Fig. 2C, blue line) again revealed a partial redistribution of Zn from both HMW-PP to HSA, but not the SMW Zn-species. At the 100:1 GSH:CP molar ratio and the 5

min time point the comparatively most pronounced re-distribution of Zn from HSA to HMW-PP was observed (we note that Zn-peak corresponding to HSA was marginally shifted to shorter retention times). At the 2 h time point, a partial back distribution of Zn was observed from both HWM-PP's and the SMW Zn-species to HSA.

Effect of GSH on the metabolism of CP in PBS-buffer

To corroborate the results that were obtained with a GSH:CP molar ratio of 100:1 in rabbit plasma, the same pharmacologically relevant dose of CP was added to PBS-buffer, incubated at 37°C and analyzed after 5 min and 2 h (Fig. 3A, red line). Although at the 5 min time point free CP corresponded to the majority of Pt that eluted from the column (97.0%), 3.0% of Pt eluted in form of a CPHP (presumably the mono-aqua Pt-species)²³. At the 2 h time point, the intensity of the parent CP peak had somewhat decreased (84.1% of total Pt) and 15.9% of total Pt eluted in form of the same CPHP peak that was detected at the 5 min time point (Fig. 3A, blue line). These dynamic changes, however, were much less pronounced compared to those obtained when the same CP dose was incubated with rabbit plasma (Fig. 1A, blue line). This difference can be rationalized in terms of the absence of PP in the PBS-buffer experiment and the associated impact that the lack of PP will have on the dynamic equilibrium that exists in plasma between CP, CPHP's and Pt-PP.²³

When GSH was added to PBS-buffer first followed 1 min later by CP (effective GSH:CP molar ratio 100:1) and the latter mixture was analyzed 5 min later (Fig. 3B, red line), a new Pt-peak (3.7% of total Pt) with a retention time of ~1200 s was detected, which eluted before the CPHP (2.4% of total Pt) and CP (93.9% of total Pt). At the 2 h time point, this new Pt-peak (77.9% of total Pt) was the most intense Pt-peak that was detectable (Fig. 3B, blue line), while the Pt-peak corresponding to CPHP (1.1 % of total Pt) was negligible compared to the second most intense Pt-peak, which corresponded to CP (21.0% of total Pt) was smaller than what was observed at the 5 min time point. These results strongly

suggested that a Pt-GSH complex had formed in PBS-buffer. Direct experimental evidence in support of the formation of this species could be obtained by plotting Pt together with the corresponding S and C-specific chromatograms for the 2 h time point (Fig. 3, pink and green lines, respectively). All attempts to structurally characterize this Pt-GSH complex by electrospray-ionization mass spectrometry (ESI-MS) failed, most likely due to the high salt content (>1%) of the PBS-buffer matrix. The reaction between CP and GSH has been previously investigated under near physiological conditions using ^1H , ^{13}C and ^{195}Pt -NMR³⁴ and it was noted that although the mono-substituted species *cis*[PtCl(GS)($^{15}\text{NH}_3$)₂] was formed first, several other species containing the $^{15}\text{NH}_3$ -Pt-S linkage were present within a few minutes. The final reaction product was a HMW polymer with a 1:2 Pt:GSH stoichiometry.³⁴ In our experiments, the retention time of the Pt-GSH complex that had formed in PBS-buffer was identical to that of the putative Pt-GSH complex in rabbit plasma (the presence of the latter was corroborated by the co-elution of a SMW S-peak with a Pt-peak in the rabbit plasma experiment; Supplementary Figure S1). Therefore, our results directly implicate the formation of Pt-GSH complexes in blood plasma as the likely biomolecular basis by which GSH ameliorated the CP-induced toxic side-effect in previously reported animal experiments.^{16,17} The fact that in previous animal experiments the administration of GSH significantly reduced the neurotoxicity without adversely affecting the anticancer efficacy¹⁶ may be rationalized in terms of the comparatively much longer residence time of the active anticancer species CP (> 2 h) in rabbit plasma in our *in vitro* experiments (Fig. 1). This is in contrast to our previous *in vitro* experiments in which free CP was essentially absent in plasma when the latter was incubated with sodium thiosulfate, N-acetyl-L-cysteine or D-methionine for 50 min.¹²⁻¹⁴ Our results must be clearly distinguished from the well-established intracellular formation of Pt-GSH complexes,³⁶ which effectively inactivate CP.³⁷ Future studies should be aimed at elucidating the structure of the Pt-GSH complex(es) as well as its/their toxicity, biochemical fate and excretion from mammalian organisms.

333

334 **Conclusion**

335 Although CP is an effective anticancer drug, it inherently exerts severe dose-limiting toxic side-effects,
336 which can dramatically reduce the quality of life in patients, sometimes permanently. It has been
337 demonstrated in animal studies, however, that GSH can reduce the neurotoxicity and nephrotoxicity of
338 CP without notably reducing its efficacy. To possibly translate these promising results to benefits for
339 patients that are being treated with CP, it is important to elucidate the underlying mechanism of action.
340 To this end, we have conducted *in vitro* experiments in which increasing GSH:CP molar ratios (25:1,
341 50:1 and 100:1) were added to rabbit plasma. The determination of the Pt-distribution in plasma after 5
342 min and 2 h revealed significant changes compared to the metabolism of CP and provided evidence for
343 the formation of a Pt-GSH complex which did not bind to plasma proteins. These results were
344 corroborated by reacting GSH and CP (molar ratio 100:1) in PBS-buffer. Analysis of the obtained
345 mixture revealed the co-elution of a species which contained C, S and Pt (2 h time point) and had the
346 same retention time as a Pt-species that was detected in rabbit plasma. These findings conclusively
347 identify the formation of a Pt-GSH complex in plasma as one possible mechanism by which GSH
348 ameliorated the CP-induced toxic side-effects in animal studies without adversely affecting the
349 anticancer effect.^{16,17} In addition, at all investigated GSH:CP molar ratios a perturbation of the Zn
350 metalloproteome – which is attributed to GSH – was observed at the 5 min time point, while at the 2 h
351 time point the Zn distribution was more similar to that observed for untreated rabbit plasma.

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356 **Figure Captions:**

Figure 1: Representative Pt-specific chromatograms that were obtained after the addition of (A) *cis*-platin (CP) to rabbit plasma and analyzed after 5 min (red line) and 2 h (blue line) of incubation at 37° C. Then, L-glutathione (GSH) was added to rabbit plasma incubated for 1 min (37° C) and subsequently CP was added and the obtained mixtures were analyzed 5 min (red line) and 2 h (blue line) later by SEC-ICP-AES at GSH:CP molar ratios 25:1 (B), 50:1 (C) and 100:1 (D). Stationary phase: Superdex 200 Increase 10/300 GL (30 x 1.0 cm I.D., 8.6 µm particle size) SEC column at 22° C; Mobile phase: PBS-buffer (0.15 M, pH 7.4); Flow rate: 1.0 mL/min, Injection volume: 500 µL; Detector: ICP-AES at 214.423 nm (Pt). The retention times of the molecular markers are depicted on top of the figure.

Figure 2: Representative Zn-specific chromatograms that were obtained for blank rabbit plasma [(A) green line], *cis*-platin (CP) spiked plasma [(A) 5 min (red line) and 2 h (blue line) of incubation at 37° C after addition of CP] and plasma which was spiked with L-glutathione (GSH) (incubated for 1 min at 37° C) and CP using a molar ratio of 25:1 (B), 50:1 (C) and 100:1 (D) [5 min (red line) and 2 h (blue line) of incubation at 37° C after addition of CP]. Stationary phase: Superdex 200 Increase 10/300 GL (30 x 1.0 cm I.D., 8.6 µm particle size) SEC column at 22° C; Mobile phase: PBS-buffer (0.15 M, pH 7.4); Flow rate: 1.0 mL/min, Injection volume: 500 µL; Detector: ICP-AES at 213.856 nm (Zn). The retention times of the molecular markers are depicted on top of the figure. HSA – human serum albumin.

Figure 3: (A) Representative Pt-specific chromatograms obtained after the addition of *cis*-platin (CP) to PBS-buffer and SEC-ICP-AES analysis after 5 min and 2 h of incubation at 37° C. (B) PBS-buffer was spiked with L-glutathione (GSH), incubated at 37° C for 1 min and subsequently spiked with CP (GSH:CP molar ratio 100:1). The obtained mixture was

analyzed after 5 min (red line) and 2 h (blue line) of incubated at 37° C by SEC-ICP-AES. Simultaneously obtained S-(pink line) and C-(green line) specific chromatograms are also depicted. Stationary phase: Superdex 200 Increase 10/300 GL (30 x 1.0 cm I.D., 8.6 µm particle size) SEC column (22° C); Mobile phase: PBS-buffer (0.15 M, pH 7.4); Flow rate: 1.0 mL/min, Injection volume: 500 µL; Detector: ICP-AES at 214.423 nm (Pt), S (180.731 nm) and C (193.091 nm). The retention times of the molecular markers are depicted on top of the figure.

Supplementary Figure S1:

Representative Pt- and S-specific chromatograms that were obtained 5 min (red line) and 2 h (blue line) after L-glutathione (GSH) was added to rabbit plasma, the latter mixture was incubated for 1 min (37° C) and *cis*-platin (CP) was added by SEC-ICP-AES. GSH:CP molar ratio 100:1. Stationary phase: Superdex 200 Increase 10/300 GL (30 x 1.0 cm I.D., 8.6 µm particle size) SEC column at 22° C; Mobile phase: PBS-buffer (0.15 M, pH 7.4); Flow rate: 1.0 mL/min, Injection volume: 500 µL; Detector: ICP-AES at 214.423 nm (Pt) and 180.731 nm (S). The retention times of the molecular markers are depicted on top of the figure.

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481 **Fig.1**

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483 **Fig. 2**

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485 Fig. 3

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Table 1: Peak areas of the Pt peaks (indicated as percentage values with respect to the total Pt area) obtained after the analysis of rabbit plasma spiked with *cis*-platin (CP), rabbit plasma spiked with L-glutathione (GSH) and CP using GSH:CP molar ratios 25:1, 50:1 and 100:1 by SEC-ICP-AES, after incubation of the mixture at 37 °C (n=3, Mean ± SD).

Experiment	Molar ratio GSH:CP	Analysis after	Assigned Pt-peaks				Total Pt area
			Plasma protein bound Pt	Pt-GSH complex	CP-derived hydrolysis products	CP	
Plasma + CP	N/A	5 min	1.5 ± 0.5	-	4.1 ± 0.6	94.4 ± 1.1	10139 ± 895
		2 h	26.6 ± 2.6	-	22.6 ± 0.7	50.8 ± 2.4	9914 ± 536
Plasma + GSH (1 min) + CP	25:1	5 min	1.9 ± 0.1	2.4 ± 0.3	3.5 ± 0.1	92.2 ± 0.4	11094 ± 1204
		2 h	22.2 ± 0.9	29.7 ± 2.7	11.3 ± 0.6	36.8 ± 1.4	10798 ± 1389
	50:1	5 min	1.8 ± 0.3	3.4 ± 0.8	3.2 ± 0.5	91.6 ± 1.1	11884 ± 709
		2 h	16.4 ± 0.37	55.5 ± 1.8	5.3 ± 0.5	22.8 ± 1.7	11774 ± 682
	100:1	5 min	2.1 ± 0.2	7.6 ± 0.5	3.1 ± 0.3	87.2 ± 0.8	11449 ± 1076
		2 h	12.2 ± 0.2	76.7 ± 1.9	1.5 ± 0.40	9.6 ± 1.3	11059 ± 660

Table 2: Peak areas of the Zn peaks (indicated as percentage values with respect to the total Zn area) obtained after the analysis of rabbit plasma, rabbit plasma spiked with *cis*-platin (CP), rabbit plasma spiked with L-glutathione (GSH) and CP using GSH:CP molar ratios 100:1, 50:1 and 25:1 by SEC-ICP-AES, after incubation of the mixture at 37 °C (n=3, Mean $\pm$ SD).

Experiment	Molar ratio GSH:CP	Analysis after	Assigned Zn-peaks		Total Zn area
			Plasma protein bound Zn	Small molecular weight Zn-peak < 17 kDa	
Plasma	N/A		100	0	24421 $\pm$ 861
Plasma + CP	N/A	5 min	100	0	22406 $\pm$ 2601
		2 h	100	0	21895 $\pm$ 2997
Plasma + GSH (1 min) + CP	25:1	5 min	89.9 $\pm$ 0.7	10.1 $\pm$ 0.7	23021 $\pm$ 3050
		2 h	92 $\pm$ 0.6	8 $\pm$ 0.6	21073 $\pm$ 2651
	50:1	5 min	88.7 $\pm$ 0.8	11.3 $\pm$ 0.8	24194 $\pm$ 1868
		2 h	88.4 $\pm$ 0.5	11.6 $\pm$ 0.5	23165 $\pm$ 1507
	100:1	5 min	86.5 $\pm$ 0.7	13.5 $\pm$ 0.7	23270 $\pm$ 2234
		2 h	88.9 $\pm$ 0.4	11.1 $\pm$ 0.4	22277 $\pm$ 1848