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Protein Binding of a Novel Platinum-Based Anticancer Agent BP-C1 Containing a Lignin-Derived Polymeric Ligand

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Abstract: Platinum (Pt) antineoplastic agents remain indispensable for the treatment of oncological disease. Pt-based drugs are mainly used in the therapy of ovarian cancer and non-small-cell lung carcinoma. A novel platinum-containing antineoplastic agent BP-C1 is a complex of diamminoplatinum with an oxygen-donor polymeric ligand of benzene-polycarboxylic acids, isolated from natural lignin. The aim of the study was to investigate ex vivo protein binding of BP-C1. Protein binding of BP-C1 was tested using equilibrium dialysis. Pooled blood plasma was used in the study. Control solutions contained the same dosages of BP-C1 in PBS (pH 7.2). Plasma and control solutions were submitted to equilibrium dialysis across a vertical 8 kDa cut-off membrane for 4 h at 37 °C under gentle shaking. Platinum was quantified in dialysis and retained fractions using inductively coupled plasma mass spectrometry after microwave digestion. The dialysis system was tested and validated; this showed no protein saturation with platinum. A medium degree of binding of platinum to macromolecular species of ca. 60% was observed. The study showed the maintenance of a high fraction of free BP-C1 in the bloodstream, facilitating its pharmacological activity.

Keywords: protein binding; free platinum; anticancer drugs; lignin; equilibrium dialysis; inductively coupled plasma mass spectrometry; benzene-polycarboxylic acids; ex vivo studies

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1. Introduction

Platinum-based drugs have been actively exploited for the treatment of oncological diseases since the middle of the twentieth century [1,2]. Platinum (Pt) antineoplastic agents remain indispensable for the treatment of ovarian cancer and non-small-cell lung carcinoma [3,4]. The mechanism of action for these drugs is not fully understood; however, the most well-accepted pathway is the binding of active platinum moieties to DNA, forming non-reparable adducts and driving cancer cell apoptosis [5]. Currently, novel platinum-binding ligands are actively investigated as they improve the pharmacokinetic profile of the platinum component and exhibit their own biological activity [6].

In the current study, a novel platinum-containing antineoplastic agent BP-C1 was tested. A platinum atom of BP-C1 originates from cisplatin (Figure 1a) as a raw material. BP-C1 contains two O-donor ligands in the coordination sphere of platinum similar to carboplatin (Figure 1b). BP-C1 is a cis-coordinated complex of platinum (II) with a polymer of benzene-polycarboxylic acids derived from lignin (gross formula $C_{83}H_{70}N_2O_{27}Pt$) [7]. A structure of BP-C1 is presented in Figure 1c. The ligand in BP-C1 has a wide spectrum of biological activity [8]; the molecular mechanism is the interaction of the lignin-derived polymer with glucocorticoid and serotonin/5-HT1 receptors, regulating inflammatory responses [9]. Clinical trials of BP-C1 were undertaken in female patients with metastatic breast carcinoma (ClinicalTrials.gov identifiers: NCT04298333, NCT03789019, NCT03603197, NCT02783794

and NCT01861509). The trials indicated that prolonged intramuscular administration of BP-C1 resulted in the stabilisation of tumour growth and normalised haematological parameters [10,11]. Chemical characterisation of the lignin-derived BP-Cx-1 ligand was previously reported [8].

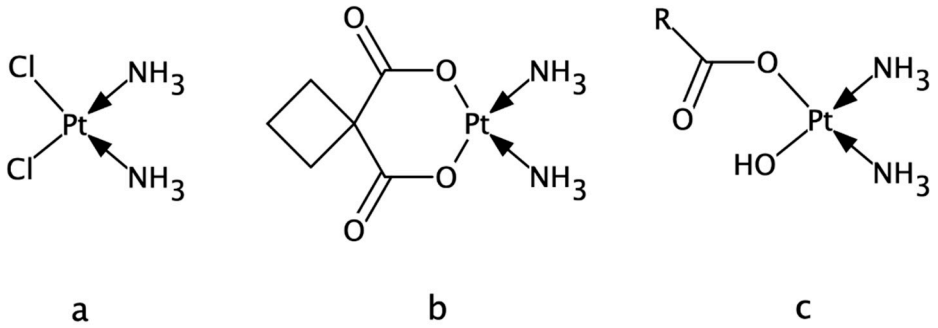

Figure 1. Chemical structures of cisplatin (**a**), carboplatin (**b**) and BP-C1 (**c**). R is a fragment of the BP-Cx-1 ligand with the average molecular weight of 1500–2000 Da.

The degree of protein binding with the drug can ultimately increase or decrease a drug's performance, depending on how much bound or unbound (free) drug there is [12]. The main target of platinum-based metal drugs at a molecular level is genomic DNA [13]. However, these drugs are able to interact with plasma proteins such as HSA. These drug–protein interactions may be the cause of drug resistance and a multitude of side effects, which may play a role in the drugs' poor therapeutic index [14]. Around 24 h after the administration of cisplatin into the bloodstream, 65–98% of this drug is bound to a variety of different plasma proteins, with the highest fraction bound to HSA [15,16]. Cisplatin can bind to and form stable complexes with two interaction sites on an HSA molecule. These involve sulphur donors that are present in methionine residues and as a free cysteine residue, cysteine-34 [17]. Unlike other xenobiotics, the binding of HSA to cisplatin has been noted as being an irreversible reaction with only 5% of the bound protein being released [18]. Thus, cisplatin binding to HSA has been considered therapeutically inactive [19]. The hydrolysis of cisplatin forms chemically active platinum species that can later convert into a cisplatin complex with HSA, which in turn can have reducing properties. This can also cause the inactivation of the cisplatin drug, also affecting the severe side effects of platinum drugs, such as nephrotoxicity [20–22].

Our recent study [23] demonstrated that increased protein binding might be crucial for the reduced side effects of cisplatin under hyperthermic intraperitoneal chemoperfusion compared to the standard infusion administration. The aim of the current study was to study the protein binding of a novel anticancer agent with the polymeric lignin-derived ligand BP-C1 using equilibrium dialysis.

2. Materials and Methods

2.1. Tested Compound, Chemicals and Reagents

BP-C1 was provided by Meabco A/S (Copenhagen, Denmark)—0.5% aqueous solution of CDBPA (batch No. P112K14A1), containing 550 mg/L of platinum, was used.

A standard sample of sterile-filtered USA-origin human donor plasma (cat No. H4522), purchased from Sigma-Aldrich (St. Louis, MO, USA), was used to test protein binding of BP-C1. Phosphate-buffered saline (PBS, pH 7.2) and warfarin (cat No. A2250) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and cisplatin was purchased from Heraeus Holding GmbH (Hanau, Germany). Concentrated 65% nitric acid of Suprapure® grade was purchased from Merck (Darmstadt, Germany). CertiPUR® Platinum ICP standard 1000 mg/L Pt (Merck, Darmstadt, Germany) and Rhodium CGRHN-1 1001 ± 5 µg/mL (Inorganic Ventures, Christiansburg, WV, USA) were used for platinum quantification and as an internal standard, respectively.

2.2. Equilibrium Dialysis

The binding was tested using a standardised protocol [24] with sterile-filtered human donor blood plasma. In brief, pooled blood plasma was mixed with PBS with pH 7.2 (dilution 1:2 per volume) containing BP-C1 with concentrations of 500, 250, 125 and 62.5 µg/L; this corresponds to ca. 55.0, 27.5, 13.7 and 6.9 µg Pt/L. Control solutions contained the same dosages of BP-C1 in PBS (pH 7.2). Fifty percent plasma and control solutions were loaded into a Teflon 96-cell dialysis plate with a vertical 8 kDa cut-off dialysis membrane. Rapid Equilibrium Dialysis (RED) Device Single-Use Plate with Inserts, 8K MWCO (cat No. 90006, Thermo Scientific, Rockford, IL, USA) was used for this purpose. The solutions were equilibrated with PBS at pH 7.2 for 4 h at 37 °C under gentle shaking. The dialysis duration was tested in a preliminary experiment with dialysis tubes in a Float-A-Lyzer G2 Dialysis Device (Repligen, Rancho Dominguez, CA, USA) with 8–10 kDa cut-off, indicating that 2 h is sufficient for stable dialysis; cisplatin was used as a control compound—see Tables S1 and S2 (Supplementary Information). Retained and diffused fractions (0.5 mL) were collected and stored at −80 °C until further analysis. For each dosage, plasma and the control buffer were tested in parallel in 6 replicates.

2.3. Platinum Quantification

Platinum was quantified in plasma and plasma dialysates using inductively coupled plasma mass spectrometry (ICP-MS) after microwave digestion, as previously reported [7,23,25]. In brief, plasma or dialysate samples were digested with concentrated nitric acid in Teflon vessels and adjusted to the final volume of 25 mL in PFA volumetric flasks. The isotope of ^{195}Pt was used for ICP-MS quantification using external calibration.

2.4. Quality Assurance

The dialysis system (the membrane and the cell) was preliminarily checked using warfarin as a standard compound. Warfarin was tested in the same setup, while its concentration was determined using high-performance liquid chromatography-mass spectrometry following a Sigma-Aldrich protocol [26]. The obtained results for warfarin's protein binding were in line with the described data for this drug [27], indicating good performance of the system used. The establishment of equilibrium within 4 h of dialysis was established beforehand in a separate experiment using dialysis tubes with 10 kDa cut-off (Tables S1 and S2).

Recovery of platinum in the dialysis cell was assessed for dialysis buffer measuring platinum concentration in the original solution and after 4 h equilibration for all dosages studied (500, 250, 125, 62.5 µg/L BP-C1). Recovery values were calculated between the original, retained and dialysed compartments, with a range of 90–106%. This showed the absence of irreversible sorption of platinum on the cell's surfaces or the membrane.

The standard plasma specimen used was preliminarily checked for platinum content, demonstrating the concentration below the limit of detection (<1 µg/L). The quantification technique was previously validated using a spiking method in a broad concentration range. For more details, the reader is referred to the publication by Navolotskii et al. [25].

2.5. Data Processing and Statistics

Dialysis data were used for the calculation of relative retention as a ratio between platinum concentration in the initial cell and total platinum concentration (sum of retained and dialysed platinum), expressed as a percentage. To compare retention between 50% plasma and the control buffer, a paired t-test was used. The Shapiro–Wilk test was used to test for normality of the distributions. A statistical level of $p < 0.05$ was considered as significant.

Average retention of Pt was calculated as follows: Equation (1) for both 1:2 diluted plasma and the control buffer:

$$\text{Retained Pt\%} = C_{\text{retained}} / (C_{\text{retained}} + C_{\text{dialysed}}) \times 100 \quad (1)$$

'Free' platinum unbound to high-molecular-weight species (including the highest-molecular-weight fraction of BP-Cx-1 ligand) was calculated (as a percentage) as the difference between 100 and average retention:

$$\text{Unbound Pt}_{\text{control buffer}}\% = (100 - \text{Retained Pt}\%) \quad (2)$$

In the case of plasma, the obtained value was divided by 2 to correct for the initial plasma dilution:

$$\text{Unbound Pt}_{\text{plasma}}\% = (100 - \text{Retained Pt}\%)/2 \quad (3)$$

The quantity of platinum bound to the macromolecular fraction (BP-Cx-1_{HMW}) of polymeric ligand BP-Cx-1 was calculated as follows:

$$\text{BP-Cx-1}_{\text{HMW}}\ \text{Pt}\% = 100 - 2 \times \text{Unbound Pt}_{\text{control buffer}}\% \quad (4)$$

The fraction of macromolecular BP-Cx-1 bound platinum was taken into account when calculating the actual binding to proteins:

$$\text{Pt bound to proteins, }\% = 100 - 2 \times \text{Unbound Pt}_{\text{plasma}}\% - \text{BP-Cx-1}_{\text{HMW}}\ \text{Pt}\% \quad (5)$$

Microsoft Excel 2011 for MacOS (Microsoft, Redmond, WA, USA) and Prism 6 (GraphPad Software, San Diego, CA, USA) packages were used for calculations.

3. Results

Equilibrium dialysis is a gold-standard technique used when determining the concentration of free, non-protein-bound drugs [28]. This process involves using drug diffusion to separate the bound and unbound drug, applying a semi-permeable membrane that allows only the unbound drug to pass through. The unbound drug will diffuse through the membrane into a buffer solution until equilibrium is reached on both sides [29]. The dialysis data are presented in Table 1 (the whole dataset is available in Supplementary Data File). The validated system was used for the assay.

Table 1. Platinum (Pt) concentration and retention in the cells after dialysis equilibrium was established. Fifty percent plasma and control PBS dialysed against PBS with pH 7.2 for 4 h at 37 °C across the 8 kDa cut-off vertical membrane. Pt was quantified by ICP-MS after microwave digestion. All dosage levels were tested in six parallel measurements ($n = 6$). Fifty percent plasma samples were compared to the control buffer for the same dosage level.

Sample Name	Average Pt Concentration, µg/L	RSD, %	Relative Retention%	SD of Relative Retention%	*p*-Value *
50% plasma + 500 µg/L BP-C1 retained fraction	32.7	8.1	77.6	6.3	0.004
50% plasma + 500 µg/L BP-C1 dialysis fraction	9.4	3.4			
Control buffer + 500 µg/L BP-C1 retained fraction	17.9	9.0	56.4	5.1	
Control buffer + 500 µg/L BP-C1 dialysis fraction	13.8	7.2			
50% plasma + 250 µg/L BP-C1 retained fraction	16.7	1.3	74.4	1.0	<0.001
50% plasma + 250 µg/L BP-C1 dialysis fraction	5.8	10.6			
Control buffer + 250 µg/L BP-C1 retained fraction	9.0	9.5	56.3	5.4	
Control buffer + 250 µg/L BP-C1 dialysis fraction	7.0	6.9			
50% plasma + 125 µg/L BP-C1 retained fraction	9.4	8.6	77.8	6.7	0.002
50% plasma + 125 µg/L BP-C1 dialysis fraction	2.7	6.6			
Control buffer + 125 µg/L BP-C1 retained fraction	4.9	7.9	58.3	4.6	
Control buffer + 125 µg/L BP-C1 dialysis fraction	3.5	4.5			
50% plasma + 62.5 µg/L BP-C1 retained fraction	4.2	11.1	76.1	8.4	0.008
50% plasma + 62.5 µg/L BP-C1 dialysis fraction	1.3	8.9			
Control buffer + 62.5 µg/L BP-C1 retained fraction	2.3	9.1	59.3	5.4	
Control buffer + 62.5 µg/L BP-C1 dialysis fraction	1.6	5.7			

* Paired *t*-test, normality was checked using Shapiro–Wilk test ($p < 0.05$); SD—standard deviation; RSD—relative standard deviation.

The dose dependency was investigated for the 50% plasma and control buffer through linear regressions (Figure 2). Good linearity ($R^2 \geq 0.98$) indicates adequate performance of the dialysis system and the absence of protein-binding saturation in the case of the plasma. The ratios between 50% plasma and control PBS buffer (Figure 3) were investigated, showing dose-stable increased retention of BP-C1 associated with macromolecular (retained) moieties in the plasma compared to the control buffer. This is supported by the low slope value of −0.0003. The average ratio is 0.77.

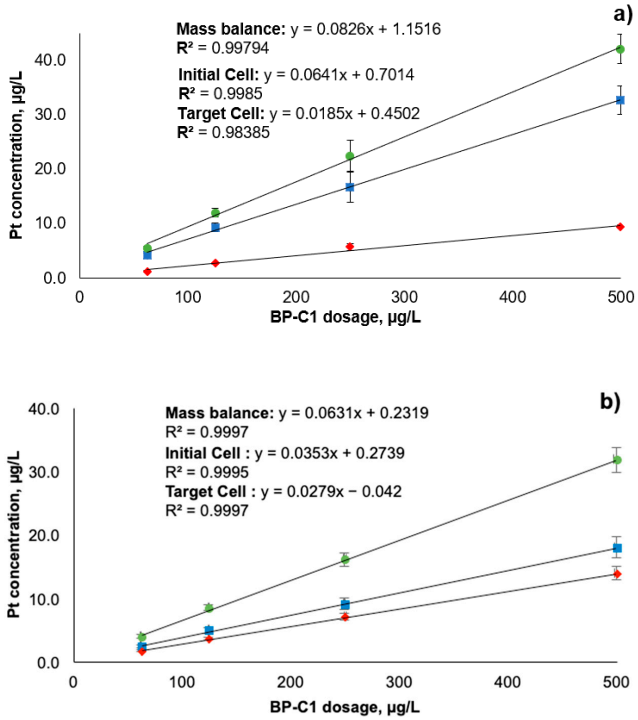

Figure 2. Dose dependence of the measured platinum (Pt) concentration in initial cell (retained fraction), target cell (dialysis fraction) and total Pt (mass balance in both cells) for: (**a**) 50% plasma and (**b**) control PBS dialysed against PBS with pH 7.2 for 4 h at 37 °C across the 8 kDa cut-off vertical membrane. Pt was quantified by ICP-MS after microwave digestion. Blue squares (■) stand for initial cell after the establishment of equilibrium; red diamonds (◆) stand for target cell after crossing the dialysis membrane; green circles (●) stand for total Pt in both cells (mass balance). Error bars represent ± standard deviation (n = 6).

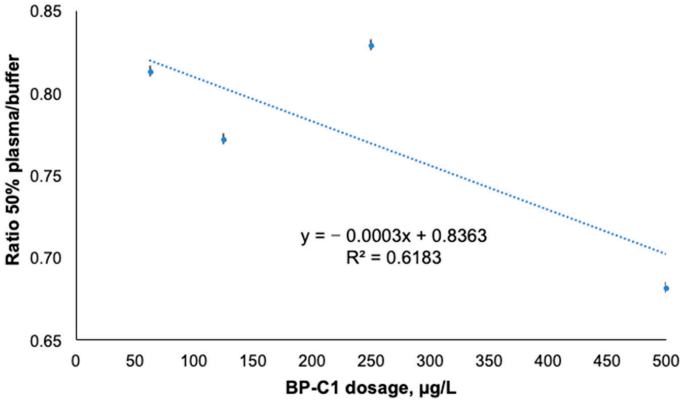

Figure 3. Ratio of 50% plasma to control buffer for the dialysed fraction of platinum (Pt), indicating plasma protein binding. Plasma and control buffer were dialysed against PBS with pH 7.2 for 4 h at 37 °C across the 8 kDa cut-off vertical membrane. Pt was quantified by ICP-MS after microwave digestion. Dashed line represents linear trend. Error bars represent ± standard deviation (n = 6).

4. Discussion

Good linearity for the dose dependency curve (Figure 2) indicates the absence of protein saturation with platinum under the experimental conditions. The slightly increased slope for the mass balance of the plasma may be attributed to the matrix effects of the residual organic carbon in the plasma samples [30–33]. A good repeatability for parallel experiments (relative standard deviations, RSDs < 10%) was observed (Table 1). Significant retention of platinum for the control buffer after 4 h of dialysis is related to the macromolecular nature of the BP-Cx-1 ligand, reducing the pure drug's capability to cross the 8 kDa membrane. This was additionally validated by independent experiments using different dialysis durations (1–8 h, Supplementary Tables S1 and S2).

The difference between 50% plasma at different dosage levels with a corresponding control PBS was evaluated, indicating a statistically significant difference (Table 1). Additionally, average binding to macromolecular species in plasma and the control buffer were calculated to be 76.5 ± 6.0% and 57.6 ± 5.0% (±standard deviation, SD), respectively. Taking into account the dilution of initial plasma 1:2 per volume with PBS before dialysis, the following fractions of 'free' (unbound to macromolecules) platinum were calculated: 42.4 ± 5.0% and 11.8 ± 3.0 % (±SD) for the control buffer and plasma, respectively. Considering the macromolecular nature of the BP-C1 lignin-derived ligand (average molecular weight of 1500–2000 Da [7]), the contribution of binding to plasma proteins may be considered to be ca. 60% (61 ± 12%, ±2 SD, n = 24), since ca. 15% of Pt is associated with polyphenolic moieties, as identified by the control buffer experiments (see Supplementary Information for more details on calculations).

Platinum-based drug plasma protein binding is known to be lower for drugs containing O-donor leaving ligands. For instance, carboplatin mostly releases active platinum moiety only intracellularly, showing ex vivo binding of 25–40% [34,35]. Previous studies of platinum drug binding to plasma/serum proteins indicated that carboplatin is primarily bound to HSA (45–96%, depending on incubation times), followed by transferrin and immunoglobulin-G (both ca. 2%) [16,36]. In vivo studies indicated the formation of reversible complexes of carboplatin with HSA [37]. Nevertheless, the intracellular distributions of cisplatin and carboplatin are similar, and their exhibited toxicity is not directly correlated with a total platinum concentration in target organs [38]. This is consistent with our previous studies for BP-C1 and other platinum drugs, indicating a high level of total platinum in kidneys and liver without the formation of toxic effects [23,25]. Thus, BP-C1 demonstrates medium protein binding, comparable to carboplatin and considerably lower than that for cisplatin.

A medium degree of binding of ca. 60% was demonstrated, indicating the maintenance of a high fraction of free drug in the bloodstream, facilitating its pharmacological activity. Further research on BP-C1 should focus on the effect of O-donor leaving ligands on its antitumour activity and the presence of potential cross-resistivity of certain tumours against platinum compounds.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app112211008/s1. Datasets for the protein-binding evaluations. The selection of optimal dialysis time is explained in Supplementary Table S1: Selection of optimal dialysis duration for cisplatin; 10 mL vertical dialysis tubes with 8-10 kDa cutoff in a Float-A-Lyzer G2 Dialysis Device (Repligen) were used for dialysis vs. 25 mL isotonic 0.9 % NaCl solution at room temperature. Pt was quantified by ICP-MS. The experiment was undertaken in triplicate for each tested duration and Table S2: Selection of optimal dialysis duration for BP-C1; 10 mL vertical dialysis tubes with 8-10 kDa cutoff in a Float-A-Lyzer G2 Dialysis Device (Repligen) were used for dialysis vs. 25 mL isotonic 0.9 % NaCl solution at room temperature. Pt was quantified by ICP-MS. The experiment was undertaken in triplicate for each tested duration.

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