In vitro and *in vivo* suppression of growth of hepatocellular carcinoma cells by novel traditional Chinese medicine – platinum anti-cancer agents

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Protein phosphatase 2A (PP2A) is a new target for platinum (Pt)-based cancer chemotherapeutic agents. A series of novel Pt complexes containing demethylcantharidin, a modified component of a traditional Chinese medicine (TCM), [Pt($C_8H_8O_5$)(NH_2R)$_2$] 1–5 have been shown to inhibit PP2A both in its purified form and in cell homogenates. In this study, the potential efficacy of compounds 1–5 in suppressing the growth of PP2A-highly expressed liver cancer was evaluated. The *in vitro* anti-proliferative activity of compounds 1–5 was investigated in human hepatocellular carcinoma (HCC) cell lines using the MTT assay. Compounds 1–5 were about 2–20 and 20–200 times more potent than cisplatin and carboplatin, respectively, in SK-Hep1 and HepG2 cells. The *in vivo* anti-tumor efficacies of 1–5 were evaluated in a s.c. inoculated SK-Hep1 xenograft model in nude mice. Compounds 1–5 demonstrated definite *in vivo* activity (giving rise to an optimal %T/C as low as 14.5%) without inducing undue toxicity, contrasting the lack of activity of cisplatin and carboplatin. In a cisplatin-resistant model established *in vivo* in human HCC, compounds 1–5 could still elicit the same level of tumor growth suppression as in the control tumors, demonstrating the circumvention of cisplatin cross-resistance. An acute toxicity study in ICR mice showed that compounds 1–5 are not nephrotoxic at LD_{10}. The high potency of the novel TCM-Pt compounds against liver cancer and the minimal toxicity suggest that they have significant potential to be developed into useful Pt-based anti-tumor drugs. *Anti-Cancer Drugs* 16:825–835 © 2005 Lippincott Williams & Wilkins.

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Introduction

Cisplatin is one of the most effective anti-tumor agents clinically used against solid tumors, but its clinical efficacy is limited by the development of resistant tumor cells [1]. However, attempts to overcome cisplatin resistance by dose escalation were hindered by its dose-limiting nephrotoxicity [2]. This then inspired different structure–activity relationship studies aimed at identifying critical modifications that might improve the drug's therapeutic profile in terms of both drug resistance and toxicity [3]. We previously reported on a series of novel traditional Chinese medicine-platinum (TCM-Pt) compounds (1–5) (Fig. 1) that exhibited anti-cancer and protein phosphatase 2A (PP2A)-inhibitory properties [4].

In vitro studies of 1–5 found them to profoundly inhibit the proliferation of mouse and a number of human cancer cell lines; the latter included human non-small cell lung, ovarian, testicular, breast, cervical, colon-rectal and liver (SK-Hep1) cancers [4]. Compounds 1–5 also demonstrated anti-tumor activity against a SK-Hep1 s.c. inoculated xenograft in nude mice in an *in vivo* pilot study [4].

TCM-Pt 1–5 were designed by integrating demethylcantharidin (DMC), a TCM-derived ligand, with a Pt moiety akin to the classical Pt-based anti-cancer agent, cisplatin [4]. Cantharidin, the active principle of *Mylabris* ("blister beetle"), has long been used as a TCM for the treatment of liver and digestive tract cancers in China [5]. However, its unacceptable toxic side-effects such as dysuria and hematemesis prevent cantharidin from becoming a more widely used therapeutic agent. DMC (or norcantharidin) is a synthetic analog of cantharidin and has potent anti-tumor activity, but without the latter's adverse effects [5,6]. Literature findings have reported that cantharidin and its derivatives have strong affinity and specificity for PP2A [7]. It has been reported that liver cytosol is one site that is rich in PP2A [8] and the level of PP2A inhibition parallels cytotoxicity [9]. Significantly, endothall, the diacid form of DMC, has also been reported to inhibit the growth of hepatocellular carcinoma (HCC) cell lines in culture more than that of normal hepatocytes or colon carcinomas [10]. We therefore postulated that incorporation of DMC into 1–5 should result in new chemical entities that are highly potent in inhibiting the growth of liver cancer, normally unresponsive to the classical Pt-based anti-cancer drugs cisplatin and carboplatin. Prior to our studies,

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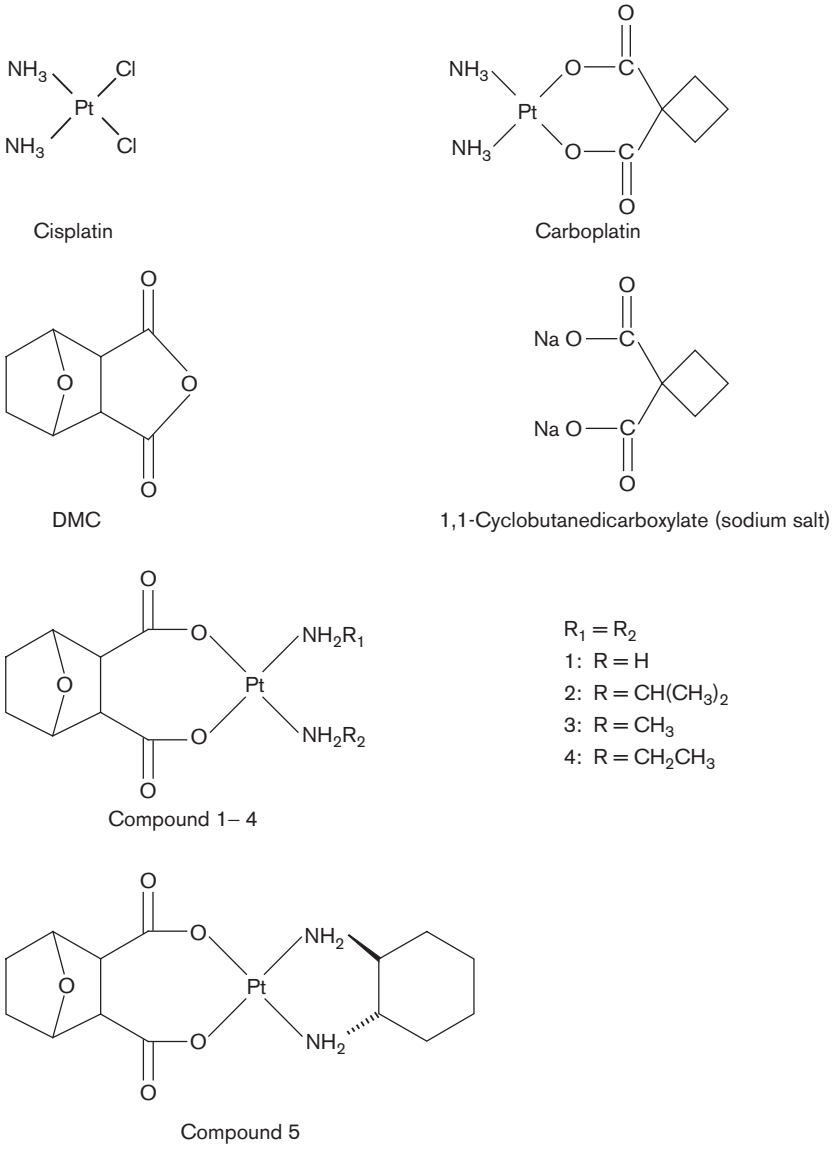
Fig. 1



Chemical structures of cisplatin, carboplatin, novel TCM-Pt compounds 1–5, DMC and sodium 1,1-cyclobutanedicarboxylate.

anti-tumor activity by inhibition of protein phosphatases had not been reported for Pt-based drugs.

The purpose of this study was to evaluate in more detail the *in vitro* and *in vivo* suppression of HCC cell growth by novel TCM-Pt anti-cancer agents and DMC.

Materials and methods

Chemicals

TCM-Pt 1–5 were prepared at the School of Pharmacy (Chinese University of Hong Kong) as previously described [4]. Cisplatin and carboplatin were obtained from Strem (Newburyport, Massachusetts, USA). 1,1-Cyclobutanedicarboxylic acid (as a sodium salt) was purchased from Aldrich (St Louis, Missouri, USA). Cisplatin, DMC and 1,1-cyclobutanedicarboxylate were dissolved in normal saline (NS), whereas carboplatin and all other novel TCM-Pt compounds were dissolved in 5% dextrose in water, immediately before use. The creatinine (Cr) assay (555-A) and blood urea nitrogen (BUN) assay (endpoint) (66-UV) kits were obtained from Sigma (St Louis, Missouri, USA). All other chemicals were of the best reagent grade available.

Cell culture

Human HCC cells SK-Hep1 and HepG2 were obtained from ATCC (Manassas, Virginia, USA). They were

maintained as adherent cultures in DMEM medium (Gibco/BRL, New York, USA) supplemented with fetal bovine serum (10%) (Gibco/BRL), penicillin (100 U/ml) (Gibco/BRL) and streptomycin (100 µg/ml) (Gibco/BRL). The cells were grown in a CO_2 incubator (5% CO_2/95% air) at 37°C.

Primary hepatocyte cell culture was established from livers dissected from ICR mice (6–8 weeks old). Suspensions of isolated liver cells were manipulated so as to remove the non-hepatic and non-viable cells from the desired primary hepatocytes according to Kreamer *et al.* [11]. The adherent primary cultures were then established on collagen pre-coated culture flasks.

Animals

All experimental animals were obtained from and bred at the Laboratory Animal Service Center (LASEC) at the Chinese University of Hong Kong. Male athymic nude mice at 6–8 weeks of age were housed in sterile microisolator cages with access to autoclaved laboratory animal diet and tap water *ad libitum*. Male ICR mice (aged 6–8 weeks, weighing 20–25 g), which were used for the acute toxicity study, were housed in groups of six in metal cages, and had free access to standard rat chow and water. Animals were exposed to 12 h cycles of light and dark. All experiments involving animals were approved by the Chinese University of Hong Kong Animal Ethics Committee and were carried out under the supervision of the University LASEC.

In vitro growth inhibition assay

The anti-proliferative activity of the Pt compounds was assayed using the colorimetric MTT assay [12]. Briefly, cells were seeded in 96-well microtiter plates (1×10^4/well; 100 µl) and allowed to equilibrate in quadruplicate wells and exposed to drugs (cisplatin, 1–5 and DMC: 2–200 µM; carboplatin: 50–3000 µM) for 72 h. After which, MTT (5 mg/ml, 20 µl) in phosphate-buffered saline was added and the cells were incubated for 4 h at 37°C. MTT/medium was then removed and the formazan product was dissolved in DMSO (150 µl) and absorbance was measured at 570 nm using a microtiter plate reader. The concentration of drug resulting in 50% growth inhibition (IC_{50}) was determined using Graphpad Prism 3.0 by fitting the graph into a sigmoidal dose–response curve.

In vivo anti-tumor activity

SK-Hep1 cells were initially cultured *in vitro* as described above. To establish s.c. xenografts in nude mice, the animals were inoculated s.c. in the flank with 0.1 ml of a cell suspension containing 6×10^6 cancer cells. After the tumors became palpable (staging day), serial growth was determined on alternate days by measurement of two perpendicular diameters using vernier calipers. Tumor volume was calculated using the formula: volume = length × width^2/2 and were normalized to the volume at the start of treatment (staging day; day 0) as the relative tumor volume (RTV). Xenografts that gave rise to no-takes or spontaneous regression were abandoned.

Chemosensitivity was assessed as described previously [13]. Briefly, mice bearing comparable-sized tumors (approximately 5 mm in diameter) were randomized into treatment groups (six animals) or control groups (15 animals) (day 1). Drugs were administered by i.p. injection at three or four dose levels up to the maximum tolerated dose (MTD), on day 1, and thereafter on days 5 and 9. The MTD was determined as the dose that gave a body weight loss nadir (mean of group) of greater than 20% or greater than 20% drug-related deaths [14]. Control animals ($n = 15$) received no active therapy, but were otherwise treated in an identical manner to the animals in the treatment groups. Tumor-free mice and drug-related deaths were excluded from all measurements.

Anti-tumor activity was assessed by two evaluation criteria: (i) ratios of T/C (%) which is equal to (median RTV of the treated group on day x/median RTV of the control group on day x) × 100, the optimal value being the minimal T/C ratio that reflects the maximal tumor growth inhibition achieved [14], and (ii) on the basis of delay in tumor growth, calculated as specific growth delay (SGD). SGD is expressed as the percentage by which the treated group tumor volume is delayed in attaining a specified number of doublings (from its staging day volume) compared with the controls using the formula: (T–C)/C, where T and C are the median times in days for treated and control groups, respectively, to attain the specified size (500 cm^3, i.e. about three doublings). According to NCI standards, the criterion for efficacy for the T/C ratio is 42% or below and a value of 10% or below is judged as attaining a high level of activity [14]. The anti-tumor activity was also scored according to Fodstad [15], on the basis of optimal %T/C and SGD values. A %T/C below 50 and SGD above 1.0 is considered active (+) whereas treatment giving a %T/C below 25 and SGD above 2.0 is graded highly active (+++). *In vivo* data were analyzed using Student's t-test. $p \leq 0.05$ was considered to be statistically significant.

To evaluate the toxicity of the compounds in the nude mice, both drug-related deaths (DRDs) and maximum percent relative mean net body weight losses were determined. Animals were weighed on alternate days until the completion of the study. Any weight loss that occurred was calculated as a percentage of the staging day weight. An animal death

was assumed to be treatment related if the animal died within 15 days of the last treatment and if either its tumor weight was less than the lethal burden in the control mice or its net body weight loss at death was 20% greater than the mean net weight change of the controls at death or sacrifice.

Induction of cisplatin resistance *in vivo* and evaluation of cross resistance

Cisplatin resistance was induced in the aforementioned s.c. xenograft model in nude mice using a protocol adapted from Caffrey and Frenkel [16]. When tumor size reached approximately 5 mm in diameter, mice were injected i.p. with 0.2 ml NS (controls) or 2 mg/kg cisplatin in 0.2 ml NS (cisplatin-resistant group) on a day 1, 5 and 9 schedule. After 2 weeks (day 23), all animals were injected i.p. with cisplatin (6 mg/kg), 1 (25 mg/kg), 2–4 (120 mg/kg) or 5 (30 mg/kg). The response of the tumor to the treatment in the control group versus the acquired cisplatin-resistance group was used as a measure of its sensitivity to the drug and cross-resistance to cisplatin. The percentage tumor growth inhibition was calculated 7 days after the treatment (day 30). Student's *t*-test was performed to compare the control group with the cisplatin-resistant group. Mean ± SD values were presented and significance was defined as $p \leq 0.05$.

To ascertain the role of DMC in the circumvention of cisplatin cross-resistance, tumor-bearing mice were treated with cisplatin (6 mg/kg) plus DMC (2 mg/kg) 2 weeks after the induction of cisplatin resistance. For comparison, cisplatin was also combined with 1,1-cyclobutanedicarboxylate (leaving the ligand in carboplatin; *cf*. DMC in the novel Pt compounds).

Acute toxicity study in male ICR mice

The animals were divided into nine groups of 18 mice each. Group 1 was the control where the mice were injected i.p. with NS. Groups 2–9 received a single dose of cisplatin, carboplatin, 1–5 and DMC at the predetermined LD_{10} doses (cisplatin, 10.99 mg/kg; carboplatin, 100 mg/kg; 1, 50 mg/kg; 2, 66.67 mg/kg; 3, 100mg/kg; 4, 94.97 mg/kg; 5, 50 mg/kg; DMC, 6.21 mg/kg). Toxicity was observed over a period of 5 days.

Mice (in groups of six) were sacrificed 1, 2 or 5 days after the drug treatments in order to study the toxic effect of the Pt compounds and DMC. Blood samples were collected by heart puncture, and the animals were weighed and sacrificed by cervical decapitation. Kidneys and liver were quickly excised, rinsed, blotted on filter paper, weighed and homogenized with ice-cold Tris–HCl buffer (pH 7.4). Serum was used for the estimation of Cr and BUN using commercially available kits (Sigma). Kidney and liver tissues were collected for the determination of Pt accumulation by the method of Pera and Harder [17]. The body weights of mice were recorded throughout. All assay samples were analyzed in triplicate for each animal.

Results

Inhibition of growth of SK-Hep1, HepG2 and primary culture of hepatocytes

In the human HCC cell lines, 1–5 were found to be significantly more potent than cisplatin and carboplatin (Table 1; Student's *t*-test, $p < 0.05$). In particular, 5 was 18 and 20 times more potent than cisplatin, and 168 and 211 times more potent than carboplatin in the SK-Hep1 and HepG2 cells, respectively. To compare the relative anti-proliferative activity between the TCM-Pt compounds and cisplatin towards liver cells, inhibition of growth in a primary culture of mouse hepatocytes was evaluated. Compounds 1–5 were found to be significantly more potent than cisplatin (4–34 times) and carboplatin (34–290 times) (Table 1). DMC was also found to be highly effective in inhibiting the growth of mouse hepatocytes, with an IC_{50} of about 10 μM.

Anti-tumor activity *in vivo*

The anti-tumor activities of 1–5, DMC, cisplatin and carboplatin were evaluated *in vivo* in the SK-Hep1 xenograft in athymic nude mice. Individual tumor growth data were censored for animal deaths and hence evaluation of drug effects was not affected.

Dose-finding studies with 1–5 identified respective doses of 25, 120, 140, 140, 30 mg/kg/injection administered i.p. every 5 days for 3 times as approximating to their MTD, either on the basis of induced mortality or major body weight loss. TCM-Pt 1–5 demonstrated definite *in vivo* activity against the human HCC xenograft model (Table 2), contrasting the minimal activity recorded for cisplatin and carboplatin. A high level of activity was noted for 2 at 120 mg/kg/injection, 3 at 140 mg/kg/injection and 5 at 30 mg/kg/injection (Table 2). Optimal %T/C

Table 1 ***In vitro*** **growth-inhibitory activity of the Pt compounds**

	Cisplatin	Carboplatin	1	2	3	4	5	DMC
SK-Hep1	54.2 ± 9.8	500.6 ± 86.6	20.6 ± 5.9	17.2 ± 8.1	15.4 ± 6.0	20.0 ± 5.5	3.0 ± 0.5	11.5 ± 4.5
HepG2	37.3 ± 2.4	392.7 ± 40.3	16.5 ± 3.2	19.2 ± 3.3	17.2 ± 4.3	18.2 ± 2.7	1.9 ± 0.1	12.3 ± 2.4
Mouse primary hepatocyte	121.4 ± 16.3	1032.2 ± 69.6	32.4 ± 7.3	30.1 ± 4.1	37.7 ± 8.2	37.3 ± 7.9	3.6 ± 1.2	12.5 ± 3.3

IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by MTT assay after a 72-h drug exposure. Data represent the mean values of three independent experiments (IC_{50} ± SD; in μM; $n = 12–16$). Results for cisplatin, carboplatin and 1–5 in SK-Hep1 cells have been previously cited in [4]. For the primary mouse hepatocyte culture, data represent the mean values from 12 samples assayed in three independent experiments.

Table 2 In-vivo anti-tumor activity of compounds 1–5 and DMC in human HCC SK-Hep1 grown as s.c. xenografts in male nude mice

Compounds	Treatment schedule	Tumor growth inhibition					
		Dose (mg/kg/dose)	Maximum percentage weight change	Optimal T/C (%)	SGD	*n* (no. of deaths)	Efficacy[a]
Cisplatin	i.p. day 1, 5, 9	4	−25	97.5	0	6 (0)	−
		6	−30	90.5	0.48	6 (0)	−
		8	−35	85.5	0.52	6 (2)	−
		10	−40	85.5	0.52	6 (3)	−
Carboplatin	i.p. day 1, 5, 9	50	+18	108.7	<0	6 (0)	−
		75	+3	95.3	<0	6 (1)	−
		100	−4	92.1	0.55[d]	6 (1)	−
		120	−21	35.2[b]	>1.5[e]	6 (2)	+ +
1	i.p. day 1, 5, 9	12.5	+19	66.3[c]	0.90[d]	6 (0)	−
		25	+8	14.5[b]	1.86[e]	6 (0)	+ +
		50	−14	10.2[b]	>2.0[e]	6 (2)	+ + +
2	i.p. day 1, 5, 9	50	+18	92.7	0.57	6 (0)	−
		7.5	+19	70.0[c]	0.50[d]	6 (0)	−
		100	+16	18.6[b]	1.24[d]	6 (0)	+
		120	+9	15.2[b]	>2.0[e]	6 (0)	+ + +
		140	−6	14.0[b]	>2.0[e]	6 (2)	+ + +
3	i.p. day 1, 5, 9	75	+17	101.2	0.26	6 (0)	−
		100	+15	75.8[c]	0.88[d]	6 (0)	−
		120	+15	38.1[b]	>2.0[e]	6 (0)	+ +
		140	+5	16.0[b]	>2.0[e]	6 (1)	+ + +
4	i.p. day 1, 5, 9	75	+16	102.9	0.59	6 (0)	−
		100	+18	73.6[c]	0.48	6 (0)	−
		120	+15	49.9[b]	>2.0[e]	6 (0)	+
		140	+4	38.6[b]	>2.0[e]	6 (1)	+ +
		160	−7	22.3[b]	>2.0[e]	6 (2)	+ + +
5	i.p. day 1, 5, 9	12.5	+16	91.9	0.95[d]	6 (0)	−
		25	+14	41.2[b]	>2.0[e]	6 (0)	+
		30	+13	24.6[b]	>2.0[e]	6 (0)	+ + +
		50	−15	13.8[b]	>2.0[e]	6 (1)	+ + +
DMC	i.p. day 1, 5, 9	2	+20	93.2	0.48	6 (0)	−
		4	+15	76.1[c]	0.52	6 (0)	−
		8	−20	44.8[b]	1.02[d]	6 (2)	+

[a]The anti-tumor activity was scored according to Fodstad [15], on the basis of optimal %T/C and SGD.
[b]$p<0.01$.
[c]$p<0.05$; relative tumor volume significantly different from control.
[d]$p<0.05$; absolute growth delay significantly different from cisplatin.
[e]The specific tumor delay was not determined because the tumor in the treatment group had not reached 500 cm^3.

values and SGD values were below 25% and greater than 2.0, respectively. These effects were achieved without any significant body weight loss and were not associated with any marked toxicities or any drug-related mortality (except one out of six premature deaths for 3 at 140 mg/kg/injection). Complete remission of the tumor xenograft in two out of six mice after 25 days was also observed by treatment with 30 mg/kg of 5. The mice in remission were re-challenged with the tumor to exclude any possible immunological problems. In contrast, cisplatin showed minimal activity even at its MTD of 6 mg/kg, yielding an optimal %T/C of 90.5% and a SGD value of 0.48 (Table 2). Carboplatin achieved a moderate retardation of tumor growth at doses of more than its MTD (100 mg/kg) and had no effect at low doses. DMC also demonstrated a moderate response: a MTD of 8 mg/kg could give an optimal %T/C of 44.8%.

The results for DRDs and body weight change are presented in Table 2. DRDs were prominent at high doses for cisplatin (8 mg/kg: two out of six) and carboplatin (120 mg/kg: two out of six). For 1–5, there were few cases of DRDs (none or one out of six) at the effective doses. Notably, DMC was also remarkably toxic at 8 mg/kg and gave two cases of DRD out of six.

Compounds 2–5, at all doses, did not result in any weight loss during the course of therapy and most of the mice were able to attain the same body weight gain of about 20% as the control tumor-bearing mice by the end of the observation period. Compound 1 gave a transient body weight loss of about 15% during the early course of treatment. In contrast, cisplatin (above 6 mg/kg) and carboplatin (above 100 mg/kg) gave unacceptable body weight losses of more than 20%, and mice in these treatment groups could not attain the same body weight

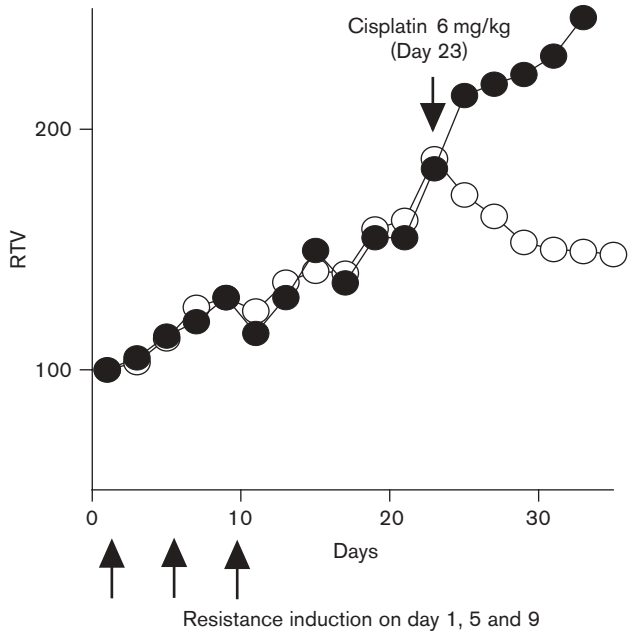
gain by the end of the observation period, as compared to the control.

Induction and evaluation of cisplatin resistance *in vivo*

To verify the cross-resistance behavior of the novel TCM-Pt compounds and corroborate the role of DMC in overcoming cisplatin resistance, an *in vivo* assay system was designed in which treatment of tumor-bearing mice with cisplatin caused a rapid development of resistance to the drug. Fourteen days after resistance induction (day 23), animals were challenged with cisplatin (6 mg/kg). Seven days later, the control group showed a 13% suppression of tumor growth, whereas the cisplatin-pre-treated tumor continued to grow by 28% ($n = 12$–14). Thus, the tumor had developed resistance as a result of the initial treatment with cisplatin and a typical individual result with this protocol is shown in Figure 2.

For both the control and the acquired cisplatin-resistant groups, 1–5 at the designated doses gave rise to similar tumor growth inhibition (25–35% at day 30), with no cross-resistance between 1–5 and cisplatin (Table 3). Concomitant treatment using cisplatin with DMC resulted in a tumor growth inhibition of about 20% in both the cisplatin-pre-treated and the control groups, demonstrating circumvention of cisplatin resistance.

Fig. 2



In vivo induction of drug resistance by cisplatin. Tumor-bearing mice were treated with three i.p. injections of either NS (open circles) or 2 mg/kg cisplatin (filled circles) on days 1, 5, and 9. Both animal groups were treated i.p. with 6 mg/kg cisplatin on day 23. Tumor sensitivity to the treatment with 6 mg/kg cisplatin was assessed on day 30.

However, combination of cisplatin with 1,1-cyclobutane-dicarboxylate (leaving group in carboplatin) was unable to inhibit tumor growth (Table 3).

Acute toxicity study in ICR mice

The toxicity properties (acute phase) in kidneys of the novel TCM-Pt compounds were examined in a mouse model, using an equitoxic dose of about LD_{10} for all compounds. The toxic effects of the drugs on the whole body of the mice were estimated in terms of body weight loss and survival rate. Deaths due to toxicity were observed for cisplatin and DMC only (two deaths out of 18 animals for cisplatin and three out of 18 for DMC). Control mice gained in body weight by about 20% 5 days after treatment. In contrast, cisplatin caused a marked body weight loss by more than 15% and carboplatin caused a weight loss of below 10%. However, all novel compounds and DMC resulted in a slight body weight gain of 5–10% (Fig. 3a and b).

Serum levels of Cr and BUN were selected as indicators of nephrotoxicity. Compounds 2–4 and carboplatin had no significant effect on the level of serum Cr and BUN. However, marked elevation of these indicators was observed in the mice treated with cisplatin. The serum Cr level in the control group was 0.43 ± 0.09 mg/dl. A significant increase in the serum Cr level was induced by treatment with cisplatin, where levels rose from 2.25 mg/dl on day 2 to 6.96 mg/dl on day 5 after drug treatment. Compounds 1, 5 and DMC also caused an increase of serum Cr, but were significant only on day 5, where levels were 0.73, 1.05 and 0.93 mg/dl, respectively (Fig. 4a).

Cisplatin also led to markedly increased BUN levels on day 2 (51.93 mg/dl) and day 5 (161.82 mg/dl) after treatment, with a control group BUN concentration of 22.49 ± 5.62 mg/dl. Compounds 1, 5 and DMC also increased BUN levels, but not to the extent shown by cisplatin, and once again results were significantly different from control only on day 5 after treatment, where BUN levels were 47.95, 51.34 and 38.15 mg/dl, respectively (Fig. 4b).

Pt accumulation in the kidney and liver was also assessed upon treatment with the novel compounds (Fig. 5a and b). All Pt compounds including the novel TCM-Pt compounds, cisplatin and carboplatin accumulated Pt more in the kidney than in the liver. In both organs, Pt accumulation due to cisplatin was the highest and its retention the longest, which could account for its remarkable toxic side-effects, at least in the kidney.

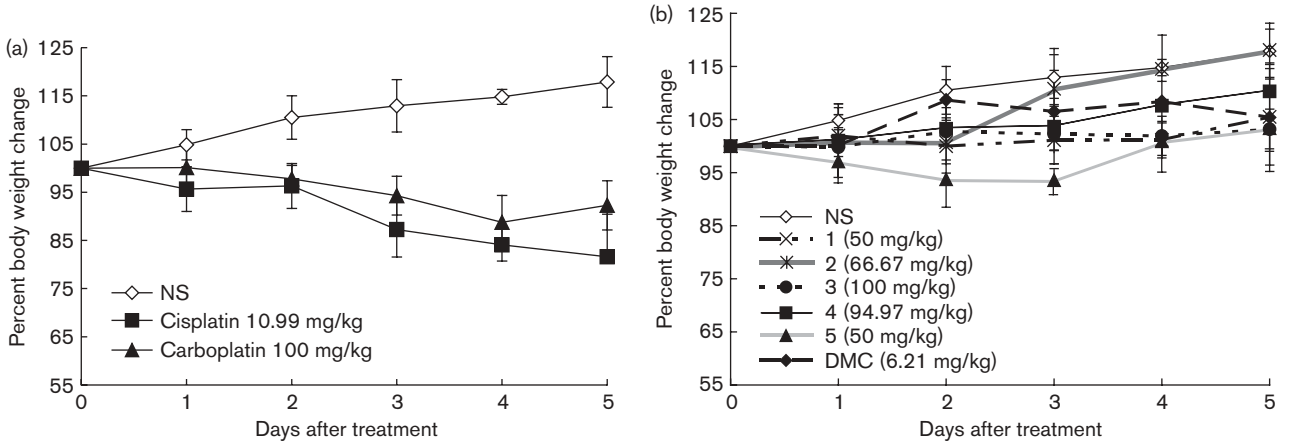
Pt accumulation due to cisplatin in kidneys was about 52 nmol Pt/g wet tissue on day 1 after treatment and remained at a level of about 34 nmol Pt/g wet tissue

Table 3 Circumvention of cisplatin cross-resistance by 1–5 and combination of cisplatin with DMC or cyclobutanedicarboxylate

Treatment on day 23	Percentage change in tumor growth on day 30[a]	
	Control group	Cisplatin-resistant group[b]
Cisplatin (6 mg/kg)	−13.1 ± 2.0	+28.5 ± 4.1[c]
DMC (2 mg/kg)	+36.5 ± 3.4	+40.2 ± 4.2
1 (25 mg/kg)	−24.3 ± 5.1	−23.3 ± 3.2
2 (120 mg/kg)	−35.8 ± 4.5	−36.3 ± 8.1
3 (120 mg/kg)	−26.8 ± 3.3	−25.2 ± 5.6
4 (120 mg/kg)	−38.7 ± 6.5	−39.7 ± 7.9
5 (30 mg/kg)	−35.7 ± 5.7	−31.5 ± 6.2
Cisplatin (6 mg/kg) + DMC (2 mg/kg)	−19.0 ± 3.1	−20.1 ± 4.9
Cisplatin (6 mg/kg) + cyclobutanedicarboxylate anion (10 mg/kg)	−14.2 ± 2.3	+24.4 ± 3.5[c]

[a]The control tumors and cisplatin-pre-treated tumors received a single dose of 1–5, cisplatin or a combination of cisplatin with DMC/cyclobutanedicarboxylate 1 week after the induction of resistance (day 23). The percentage change in tumor growth is defined as the ratio of the relative tumor volume on day 30 to that on day 23. Cross-resistance was evaluated by comparing the percentage change in tumor growth in the control tumors with that in the cisplatin-pre-treated tumors.
[b]Induction of cisplatin resistance is illustrated in Figure 2.
[c]Significant different from control tumor ($p < 0.05$).

Fig. 3



Body weight changes due to treatment with Pt compounds and DMC. (a) Control, cisplatin and carboplatin. (b) Control, novel TCM-Pt 1–5 and DMC. Body weight changes are expressed with respect to body weight on day 0; day 0 = 100%.

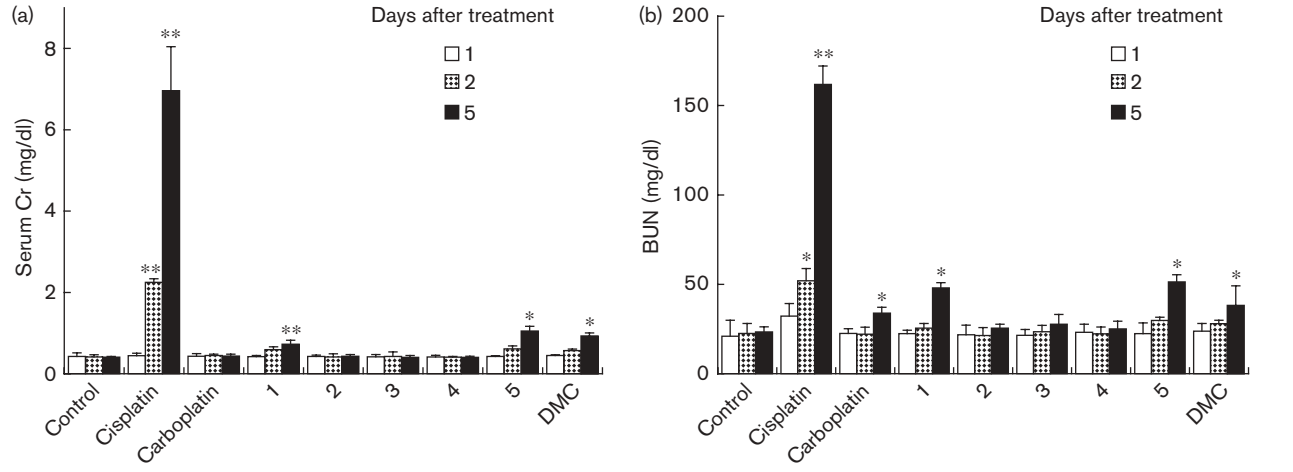
thereafter. Second to cisplatin, 5 caused a Pt accumulation of about 43 nmol Pt/g wet tissue on day 1, but the level dropped to below 20 nmol Pt/g wet tissue on day 5. Other novel compounds and carboplatin produced Pt accumulations of about 30 nmol Pt/g wet tissue on day 1; on day 5 after treatment, this level dropped to around 5–15 nmol Pt/g wet tissue. The novel compounds did not cause a long retention of Pt as demonstrated by cisplatin. Similarly, in liver, Pt accumulation due to cisplatin was the highest among all the Pt compounds tested and its retention was also the longest.

Discussion

The anti-tumor activity of TCM-Pt anti-cancer agents 1–5 in a panel of murine and human cancer cell lines has been previously reported [4]. Spearman rank-order analysis comparing the sensitivity pattern of the TCM-Pt compounds with that of established Pt-based anti-cancer drugs found that cisplatin sensitivity correlated only with that of carboplatin [18]. This is consistent with clinical findings that these two drugs share the same spectrum of anti-tumor activity [19]. However, sensitivity to cisplatin and the novel TCM-Pt compounds was not correlated, mainly due to the fact that 1–5 were also active against cisplatin-unresponsive cancer cells such as HCC [18].

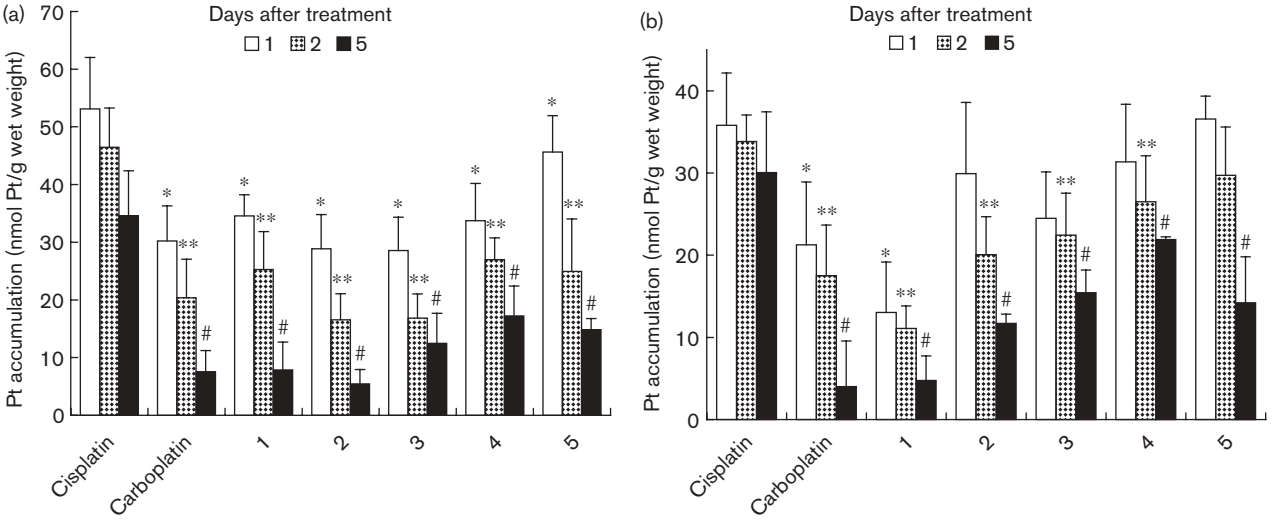
Historically, cantharidin has been used in China to treat liver and gastric cancers for many years [5]. An early hypothesis by our research team was that integration of the modified TCM component, DMC, into 1–5 might introduce a particularly high anti-tumor activity toward liver cancer cells [4]. If this was indeed the case, it would mean a substantial advancement in the development of Pt anti-cancer agents, as to date there have been limited

Fig. 4



Changes in (a) Cr levels and (b) BUN levels in male ICR mice ($n=6$) treated with a single i.p. injection of cisplatin, carboplatin, novel TCM-Pt 1–5 and DMC. Each bar represents the mean ± SD in each treatment group. Control mice ($n=10$) were injected with NS. *$p<0.05$, ** $p<0.001$; compared with the control group.

Fig. 5



Changes in Pt accumulation in the (a) kidney and (b) liver in male ICR mice ($n=6$) treated with a single i.p. injection of cisplatin, carboplatin and novel TCM-Pt 1–5. Each bar represents the mean ± SD of Pt accumulation in each treatment group. *, **, #$p<0.05$; compared with the cisplatin-treated group on days 1, 2 and 5, respectively.

chemotherapeutic options available for the treatment of HCC [20,21].

The present study provides further evidence that 1–5 do exert a superior anti-tumor activity towards liver cancer (SK-Hep1 and HepG2), compared with the poor response shown by cisplatin and carboplatin, which is consistent with our previous preliminary cytotoxicity results [4]. HepG2 cells are well-differentiated polygonal cells that resemble normal hepatocytes, while SK-Hep1 cells are poorly differentiated [22]. Our results that SK-Hep1 is less sensitive than HepG2 towards cisplatin and carboplatin is consistent with those published elsewhere [23]. Cell differentiation status may play a role in the distinct chemosensitivity towards cisplatin and carboplatin. Interestingly, the novel compounds were found to be equally

effective against the two cell lines, again suggesting a different mechanism of anti-tumor activity compared with cisplatin.

Additional evidence that liver cells are more sensitive towards the TCM-Pt compounds than cisplatin and carboplatin was obtained by assaying the growth inhibition in a primary culture of mouse hepatocytes. As mentioned earlier, liver cytosol is one site that is rich in PP2A and the level of its inhibition has been found to parallel cytotoxicity [9]. Therefore, the results appear to further substantiate our hypothesis that the incorporation of PP2A-inhibitory DMC into 1–5 should bring about a higher potency towards the liver, when compared with the classical Pt drugs (i.e. cisplatin and carboplatin). However, reasons for this higher sensitivity of SK-Hep1, HepG2 and mouse hepatocytes towards 1–5 are still unclear. It has been suggested that PP2A inhibition in primary hepatocytes is less well tolerated for disruption in DNA replication than in other cell types [24]. PP2A is the major class of dephosphorylating enzymes active in gluconeogenesis in the liver [25]. Treatment with okadaic acid, a specific PP2A inhibitor, leads to hyperphosphorylation of such enzymes [26,27]. Based on evidence from the regenerating liver [28] and cultured hepatocytes [29], it has been postulated that signals evoking maximal gluconeogenesis in the hepatocyte lead to abrupt cessation of the G_1/S transition. The teleological explanation may be that if the organism acutely needs glucose produced by the hepatocytes, the latter should not divert their energy toward proliferation. However, the higher anti-proliferative activity of 1–5 in the mouse hepatocytes *in vitro* does not necessarily lead to higher organ toxicity *in vivo*. Evidence of clinically significant toxic side-effects should come from toxicity studies performed in animals.

SK-Hep1 was chosen for the *in vivo* study in nude mice because it has been reported to be the most tumorigenic human liver cancer cell line in this animal model [22]. Moreover, among a number of liver cancer cell lines, SK-Hep1 is the most resistant to cisplatin *in vitro* [23]. Consistent with our preliminary result in a SK-Hep1 xenograft in nude mice reporting tumor growth up to 60 days post-treatment [4], a good correlation between the *in vitro* and the *in vivo* anti-tumor activity of the novel TCM-Pt compounds apparently does exist in our present detailed study. It is likely that the superior *in vivo* efficacy of the novel Pt compounds over cisplatin reflects the contribution of improved tolerability, thus allowing a more intensive treatment schedule and a higher cumulative dose.

Interestingly, the human liver cancer xenograft was only moderately sensitive to DMC (optimal %T/C = 44.8% at its MTD). This result excludes the use of DMC alone as an effective anti-tumor agent. However, there is strong evidence that an enhanced anti-tumor effect may be achieved by incorporating DMC into a Pt moiety and, importantly, the combined chemical entity does demonstrate high potency towards HCC in the model used.

With regard to toxicity, the activities of the TCM-Pt compounds were obtained at doses which are not associated with any undue toxicity, as judged by body weight monitoring of tumor-bearing mice. The progressive release of the cytotoxic PP2A-inhibitory moiety (hydrolyzed DMC) from 1–5, as previously reported [30], may allow the novel compounds to be better tolerated.

It has been reported that nephrotoxicity is the most significant clinical side-effect of cisplatin and the proximal tubule has been described as the main target site [31]. It has also been established that the elevation of serum level of BUN and Cr upon treatment with cisplatin increased progressively from 48 to 120 h, reaching a maximum about 5 days after the drug treatment [32]. Therefore, in our acute toxicity study using ICR mice, toxicity was monitored for 5 days. Cisplatin was found to be highly nephrotoxic, causing significant elevations of the two parameters at its LD_{10}. However, all novel TCM-Pt compounds and carboplatin were much less nephrotoxic, and did not cause significant changes in BUN and Cr. Exceptions were 1, 5 and DMC, where an increase in the two parameters on day 5 after treatment was observed, but the levels were much lower than that due to cisplatin. The results of this animal study were consistent with the clinical results obtained with cisplatin and carboplatin [33]. We also conducted a preliminary study on drug disposition in ICR mice which indicated a lower tissue content of 1–5 than cisplatin in organs targeted for toxicity, i.e. the kidney, and a faster clearance from plasma (data not shown). In another related study, tissue slices (kidney and liver) were dissected from ICR mice treated i.v. q24 h for 5 days with a projected human MTD (1/12 LD_{10} in mice) of cisplatin, carboplatin and the novel compounds for histopathological examination [34]. Histopathology was carried out and data validated by qualified specialists at the Victorian Veterinary Pathology Services, South Yarra, Victoria, Australia. No undue toxicity was recorded for either kidney or liver with the novel compounds. Such findings are in line with a better tolerability and provide evidence of a lower nephrotoxicity in animals.

To date, there have been relatively few studies of Pt resistance in an *in vivo* setting, either involving primary human tumor tissue or murine-based tumor models. A key finding from literature was a recent report describing the rapid induction of cisplatin resistance in an *in vivo* model of human ovarian tumor xenograft after treatment with only a low dose of cisplatin [16]. Since circumvention of cisplatin resistance has been successfully

demonstrated *in vitro* by the TCM-Pt compounds [18], we embarked on obtaining further proof *in vivo* with a cisplatin-resistant human liver cancer xenograft model, using a protocol adapted from Caffrey and Frenkel [16]. In our *in vivo* resistant model, cisplatin-pre-treated tumors became resistant to subsequent treatment with cisplatin, but **1–5** could still elicit the same level of tumor growth suppression as in the control tumors. Moreover, a combination of cisplatin and DMC also demonstrated circumvention of resistance. A parallel experiment using a combination of cisplatin and 1,1-cyclobutanedicarboxylate, the leaving group in carboplatin, showed an absence of this effect. The result reinforces the importance of PP2A inhibition in the circumvention of cisplatin cross-resistance in the HCC model because 1,1-cyclobutanedicarboxylate does not posses the inhibitory effect [18]. This resistance model overcomes the problem of tumor heterogeneity because the evaluations of Pt accumulation, intracellular GSH content and DNA repair capacity were carried out on primary cultures generated from the cisplatin-resistant tumor cells selected *in vivo* (data not shown). We found that the 2.6-fold resistant primary cultures obtained from tumors subjected to three doses of cisplatin did not contain elevated levels of GSH. Other observations were: no apparent decrease in cisplatin accumulation, and a greater DNA repair capacity as shown from a lowered level of DNA platination and a faster removal rate of DNA–Pt adducts when compared with the control tumors (data not shown).

This current study has demonstrated significant anti-tumor activity of the TCM-Pt compounds in human HCC cell lines and in xenografts grown in nude mice that are intrinsically unresponsive to cisplatin treatment. Taken together, the data does support the potential value of the TCM-Pt compounds in the treatment of human HCC. The importance of PP2A inhibition is implied, and further studies exploring the relationship between cytotoxicity and PP2A sensitivity of the liver cancer cells are warranted.

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