Differential nephrotoxicity of cisplatin and a novel series of traditional Chinese medicine–platinum anticancer agents correlates with their chemical reactivity towards sulfur-containing nucleophiles

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A series of novel traditional Chinese medicine–platinum compounds has been found to be active against a number of murine and human cancers both *in vitro* and *in vivo*. Their high potency and the lack of cisplatin cross-resistance are believed to be due to the inclusion of the protein phosphatase 2A-inhibiting demethylcantharidin in the novel structures. A simple reversed-phase high-performance liquid chromatographic method was developed and validated as a stability-indicating assay for the platinum compounds. Using cisplatin and carboplatin as reference compounds, the stability study agrees well with the literature-reported findings. The novel traditional Chinese medicine–platinum compounds were more stable than cisplatin in water and dextrose, but became unstable in normal saline, a characteristic similar to that of carboplatin. The developed assay was further applied to study the chemical reactivity of the novel platinum compounds towards physiologically important nucleophiles such as glutathione and cysteine. The novel compounds were considerably less reactive to the sulfur-containing nucleophiles than cisplatin. In-vitro cytotoxicity assay was performed in a porcine kidney LLC-PK1 cell line model to investigate the nephrotoxicity potential of the platinum compounds. The lower rate of hydrolysis and the decreased reactivity of the novel traditional Chinese medicine–platinum compounds towards sulfur-containing bionucleophiles appear to have reduced their toxicity when compared with cisplatin, yet the antitumor activities of the novel compounds have not been compromised. *Anti-Cancer Drugs* 17:673–683 © 2006 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2006, **17**:673–683

Keywords: high-performance liquid chromatography, nephrotoxicity, sulfur-containing bionucleophiles, traditional Chinese medicine–platinum compounds

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Sponsorship: This work was supported by the Chinese University of Hong Kong (CUHK) (studentship for K.K.W.T.) and in part by the Patent Committee of CUHK.

Received 25 January 2006 **Revised form accepted** 3 March 2006

Introduction

Cisplatin is a commonly used antitumor drug for the treatment of human solid tumors. Severe toxic side-effects, particularly dose-limiting nephrotoxicity, however, seriously hinder its clinical usefulness. The low aqueous solubility and poor solution stability of cisplatin have also presented pharmaceutical-related problems.

The solution chemistry of cisplatin and its analogs has been extensively explored, and is considered important for understanding its pharmacology [1]. The hydrolysis reactions of cisplatin have been studied in detail [2–5] and are summarized in Scheme 1(a). In aqueous solutions, cisplatin decomposes by a reversible chloride ligand exchange reaction, which is dependent upon both chloride ion concentration and pH. The chloride ligands are displaced by water molecules in a stepwise manner, yielding monoaqua and diaqua complexes as the more reactive hydrated species [6,7].

It is commonly believed that DNA is the critical intracellular target for the antitumor activity of cisplatin [8]. Cisplatin is present as an intact drug in the plasma, where there is a high chloride concentration (around 110 mmol/l). The chloride concentration, however, is considerably lower inside the cell (around 10 mmol/l), hence favoring the formation of the aqua complexes [9]. The monoaqua complex formed inside the cell represents the apparent cytotoxic agent that reacts with the target DNA [10,11]. The aqua complexes have also been implicated in the renal toxicity of the drug [12] by reaction with proximal tubule membrane enzymes [13]. Toxicity may result from the high reactivity of cisplatin with bionucleophiles, and from the formation of the active aquated metabolites $(NH_3)_2PtCl(OH_2)^+$ and $(NH_3)_2Pt(OH_2)_2^{2+}$, which are also subject to irreversible binding to sulfur-containing bionucleophiles, in particular with plasma protein albumin [14]. It was originally thought that, as platinum (Pt)–thiol does not interact well with DNA, the formation of such complexes would

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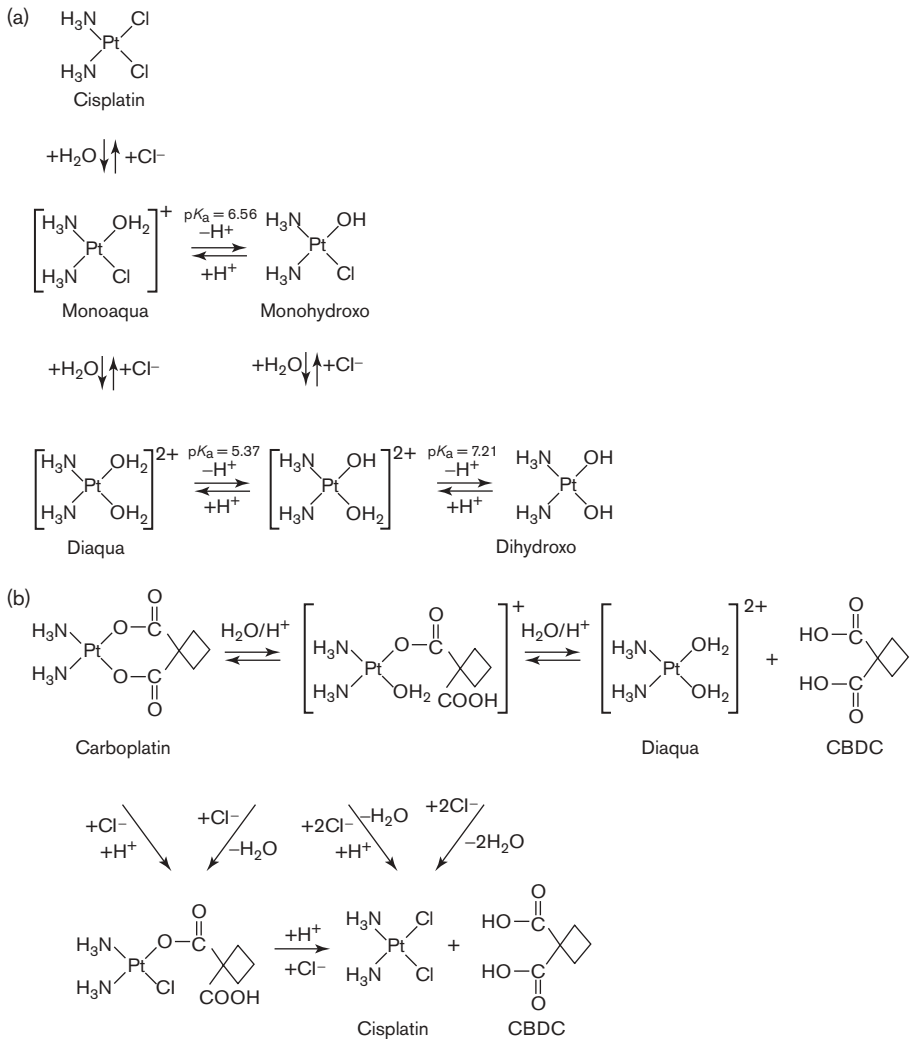
Scheme 1

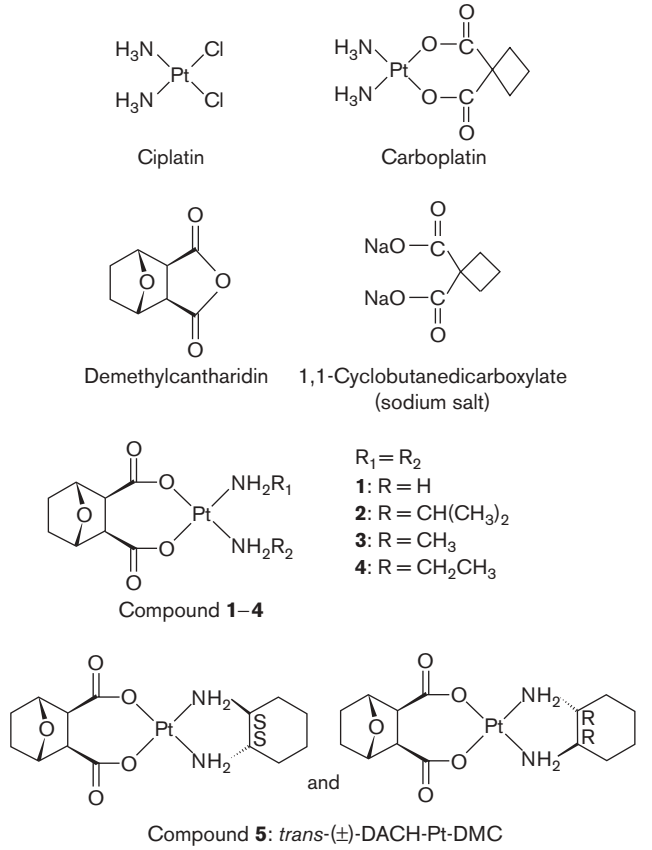


(a) Equilibrium for cisplatin and its monohydrated complex in aqueous solution. (b) Hydrolysis of carboplatin in aqueous solution and its reaction with chloride ions.

limit the amount of drug available for binding to DNA [15]. A more recent school of thought proposed that Pt–sulfur interactions could serve as a drug reservoir for platination at DNA, thus affording an additional pathway towards DNA platination [16]. This is supported by the finding that the N^7 donor atom of guanine in DNA can intramolecularly replace the sulfur atom of a Pt–sulfur adduct obtained from protein molecules [17]. The known affinity of sulfur for Pt complexes, however, has resulted in studies of the so-called 'protecting agents' to ameliorate the side-effects of Pt therapy, without reducing its antitumor activity. Such nucleophilic sulfur compounds include sodium thiosulfate, sodium diethyldithiocarbamate, glutathione, amifostine and mesna. The protective effect of these compounds is due to prevention or reversal of Pt–S adduct formation in proteins [18]. Furthermore, continued exposure to cisplatin can increase the cytosolic sulfhydryl level [19], which may give rise to cisplatin resistance. It is therefore of vital importance to understand the nucleophilic substitution reactions of Pt compounds that are responsible for the associated tumor and host toxicities and amelioration of toxicity.

We previously reported on a series of novel traditional Chinese medicine (TCM)–Pt compounds (**1–5**) (Fig. 1) that exhibited anticancer and protein phosphatase 2A-inhibitory properties [20–22]. TCM–Pt compounds **1–5** were designed by integrating demethylcantharidin (DMC), a TCM-derived ligand, with a Pt-moiety akin to

Fig. 1



Chemical structures referred to in this study.

the classical Pt-based anticancer agent, cisplatin [20]. The compounds were found to be more potent than cisplatin and carboplatin in a number of cancer cell line models, with the order of potency following a general trend of $\mathbf{5} > \mathbf{1} > \mathbf{2} \sim \mathbf{3} \sim \mathbf{4} >$ cisplatin $\gg$ carboplatin. The inclusion of DMC in the design strategy has apparently led to the novel compounds' ability to circumvent cisplatin cross-resistance [21]. The novel TCM–Pt compounds share basic structural characteristics with cisplatin, i.e. two *cis* ammine groups that are bound firmly to the Pt atom and a labile bidentate DMC leaving group. Therefore, it is logical to expect these compounds to undergo reactions fundamentally similar to those of cisplatin. It follows that valuable insights could be gained if the aqueous chemistry, in particular the nucleophilic substitution reaction by sulfur-containing biomolecules, of the novel compounds could be ascertained. In this regard, very few studies related to such nucleophilic reactions for new generations of Pt antitumor agents have been reported.

In this study, chromatographic separations of the novel TCM–Pt compounds and some of the possible degradation or biotransformation products were investigated. A simple and reliable reversed-phase high-performance liquid chromatography (HPLC) method using a phenyl-bonded-phase column and UV detection was developed as a stability-indicating assay. The method was validated for specificity, limit of detection, limit of quantitation, linearity, assay accuracy and precision. It was subsequently used for the determination of aqueous stability and chemical reactivity of the Pt compounds towards sulfur-containing nucleophiles. The value of utilizing the chemical reactivity of the Pt compounds in predicting their nephrotoxicity potential was ascertained in an in-vitro porcine kidney LLC-PK1 model.

Materials and methods

Chemicals and reagents

Cisplatin and carboplatin were obtained from Strem (Newburyport, Massachusetts, USA). The novel TCM–Pt compounds were synthesized by the School of Pharmacy and Department of Chemistry at the Chinese University of Hong Kong. Glutathione, various amino acids, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Ellman's reagent [5-5′-dithiobis(2-nitrobenzoic acid)] (DTNB) were obtained from Sigma (St Louis, Missouri, USA). Methanol was of HPLC grade, while all other reagents and solvents were of AnalaR quality. Deionized water used in the HPLC mobile phase was obtained by a Milli-Q purification system (Millipore, Bedford, Massachusetts, USA). All solutions were filtered before analysis through a 0.2-mm filter (Millipore) and degassed using ultrasonication.

Chromatography apparatus and conditions

All assays were performed using HPLC equipment equipped with a reciprocating-piston pump (Waters 2690; Millipore, St Quentin, France) and an autosample injector (Waters 717; Millipore). Isocratic reversed-phase chromatography was performed at ambient temperature with a Spherisorb phenyl column (250 × 4.2 mm internal diameter, 5 µm particle size; Alltech, Deerfield, Illinois, USA). The column effluent was monitored with a dual wavelength ultraviolet (UV) detector (Waters 2487; Millipore) or a photodiode array detector (Waters 996; Millipore). Peak retention time and peak area were monitored at 210 nm using a computing integrator (Millenium Software; Millipore). Carboplatin (1 mg/ml) was used as the internal standard (IS). The ratio of the area of the drug peak to the area of the IS peak was used for quantitation. The chromatography conditions are summarized in Table 1. The column dead time (t_0) was determined from the time of negative peak from water injection.

Validation of high-performance liquid chromatography assay

Validation of the method was performed according to International Conference on Harmonization of Technical

Requirements for Registration of Pharmaceuticals for Human Use guidelines [23]. The stability-indicating capacity of each of the chromatographic methods was demonstrated using decomposed and partially decomposed solutions of each compound. Decomposition was forced by (a) heating samples in sterile water (SW) at 70°C for 1 week, (b) incubating samples (except cisplatin) in normal saline (NS) at 37°C for 1 week, (c) adding hydrochloric acid (0.1 mol/l) to samples with the final pH being 2.0, and (d) adding sodium hydroxide solution (0.1 mol/l) to samples with the final pH being 12.2.

Stability study of the novel traditional Chinese medicine–platinum compounds

The concentrations of cisplatin and carboplatin selected for this study were based on current clinical protocols: cisplatin, 0.5 and 0.1 mg/ml; carboplatin, 1 and 0.1 mg/ml. The concentrations used for the novel TCM–Pt compounds were 0.5 and 0.1 mg/ml. The Pt compounds were reconstituted and further diluted to desired concentrations with various aqueous media such as SW, 0.9% sodium chloride (NS) and 5% dextrose solution (D5W).

The sample solutions were stored in silylanized glass bottles and kept at 4°C in a refrigerator or in a water bath at 25 ± 1, 37 ± 1, 50 ± 1, 60 ± 1 and 70 ± 1°C. Sample solutions were either illuminated under normal laboratory light or protected from light. At appropriate intervals, samples were removed and observed visually for discoloration or precipitation, and the pH values were immediately measured. After combining with internal standard solutions, samples were immediately subjected to chromatographic analysis. Three independent experimental runs were carried out for each concentration–vehicle–temperature variation. Drug concentrations were determined in duplicate by HPLC. The initial concentration of each drug was designated as 100%, and subsequent concentrations were expressed as a percentage of the initial concentration. All experiments used cisplatin and carboplatin as standard compounds for comparison.

Chemical reactivity of the novel traditional Chinese medicine–platinum compounds

A screening for the more reactive nucleophiles was performed using the developed HPLC method. Unreacted cisplatin, carboplatin and the novel TCM–Pt compounds after incubation with the respective nucleophiles were measured. In these experiments, the concentration of the Pt compounds were 0.5 mmol/l; various bionucleophiles such as glutathione, various amino acids with different side-chains, chloride ions, hydrogen carbonate ions and phosphate ions were used at their physiological concentrations [24]. Therefore, the approximate ratio of Pt:nucleophile ranged from 10:1 (in reduced glutathione) to 1:4 (in glutamine). Reactions were performed in *N*-2-hydroxyl piperazine-*N*′-2-ehane sulfonic acid buffer (5 mmol/l, pH 7.4, 37°C). Ionic strength was maintained at 300 μmol/l by the addition of $NaClO_4$. The reaction mixtures were incubated at 37°C in the dark and aliquots were removed for analysis after 24 h. Reactivity of the bionucleophiles was defined as the percentage of Pt compounds consumed after a 24-h incubation.

Cell culture, drug treatment and cytotoxicity study

LLC-PK1 porcine kidney cell line was purchased from the American Type Culture Collection (Rockville, Maryland, USA) and the procedure used for cell culture was described previously [25]. In brief, cells were grown to confluence (2.5×10^5 cells/cm^2) over 8 days in medium containing 10% fetal bovine serum and then maintained in serum-free medium for 2 days before drug treatment. LLC-PK1 cells postconfluency and demonstrating active dome formation were exposed to the Pt compounds or control medium for 1, 4, 24, 48 or 72 h (followed by a washing step with phosphate-buffered saline). All Pt compounds were prepared in sterile Hank's balanced salt solution. Less than 10 min elapsed between solution preparation and application to cell monolayers. The cultures were then replenished with serum-free medium and placed back in the incubator for a further 72 h, after which cell viability was determined. MTT assay was employed for the cytotoxicity study [26].

Table 1 Chromatographic conditions

	Cisplatin	Carboplatin	1	2	3	4	5
Column	phenyl-bonded column (Spersorb 5 mm, 250 × 4.6 mm; Alltech)						
Mobile phase	100% H_2O	2% methanol–H_2O (v/v)	2% methanol–H_2O (v/v)	30% methanol–H_2O (v/v)	10% methanol–H_2O (v/v)	20% methanol–H_2O (v/v)	30% methanol–H_2O (v/v)
Flow rate (ml/min)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Wavelength (nm)[a]	210	210	210	210	210	210	210
Internal standard	carboplatin in H_2O 1 mg/ml						
Retention time (min)							
platinum compound	5.39	11.18	8.92	11.22	9.57	10.42	8.65
internal standard	13.55	NA	11.18	5.84	7.77	6.42	5.84

[a]UV spectrum of peaks was monitored using a diode array detector (190–600 nm) to check for peak purity.
NA, not available.

Determination of protein-bound sulfhydryl

Protein-bound sulfhydryl (P-SH) level was measured using the method of Sedlak and Lindsay [27]. Briefly, cells (1×10^6) were harvested and homogenized in phosphate-buffered saline (about 1 ml) after treatment with the Pt compounds. For the determination of total SH content, cell homogenates (0.5 ml) were added to Tris-ethylene diaminetetraacetic acid buffer (pH 8.2, 2.0 ml) and DTNB (0.1 ml) was added to start the reaction. The solution was kept at room temperature for 30 min and the resulting absorbance was read at 412 nm on a UV spectrophotometer (Genesys, Spectronic Instruments, Rochester, New York, USA). The results were expressed in nmol SH/mg protein with an SH calibration curve. Protein content was determined by using the Lowry assay [28]. Non-protein sulfhydryl (NP-SH) content was determined similarly as mentioned above except that the cell homogenates were precipitated with trichloroacetic acid and centrifuged (1000 *g* for 10 min). The supernatant (0.5 ml) thus obtained was subjected to colorimetric reaction with DTNB. The amount of P-SH content was then obtained by subtracting NP-SH from the total sulfhydryl content.

Statistical analysis

For the determination of P-SH, statistical analysis was performed using Student's *t*-test. Results were expressed as mean ± SD and a statistical probability of $P < 0.05$ was considered to be significant.

Results

Separation and validation of the high-performance liquid chromatography method

Precision and accuracy

Intraday and interday precision and accuracy were assessed by analyzing quality control samples at three concentrations within the calibration curve range, on the same or different days, respectively. Assay precision was determined by calculating the relative standard deviation (RSD) (coefficient of variation [standard deviation/mean × 100%]) for each concentration. Similarly, the accuracy of the HPLC method was determined by calculating the percentage relative error (RE%) [(mean observed concentration – nominal concentration) × 100/nominal concentration] at each concentration. The RSD% and RE% obtained for the three quality control concentrations in different media (SW, NS, D5W) are summarized in Table 2.

Linearity of analytical response and limit of detection/quantitation

All standard curves were linear ($r > 0.99$) in the following working ranges: cisplatin, 12.5–500 μg/ml; carboplatin, 6.25–200 μg/ml and 200–1000 μg/ml; compounds **1–5**, 6.25–200 μg/ml and 200–1000 μg/ml. On the basis of a signal-to-noise ratio of at least 3:1, the limit of detection for cisplatin, carboplatin and compounds **1–5** were found to be 0.25, 0.31, 0.52, 0.26, 0.44, 0.38 and 0.29 μg/ml, respectively. According to a RSD of below 20% for the response of three different solutions at the same concentration, the limit of quantitation for the compounds were 21, 5, 5, 8, 8, 12 and 11 μg/ml, respectively.

Stability indicating capacity

Degraded samples of all Pt compounds were assayed to confirm separation of the parent compounds from their degradation products. In all cases, the decomposition product peaks were resolved from the peak of the intact compound. Peak purity was confirmed by monitoring the UV spectrum of a peak with a photodiode array detector (190–600 nm). Concentrations of degradation products were not determined but are easily detectable and identified. Representative chromatograms are shown in Fig. 2.

Stability of the novel traditiona Chinese medicine–platinum compounds

The stability of each compound subjected to different conditions was assessed by the HPLC method. Stability profiles for each Pt compound as indicated by the remaining drug concentration at each time point are shown in Fig. 3 and a summary of the results is tabulated in Table 3.

Table 2 Intraday and interday precision and accuracy of the high-performance liquid chromatography method

All media (SW, NS, D5W)	Nominal concentration (μg/ml)	Intraday variation[a] ($n=12$)		Interday variation[b] ($n=12$)	
		Accuracy RE (%)	Precision RSD (%)	Accuracy RE (%)	Precision RSD (%)
Cisplatin	20	96.5	3.5	96.7	7.9
	200	99.8	1.5	100.2	6.3
	500	101.1	1.2	101.3	5.8
Carboplatin	10	97.3	2.9	97.5	6.8
	200	99.6	1.1	99.2	4.1
	750	101.8	0.4	101.7	3.2
1	10	97.1	3.1	96.9	6.8
	200	99.6	1.2	98.9	3.2
	750	101.2	0.8	101.4	4.8
2	10	96.9	3.2	97.2	5.9
	200	100.8	1.5	98.6	5.6
	750	101.1	0.7	101.0	5.4
3	10	96.5	2.9	97.1	7.8
	200	99.8	0.9	99.7	5.3
	750	101.3	0.6	101.1	4.9
4	10	97.8	3.1	97.8	7.5
	200	99.7	1.2	98.6	6.3
	750	101.0	0.4	101.4	5.8
5	10	96.3	3.2	97.5	7.2
	200	100.2	1.8	99.8	5.1
	750	101.4	0.5	101.0	4.7

SW, sterile water; NS, normal saline; D5W, 5% dextrose in water; RSD, relative standard deviation; RE, relative error.

[a]Determined by analyzing replicate sets of quality controls for three concentrations on 1 day of analysis in the three media.

[b]Determined by analyzing replicate sets of quality controls at three concentrations on three independent days of analysis in the three media.

Rapid hydrolysis occurs when cisplatin is reconstituted in solution without any chloride ions, as in SW and D5W, giving the monoaqua and diaqua complexes as the degradation products [29]. The half-life for the displacement of the chloride ligand in water has been reported as approximately 2 h at 37°C [30], which is consistent with the results of our stability study. The monoaqua formed was confirmed by an authentic sample produced by reacting cisplatin with silver nitrate overnight using retention time indexing [31]. Carboplatin and the novel TCM–Pt compounds were much more stable, in which the extraordinary stability of carboplatin was observed in a number of previous reports [32,33]. The general trend for stability of different Pt compounds in SW and D5W was found to be the same: carboplatin ~ **2** > **4** > **3** > **1** > **5** > cisplatin.

In NS, cisplatin remained stable while a small amount of carboplatin was transformed into cisplatin (Fig. 2 and Scheme 1b), which concurred with the finding from Frey *et al.* [34]; likewise, compound **1** was also found to give rise to cisplatin. The formation of cisplatin from carboplatin and compound **1** was confirmed by retention time indexing. All novel Pt compounds were found to be less stable than cisplatin in NS in the following order: cisplatin > carboplatin > **2** > **4** > **3** > **15**.

Our studies demonstrated that, for compounds **1–4**, ascending the homologous series of amine group from **1** to **2** increased the stability. Compound **5** was the least stable, particularly in saline in which precipitation of the [$PtCl_2$(1,2-dach)] accelerated the ligand substitution reaction. A similar remarkable degradation of Pt compounds carrying a dach ligand, especially in NS, has also been reported [35].

Carboplatin and all novel compounds did not undergo an appreciable degradation in SW and D5W after more than a 3-month storage at 37°C. Therefore, attempts were made to study degradation at a higher temperature (an accelerated stability study in SW), the intention being to predict the shelf-life by using the Arrhenius equation. The compounds degraded faster at higher temperatures but the stability order of the Pt compounds was maintained at the same. The predicted t_{90} or t_{50} for carboplatin or the novel TCM–Pt compounds by extrapolation using the Arrhenius equation is tabulated in Table 3. Our predicted t_{50} for carboplatin in NS is in the same range (about 300 h) as that reported by Gust *et al.* [36], once again reinforcing the reliability of our HPLC method.

Screening for reactive bionucleophiles

The reactivity order of novel TCM–Pt compounds towards a number of bionucleophiles was evaluated using the developed HPLC method. Again, peaks corresponding to the biotransformation products were all well resolved from the intact compounds. *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid buffer was used in the experiments because it does not interact with cisplatin [37], or only to a significantly smaller extent when compared with Tris buffer [38]. Reactivity of the nucleophiles was defined as the percentage of Pt compounds consumed after a 24-h incubation at 37°C. The results are summarized in Table 4. The sulfur-containing bionucleophiles/amino acids were the most reactive, with glutathione > methionine > cysteine. The hydrophobic amino acids such as alanine and valine were the least reactive. Bicarbonate and phosphate ions at their intracellular concentrations may also represent important nucleophiles.

With the more reactive S-containing amino acids and nucleophiles, cisplatin was found to be the most reactive among all Pt compounds, and the following trend (in descending order of reactivity) was established: cisplatin > **5** > **1** > **3** > **4** > **2** > carboplatin.

In-vitro evaluation of nephrotoxicity

The LLC-PK1 porcine kidney cell line was employed for the in-vitro evaluation of nephrotoxicity of the novel TCM–Pt compounds.

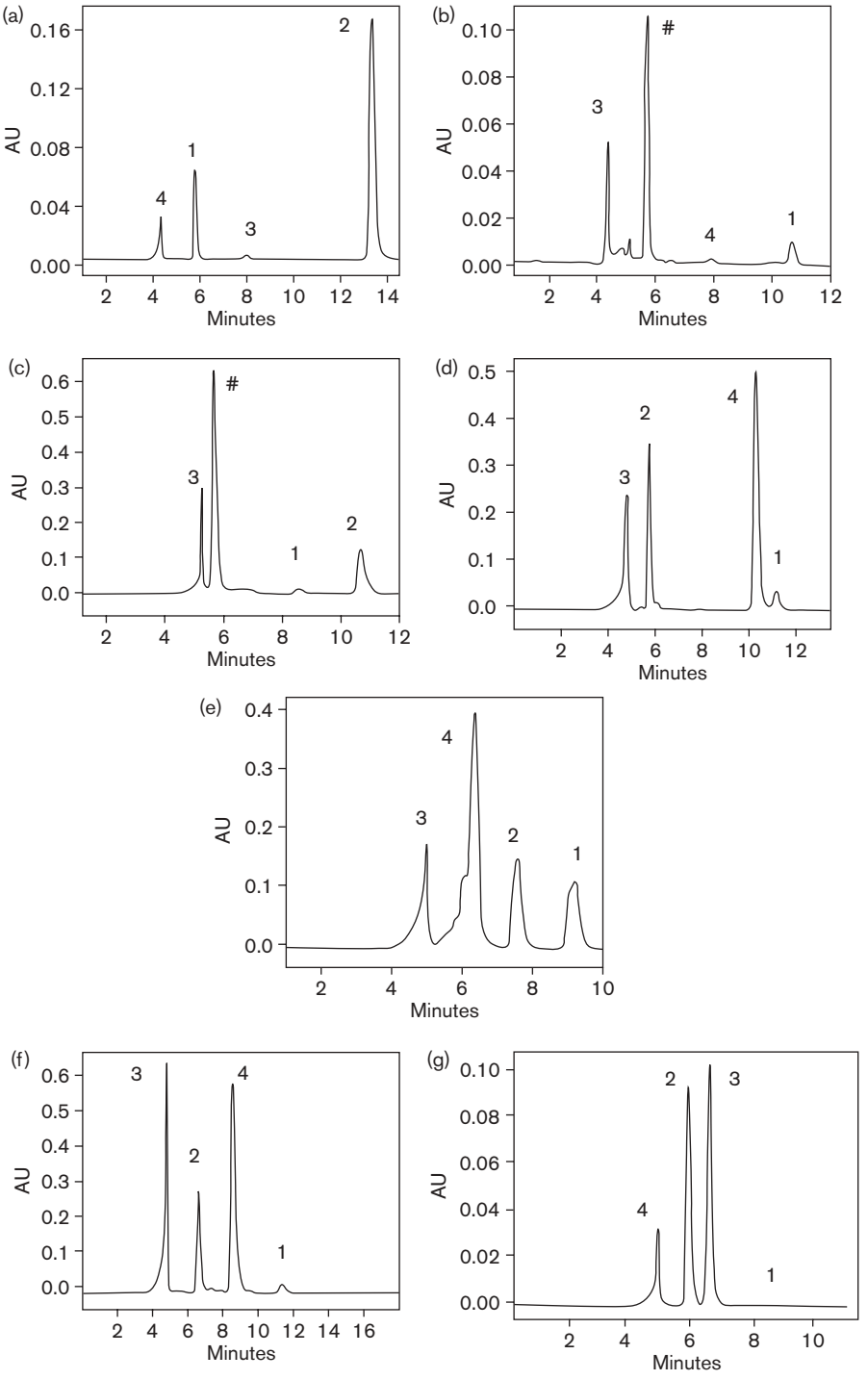
MTT assay was performed 72 h after drug exposure because it has been reported that no significant decrease in viability developed over the 10 h immediately following exposure to cisplatin. Viability began to decrease between 10 and 15 h after removal of cisplatin, and continued to 72 h in a dose-dependent fashion [39].

Compounds **1–4** and carboplatin were all much less toxic to the LLC-PK1 cells than cisplatin, as shown in Table 5. After a 1-h exposure, cisplatin gave an IC_{50} of about 350 μmol/l, whereas the IC_{50}s of compounds **2–4** were greater than 2 mmol/l and compound **1** was about 1 mmol/l. Upon prolonged exposure of the Pt compounds (72 h), the cytotoxic effects of all Pt compounds increased and cytotoxicity was found to be time-dependent. Compounds **1–4** were found to be at least 2–10 times less toxic than cisplatin by comparing their IC_{50}s. Among the novel compounds, compound **5** was the most toxic in this in-vitro model. It gave a similar IC_{50} to cisplatin (14 μmol/l) after a 72-h drug exposure, which is consistent with its high reactivity to the sulfur-containing nucleophiles.

Changes in protein-bound sulfhydryl levels

The effect of the Pt compounds on P-SH levels was examined. It was found that after 4 h of drug exposure, cisplatin (1 mmol/l) significantly decreased P-SH (Fig. 4). In general, the novel TCM–Pt compounds did not demonstrate such an effect except

Fig. 2



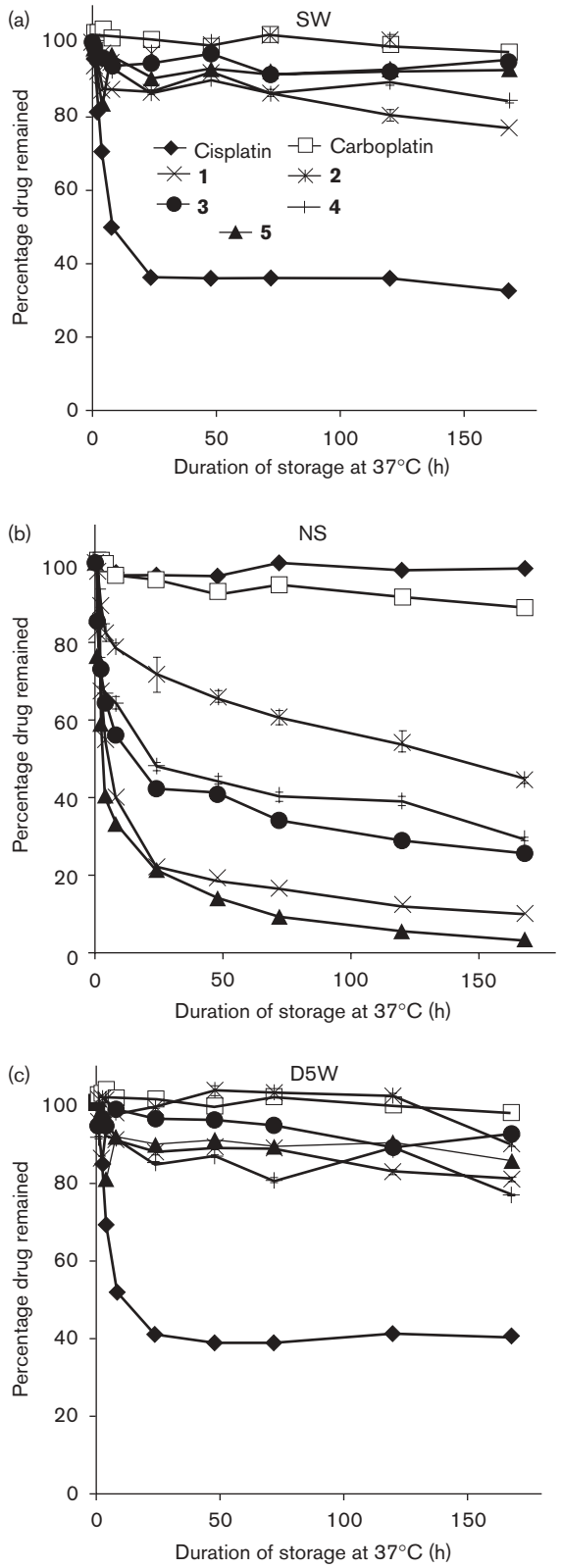
Representative liquid chromatography chromatograms. (a) Cisplatin in sterile water heated at 70°C for 1 week; (b)–(g) Carboplatin and compounds **1**–**5**, respectively, in normal saline (NS) incubated at 37°C for 1 week (peak designation: 1 = parent compound; 2 = internal standard (carboplatin); 3,4 = possible degradation products in aqueous solution; # = cisplatin transformed from carboplatin (b) or compound **1** (c), respectively, in NS.

for compounds **1** and **5**, which were found to deplete P-SH in LLC-PK1, although the degree was less than that of cisplatin.

Discussion

The stability of cisplatin and carboplatin in pharmaceutical preparations has been well documented [32,33,40].

Fig. 3



Stability of cisplatin, carboplatin and the novel traditional Chinese medicine–platinum compounds in (a) sterile water (SW), (b) normal saline (NS) and (c) 5% dextrose in water (D5W) at 37°C.

Many new Pt drugs have been synthesized in attempts to increase water solubility and drug stability of novel compounds, but stability data for these second-generation and third-generation antitumor agents are rare.

The proposed reaction pathway for cisplatin in aqueous solutions was previously described (Scheme 1). It is generally believed that aqueous stability could reflect the tendency of the Pt compounds to undergo nucleophilic reactions. The lability of the chloro ligands in cisplatin allows rapid hydrolysis, giving rise to monoaqua and diaqua adducts as the degradation products. Carboplatin is structurally similar to cisplatin except that 1,1-cyclobutanedicarboxylate, an inert bidentate ligand, replaces the two chloride ions of the original drug, leading to its enhanced stability [41]. By analogy, the relatively higher stability demonstrated by the novel TCM–Pt compounds could also be explained by their bidentate DMC ligand. The compounds, however, became unstable in NS, in which the presence of the nucleophilic chloride ions readily displaced the bidentate ligands. Notably, the aqueous stability of the novel TCM–Pt compounds was found to fall between that of cisplatin and carboplatin.

Given the avidity of cisplatin for sulfhydryl groups and the much slower reaction of carboplatin, as demonstrated here and from the literature, it has been postulated that reaction with sulfhydryl-containing enzymes is a crucial mechanism of nephrotoxicity for cisplatin [13]. The degree of enzyme inhibition and damage by Pt compounds paralleled their reactivities with the nucleophiles [13]. Moreover, literature findings suggested that sulfhydryl groups are depleted in the kidneys after cisplatin administration [42]; in-vivo studies have indicated that a reduced glutathione conjugate of cisplatin is metabolized within the renal proximal tubules to a nephrotoxic species [43,44]. Therefore, the chemical reactivity of Pt compounds, particularly towards sulfur-containing nucleophiles, represents a reasonable correlation for structure–toxicity relationships.

A number of papers have described reversed-phase HPLC conditions for detecting and monitoring cisplatin hydrolysis products. In some of these studies, however, the mobile phases used were chosen without sufficient regard for the high reactivity of cisplatin in aqueous solutions. Phosphate, acetate, formate and the organic modifier, acetonitrile in the mobile phase may all react with Pt aqua complexes [45–47]. In addition, the possibility of accidental superposition of peaks from different species was usually not considered.

In this report, a reversed-phase HPLC method using a phenyl-bonded-phase column and UV detection was developed and validated for use as a stability-indicating assay. Water and methanol was employed in the mobile phase, thereby eliminating the need for ion-exchangers or

Table 3 Stability of various platinum compounds in SW, NS and D5W[a]

Compounds	Storage temperature (°C)	t_{90} (h)[b]			t_{50} (h)[b]		
		SW	NS	D5W	SW	NS	D5W
Cisplatin	4	2.83	>2000	3.02	168	>2000	159
	25	1.22	>2000	1.65	8.2	>2000	12
	37	0.92	>2000	0.92	2.6	>2000	2.3
Carboplatin	4	>2000	230	>2000	>2000	>2000	>2000
	25	>2000	120	>2000	>2000	>2000	>2000
	37	>2000	90	>2000	>2000	210	>2000
1	4	8.2	0.5	8.8	>2000	28	>2000
	25	6.2	0.2	7.2	>2000	5.2	>2000
	37	5.4	0.1	6.4	1920	3.2	>2000
2	4	>2000	60	>2000	>2000	>2000	>2000
	25	>2000	1.5	>2000	>2000	120	>2000
	37	>2000	0.8	>2000	>2000	100	>2000
3	4	672	0.8	705	>2000	80	>2000
	25	573	0.4	650	>2000	12	>2000
	37	350	0.3	420	>2000	8	>2000
4	4	820	1	840	>2000	140	>2000
	25	672	0.8	720	>2000	18	>2000
	37	452	0.6	510	>2000	12	>2000
5	4	5.2	0.4	6	>2000	7.5	>2000
	25	4.2	0.2	5.1	>2000	2.3	>2000
	37	4.0	0.1	4.8	>2000	0.8	>2000

SW, sterile water; NS, normal saline; D5W, 5% dextrose in water.
[a]All solutions were protected from light.
[b]t_{90}=time required for the concentration to drop to 90% of its initial value. t_{50}=time required for the concentration to drop to 50% of its initial value.

Table 4 Screening for reactive bionucleophiles against the novel traditional Chinese medicine–platinum compounds

Nucleophile	Plasma concentration (mmol/l)[a]	Cisplatin	Carboplatin	Reactivity (%)[b]				
				1	**2**	**3**	**4**	**5**
S-containing amino acid/bionucleophiles								
GSH	0.05	100	1.5	19.5	2.1	14.2	3.6	38.5
GSSG	0.05	89.5	0.5	5.5	2.1	2.3	3.4	19.3
cysteine	0.05	100	2.6	12.5	2.5	10.3	3.4	25.2
methionine	0.05	100	1.2	16.7	3.8	12.4	0.5	32.2
OH-containing amino acids								
serine	0.19	3.5	2.1	2.7	2.7	1.5	2.7	3.2
threonine	0.40	5.3	2.0	3.2	2.4	2.1	1.9	3.1
tyrosine	0.10	6.2	2.8	2.6	2.6	1.3	2.4	3.5
Amide amino acids								
asparagine	0.40	4.6	2.7	3.5	1.9	2.6	2.2	2.6
glutamine	2.00	3.9	3.1	2.1	2.1	1.9	3.6	2.1
Basic amino acids								
histidine	0.10	22.6	2.1	9.8	1.7	4.8	3.1	2.3
lysine	0.40	2.5	0.2	4.4	2.9	1.5	1.8	2.1
arginine	1.00	5.6	1.4	4.1	1.6	2.9	1.6	2.5
Acidic amino acids								
aspartate	0.05	3.2	1.4	2.1	2.5	3.1	2.5	3.4
glutamate	0.10	3.5	1.9	2.0	3.8	1.3	1.8	2.9
Non-polar amino acids with aliphatic R groups								
glycine	0.10	3.8	1.6	1.9	1.6	1.8	2.0	2.5
proline	0.10	4.1	1.7	2.0	0.6	2.5	2.2	2.4
alanine	0.10	4.1	1.5	1.4	1.9	2.1	1.7	2.8
Other(s)								
chloride	108	2.2	5.6	34.2	6.2	23.2	9.5	42.6
Selected anions at intracellular levels								
10 mmol/l $NaHCO_3$		6.4	2.6	12.3	2.5	8.2	3.4	14.4
45 mmol/l (pH 7)		75.2	4.5	100	5.6	85.2	6.8	72.3
Na_2HPO_4/NaH_2PO_4		100	7.6	100	8.2	100	12.3	75.1
90 mmol/l (pH 7)								

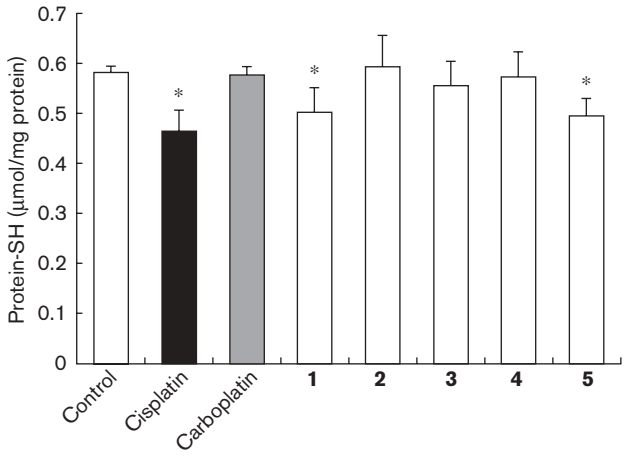
GSSG, oxidized glutathione.
[a]Values were obtained from Altman [24].
[b]Reactivity of nucleophiles was defined as the percentage of platinum compounds consumed after a 24-h incubation at 37°C. Incubation was performed in standard incubation buffer containing 0.05 mol/l $NaClO_4$, pH adjusted to 7.4 with 0.5 mol/l NaOH.

Table 5 Cytotoxicity of Pt compounds in the quiescent LLC-PK1 kidney model[a]

Pt compounds	Drug exposure time (h)				
	1	4	24	48	72
Cisplatin	356.26 ± 93.89	96.48 ± 43.68	15.65 ± 1.31	14.09 ± 1.63	12.26 ± 2.94
Carboplatin	not toxic[b]	not toxic[b]	209.35 ± 95.32	246.48 ± 48.13	217.5 ± 26.69
1	1049.28 ± 43.60	751.41 ± 21.25	43.41 ± 3.50	50.45 ± 9.43	24.43 ± 9.39
2	not toxic[b]	not toxic[b]	113.45 ± 34.13	133.1 ± 21.56	112.12 ± 25.63
3	not toxic[b]	not toxic[b]	94.03 ± 19.06	83.56 ± 3.21	65.55 ± 6.21
4	not toxic[b]	not toxic[b]	85.04 ± 6.33	89.19 ± 2.46	92.12 ± 8.61
5	72.19 ± 10.12	26.58 ± 1.11	22.44 ± 1.07	18.66 ± 3.33	14.57 ± 1.52

Pt, platinum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
[a]Effect of the Pt compounds on LLC-PK1 cell viability was expressed as IC_{50} ± SD (μmol/l) as determined by the MTT assay. Cells were exposed to the Pt compounds for 1, 4, 24, 48 or 72 h, washed and then returned to the incubator with fresh serum-free medium until assayed for viability 72 h later with the MTT assay. Data represent mean from at least three independent experiments with six replicates each time.
[b]IC_{50} values greater than 2 mmol/l were assigned 'not toxic'.

Fig. 4



Effect of platinum compounds on protein-bound sulfhydryl (P-SH) in LLC-PK1 cells. Results represent mean of three independent experiments ± SD (*$P < 0.05$, compared with untreated control).

ion-pairing systems, which may react with the Pt species. Peak purity was ascertained by monitoring its UV spectrum with a photodiode array detector.

The TCM–Pt compounds were found to be less reactive towards the sulfur-containing amino acids and glutathione than cisplatin. The higher aqueous stability of the novel compounds could also account for their decreased reactivity towards the sulfur-based nucleophiles, and, thus, may help to predict and account for the different toxic properties. On the basis of this hypothesis, the decreased reactivity of the novel Pt compounds may be associated with lower host toxicity.

In-vitro plasma protein binding was also performed to verify the physiologic relevance of Pt reactivity to bionucleophiles (data not shown). The Pt compounds were incubated with mouse plasma at 37°C for up to 24 h and the percentage of protein binding was determined by atomic spectroscopy following ultrafiltration. Cisplatin bound rapidly to plasma protein with a half-life of 1.33 h. After 24 h incubation, more than 95% of cisplatin was protein bound. Carboplatin, however, bound to plasma protein at a much slower rate and to a lesser extent ($t_{1/2}$ of free Pt = 10.38 h; about 50% protein bound at 24 h). Protein binding of the novel TCM–Pt compounds were between that of cisplatin and carboplatin, in the order cisplatin > **5** > **1** > **3** > carboplatin, which coincided with the order of their chemical reactivity towards sulfur-containing nucleophiles.

The present study showed that the in-vitro toxicity in the porcine kidney cell line LLC-PK1 paralleled the extent of P-SH depletion by the Pt compounds. When grown to confluence, LLC-PK1 cells express many characteristics of proximal tubule epithelia [25]. It has been shown to be a good model of cisplatin-induced nephrotoxicity and those caused by other nephrotoxicants such as aminoglycosides [48,49]. Cisplatin, compounds **1** and **5** were more toxic and caused significant reductions in the protein sulfhydryl levels. Depletion of P-SH levels was found to be both dose-dependent and time-dependent (data not shown), and may be a result of direct binding of the Pt compounds with –SH. The other novel compounds had no effect on cell viability even at very high doses nor did they have an effect on P-SH reduction. The results coincided well with the reactivity of the Pt compounds towards the sulfur-containing biomolecules.

In a related acute toxicity study in ICR mice, the novel Pt compounds were also found to be much less nephrotoxic than cisplatin as reflected by changes in blood urea nitrogen and serum creatinine [22]. In summary, the chemical reactivity of the novel compounds in aqueous solutions does represent a useful predicting parameter for the nephrotoxicity potential of the Pt compounds. This may serve as a valuable guide for the design of new antitumor Pt(II) compounds.

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