Evaluation of cisplatin and a novel platinum polymer conjugate for drug toxicity and drug distribution in mice

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The toxicity and distribution of cisplatin and two novel platinum (Pt) polymer conjugates (Pt-6 and Pt-7) were determined in serum and tissue of BALB/c mice at specific time points after i.p. administration of a drug bolus containing identical Pt concentrations. Pt concentrations were determined in serum, liver and kidney at 5 and 15 min, respectively, after drug administration by inductively coupled plasma mass spectrometry. It was found that the Pt polymer Pt-7 gave rise to a considerably lower Pt concentration in serum and considerably higher concentration in liver and kidney than cisplatin. LD_{25} measurements indicated that the Pt-7 polymer is considerably less toxic than cisplatin. *In vitro* experiments and determination of IC_{50} values in a variety of human tumor cell lines, normal lymphocytes and fibroblasts confirmed that Pt-6 and Pt-7 polymers are 40–500 times more toxic for tumor cells than for normal cells, perhaps reflecting preferential uptake. The toxicity of cisplatin was found to be only 1.6–40 times more effective in tumor cells. These inter-relationships are supported by the observation that the tumor enrichment factor (TEF) for cisplatin is only in the region of 6, and much lower than for Pt-6 and Pt-7, where TEFs are in the region of 40 and 150, respectively. These results demonstrate that the Pt polymer conjugates exhibit greater tumor specificity than cisplatin, killing tumor cells more effectively while being considerably less toxic for normal cells. It is concluded that the Pt polymer conjugates may be superior for cancer therapy and warrant further testing to assess their full clinical potential.
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Introduction

The effectiveness of cisplatin as an anti-tumor drug would require rapid distribution in the circulation and uptake into the tumor parenchyma. Since cisplatin toxicity is not tumor specific, targets involved in drug metabolism such as liver and kidney may act as representative sinks, which could give an indication of the ability of the drug to enter peripheral structures, including tumor tissue.

In the search for improved relatives of cisplatin it is therefore of interest to assess the *in vivo* toxicity, distribution and clearance of the new homolog in serum and tissues. Work on mice has shown that an i.p. injection of 7 mg/kg cisplatin distributes rapidly within 5 min, reaching 12.2 and 13.6 μg/ml in whole blood and in serum, respectively, while the highest platinum (Pt) concentration in liver and kidney is reached after 15 min, showing values of 20 and 40 μg/g tissue, respectively. In the same study, elimination of Pt from serum was found to be biphasic, declining rapidly within 1 h to 0.6 μg/ml and thereafter very slowly over 168 h to 0.15 μg/ml, showing good correlations between Pt levels in serum and peak levels in tissue [1]. The toxicity of Pt drugs is a particularly critical issue, as the optimum dose for cancer treatment must be kept high without causing undue toxicity to vital organs. In the case of cisplatin the therapeutically effective optimum dose was found to be 7 mg/kg, while 3 mg/kg was ineffective and 14 mg/kg constituted the LD_{50} [1,2]. The clinical application of new Pt derivatives thus would require careful pharmacokinetic measurements to determine the LD_{50}, drug tolerance and therapeutically effective dose. In the following we present a comparison of cisplatin and two Pt-containing polymer conjugates called Pt-6 and Pt-7 with regard to *in vivo* toxicity and organ distribution. In particular, we wish to test the hypothesis that a water-soluble Pt-containing polymer conjugate may be less toxic, and show a better tissue distribution and better tumor uptake than the well-established drug cisplatin.

Materials and methods

Polymer conjugates

Cisplatin was obtained from Sigma (St Louis, MO). Polymer Pt-6, a Pt conjugate derived from a linear, aliphatic polyamide, is an experimental conjugate for

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which structural details remain to be determined; Pt content is 5% by weight. The Pt-7 polymer, with a Pt content of 7%, was synthesized through polycondensation of aspartic acid to yield the intermediates of poly-DL-succinimide, which was converted to polyaspartamide and further to the Pt conjugate as described [3,4]. Both polymers were purified by aqueous dialysis in membrane tubing with a molecular weight cut-off of 25 000. The molecular size of the polymers was accordingly estimated to be in excess of 20 000 Da.

Serum and tissue distribution of Pt

Drugs were diluted in saline and female BALB/c mice (6–7 weeks old and weighing 20–25 g) were given 0.3-ml drug dilutions containing 0.33 mg Pt/ml to produce the same Pt concentration of 4.9 mg Pt/kg. The dilutions were made up fresh every day and injected soon afterwards to prevent the regeneration of the dichloro complex. In treatment group 1, consisting of 18 animals, nine animals received cisplatin only, while the remaining nine animals received Pt-7 polymer only. In the cisplatin group, three animals were sacrificed after 5 min to provide blood samples, three animals were sacrificed after 15 min to produce kidney samples and the remaining three animals were sacrificed to produce liver samples. Collection times had been ascertained in pilot experiments (not shown). Blood was allowed to clot and then centrifuged to produce serum. Pt was assayed by inductively coupled plasma mass spectrometry (ICP-MS) by AMPATH Laboratories (Pretoria, South Africa). The method is well established, and has been successfully applied in biotransformation studies showing excellent sensitivity and high specificity [5,6]. Animal experiments were conducted at the University of Pretoria Biomedical Research Centre (UPBRC). Ethical approval was granted by the Animal Use and Care Committee (AUCC) of the University of Pretoria. Statistical analysis was done by analysis of covariance (ANCOVA). The mice were kept in standard mouse cages, under controlled environmental conditions and day/light cycles of 12 h. Standard Epol mice cubes and water were given *ad libitum*.

LD_{25} measurements

In a group of 60 animals, 40 animals received escalating doses of cisplatin to generate Pt concentrations of 4.5, 6.5, 7.8 and 9.1 mg/kg. Animals were given a 0.3-ml i.p. bolus injection every 7 days for 4 weeks [2]. When 25% of the animals in a specific dose group showed any adverse reactions such as anemia or diarrhea, all animals were euthanized and the study was repeated at the next lower dose level. A group of 10 animals received Pt-7 polymer and the polymer ligand alone without any Pt, using 0.3-ml bolus i.p. injections containing the same amount of carrier, but without cisplatin, as injected in the animals receiving Pt-7 polymer. In view of the limited amount of Pt-7 polymer available, only one drug concentration was tested and no statistical data analysis was performed for this group.

In vitro experiments

Tumor cells were grown under standard tissue culture conditions using media supplemented with 10% fetal bovine serum and addition of penicillin/streptomycin and 2 mM glutamine if required. CoLo 320DM cells (ATCC; CCL-220) were grown in RPMI 1640 medium. HeLa cells (ATCC; CCL-2) were grown in EMEM medium, and DU145 (ATCC; HTB-81) were grown in RPMI medium. Cytotoxicity was determined in 96 multi-well plates at an initial cell concentration of 6×10^4 cells/well and incubated with the toxin for 4 days when cell concentrations were determined by the MTT assay [7,8] by reading at 540 nm as previously described [8]. Blood obtained from healthy volunteers was used to prepare lymphocytes using Histopaque-1077 (Sigma-Aldrich, St Louis, MO) and granulocytes to obtain a preparation of neutrophils in HBSS (Highveld Biologicals, Johannesburg, South Africa) which were counted in a hemocytometer. Lymphocytes were stimulated with phytohemagglutinin (PHA; BIOWEB, Johannesburg, South Africa) and then incubated with the toxin for 4 days when the plates were subjected to MTT assay. Normal human fibroblasts (ATCC; CCL-171) were grown in EMEM medium.

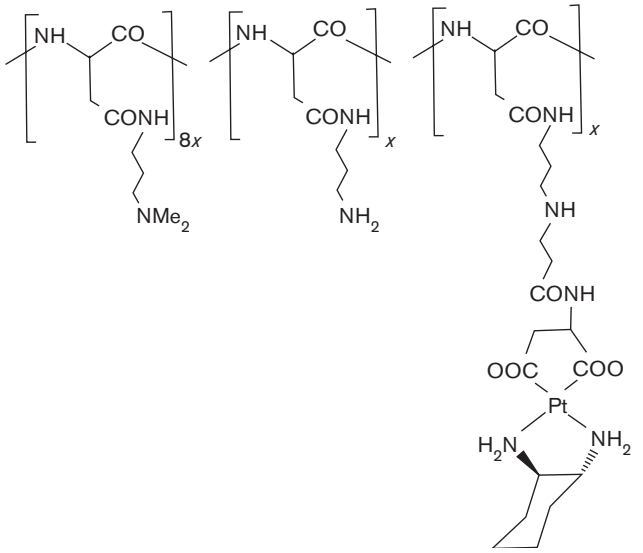
The drugs were made up as stocks of 2 mg Pt/ml using DMSO for cisplatin and water for the polymers, and then diluted as indicated. Results of cell growth inhibition were expressed as mean percentage ± SEM of untreated controls. The p value compares the experimental values with the controls and was obtained by paired Student's t-test. The IC_{50} value represents the drug concentration that inhibits 50% of cell growth.

Results

Polymer conjugates

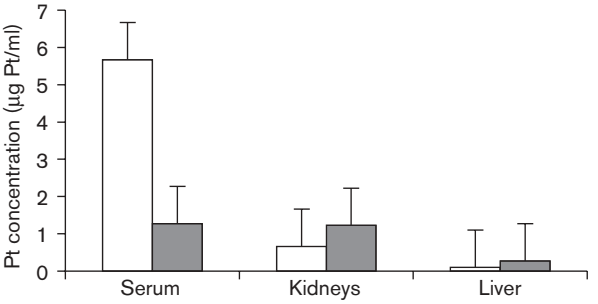
The Pt-7 polymer is properly designated poly-α,β-DL-[*N*-(3-(dimethylamino)propyl)-aspartamide (80)-*co*-*N*-(3-aminopropyl)-aspartamide (10)-*co*-*N*-(4-aza-7-oxo-7-(*N*-aspartato-*trans*-cylcohexane-1,2-diamineplatium (II))-heptyl)aspartamide (10)]. The structure, confirmed by ^{1}H-NMR spectroscopy, is given in Figure 1. It is apparent that Pt is coordinated by two amino groups of cyclohexanediamine and by two carboxylato ligands which are attached to the polyaspartamide carrier. As the polymer was isolated from the retentate after aqueous dialysis in membrane tubing with a molecular weight cut-off limit of 25 000, its average molecular weight is assumed to be within that limit. The Pt contents of the Pt-6 and Pt-7 polymers are 5 and 7%, respectively, as compared to cisplatin, where the Pt content is 65%. The Pt contents served to calculate drug concentrations, which would

Fig. 1



Structure of Pt-7 Pt polymer conjugate (see text).

Fig. 2



Mean serum and tissue concentration of Pt in mouse serum and mouse tissues after application of equal doses of Pt in the form of cisplatin (open bars) and Pt-7 polymer conjugate (shaded bars). Error bars reflect standard deviation of the mean. $p < 0.05$ indicates differences between means were significant

Table 1 Pt concentration in serum and tissues in response to application of equal doses of Pt as cisplatin and as Pt-7 polymer conjugate

	Cisplatin	Pt-7
Serum (5 min)	5.3 µg Pt/ml	1.81 µg Pt/ml
	6.8 µg Pt/ml	1.16 µg Pt/ml
	4.9 µg Pt/ml	0.86 µg Pt/ml
Kidneys (15 min)	0.68 µg Pt/g	1.49 µg Pt/g
	0.69 µg Pt/g	1.10 µg Pt/g
	0.61 µg Pt/g	1.08 µg Pt/g
Liver (15 min)	0.07 µg Pt/g	0.21 µg Pt/g
	0.18 µg Pt/g	0.37 µg Pt/g
	0.06 µg Pt/g	0.24 µg Pt/g

generate identical Pt concentrations in the animal (see Materials and methods).

In vivo drug toxicity

In the lowest dose group receiving 4.5 mg Pt/kg no abnormalities were detected after 1 month when the experiment was terminated. The group receiving 6.5 mg Pt/kg was also terminated after 1 month, showing no abnormalities except a small weight loss. At 7.8 mg Pt/kg, three animals out of 10 died after 3 days and the experiment was terminated. The dose level of 9.1 mg Pt/kg proved to be lethal to all mice within 3 days. All animals were examined for liver and kidney abnormalities, and none were detected. From these results the LD_{25} was estimated to be 7.8 mg Pt/kg. A Pt-7 polymer dose generating 7.8 mg Pt/kg was then used for toxicity response—none was detected after 1 month and there were no adverse reactions. However, there was one unrelated exitus in the carrier group not containing Pt and this is attributed to unknown unspecific causes. It is concluded therefore that the LD_{25} for the polymer must be lower than for cisplatin.

Serum and tissue distribution of Pt in mice

Figure 2 based upon data in Table 1 shows that administration of cisplatin generates Pt serum levels in the region of 4.9–6.8 µg/ml 5 min after injection, whereas administration of the same concentration of Pt in the form of the Pt-7 polymer only generates 0.85–1.81 µg Pt/ml. It is apparent therefore that the clearance of Pt-7 from the serum is 3–5 times faster than cisplatin. The kidney and liver data assayed 15 min after drug application corroborate these results, showing Pt levels in kidney of 1.09–1.49 µg Pt/g tissue, as compared to 0.61–0.69 µg Pt/g when cisplatin was given (Table 1). The liver data also show a 2–3 times higher Pt concentration with 0.21–0.37 µg Pt/g for Pt-7 polymer as compared to 0.06–0.175 µg Pt/g for cisplatin application (Table 1). The histogram (Fig. 2) illustrates that there are indeed marked differences in the Pt concentration in the target tissues and that these differences are statistically significant.

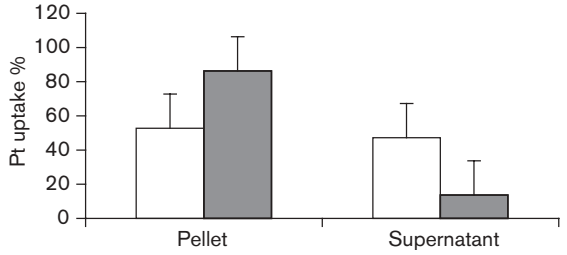
Uptake of Pt drugs in HeLa cells

When HeLa cells in EMEM medium were incubated with cisplatin and Pt-7 at 5 µg Pt/ml for 30 min, and Pt was analyzed in the supernatant and in the cell pellet by ICP-MS, it was found that cisplatin distributes fairly evenly at a 55:45% ratio between the pellet and the supernatant. In the case of Pt-7, however, the distribution of Pt was highly displaced in favor of the pellet, showing that nearly 90% of the Pt is concentrated in the cell pellet and less than 10 % in the medium (Fig. 3).

In vitro drug toxicity

The indication of preferential cellular uptake of the Pt-7 polymer prompted *in vitro* experiments to test drug

Fig. 3



Pt uptake in response to a 30-min incubation of HeLa cells with 5 μg Pt/ml as cisplatin (open bars) or as Pt-7 polymer conjugate (shaded bars) and Pt assay by ICP-MS.

Table 2 Cytotoxicity of Pt polymers compared to cisplatin as measured by the MTT assay in various cell lines

Cell line	IC_{50} (μg Pt/ml)		
	Cisplatin	Pt-6	Pt-7
CoLo 320DM	1.00	0.40	0.02
HeLa	0.11	0.04	0.0002
DU145	0.14	1.52	0.10
Mean	0.42	0.65	0.04
Resting lymphocytes	1.66	21.71	5.71
PHA-stimulated lymphocytes	2.14	24.09	4.63
CCL-171 fibroblasts	4.42	35.52	7.41
Mean	2.74	27.11	5.91
Tumor enhancement factor	6.52	41.70	147.80

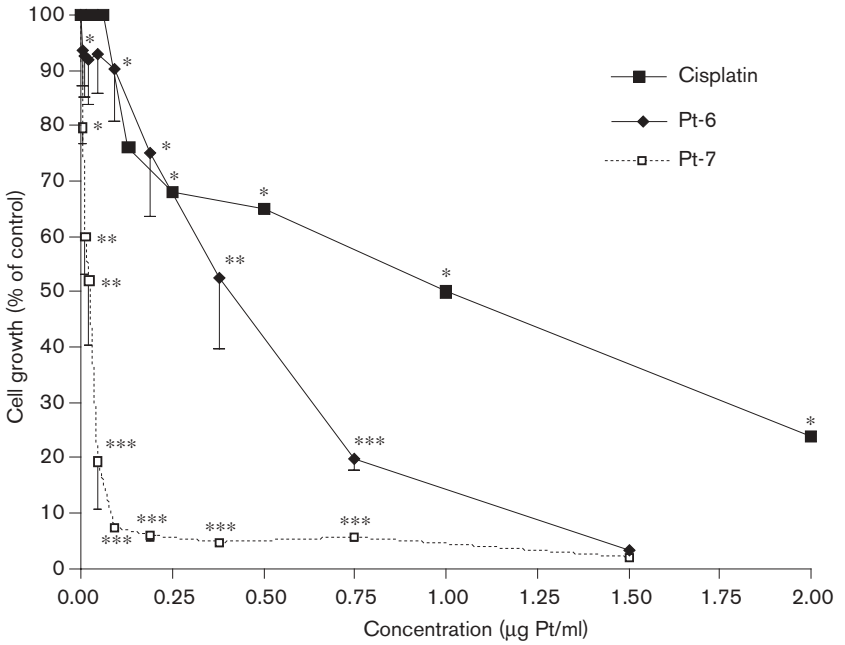
cytotoxicity in a variety of tumor cell lines, and in normal human lymphocytes and in fibroblasts. Table 2 shows that the IC_{50}s for cisplatin in HeLa cervical carcinoma, CoLo 320DM colon carcinoma cells and DU145 prostate carcinoma cells are rather high, and in the region of 0.1–1.0 μg Pt/ml, whereas Pt-6 and Pt-7 polymer are 1–3 orders of magnitude more cytotoxic, showing IC_{50} values between 0.0002 and 0.40 μg Pt/ml. The IC_{50} values of cisplatin shown here are in the same range as those found for melanoma and squamous carcinoma lines using clonogenic assays [9]. The Pt polymer conjugates enter tumor cells more effectively than normal cells (Fig. 4). Consistent with this we find the IC_{50} for tumor cells to be 2–40 times lower than for normal cells (Table 2). The high specificity of Pt drugs to kill tumor cells is also seen with the Pt-6 and Pt-7 Pt-containing polymers which show very low IC_{50}s of 0.04–0.40 μg Pt/ml for Pt-6 and 0.02–0.002 μg Pt/ml for Pt-7. Tumor specificity factors (TSF) calculated from these data show that the cytotoxicity of cisplatin for tumor cells is rather low and in the region of 6, whereas Pt-6 and Pt-7 exhibit a much greater tumor cell toxicity indicated by TSFs in the region of 40 and 150, respectively (Table 2).

Discussion

The comparison between cisplatin and the Pt-containing polymers was undertaken to confirm the finding that large water-soluble polymers may be less toxic, and show a superior tissue and plasma distribution [10,11]. The rationale for this assumption is that water-soluble polymer conjugates may be more stable than corresponding monomeric Pt complexes and do not undergo ligand-exchange reactions with water as rapidly as cisplatin; hence, they will accumulate faster in tissue without undergoing early biodegradation. In addition, utilizing the enhanced permeability and retention (EPR) effect [11], they will be preferentially retained in tumorous tissue. Replacement of the reactive chloro ligand in cisplatin by a dicarboxylate group and protection by chelate ring closure (Fig. 1) is reminiscent of carboplatin and oxaliplatin [12]. In this scenario, Pt delivery to the cancer cell may be enhanced and cell inactivation improved. It could also be argued that such exchange reactions would diminish the cisplatin concentrations in plasma, and affect clearance and bioavailability in the target cells. The absence of exchangeable Cl ligands in the Pt polymer conjugate thus may contribute to better bioavailability and greater cytotoxicity. The observation that the serum concentration of Pt offered as Pt polymer conjugate is surprisingly low (Fig. 2) could be the result of trapping of the Pt polymer conjugate in the clot. It is therefore possible that the serum levels reported here may not be representative of the clearance of the Pt polymer conjugate. On the other hand, we find that the Pt-7 polymer conjugate is taken up much more effectively by HeLa cells as compared to cisplatin (Fig. 3). Addition of equal Pt concentrations to the growth medium as Pt polymer conjugate showed a 2-fold higher Pt concentration in the cell pellet and a 3-fold lower concentration of Pt in the growth medium as compared to cisplatin (Fig. 3), while cisplatin generated almost equal concentrations of Pt in the medium and in the cell pellet (Fig. 3). The results of the toxicity study, imperfect as they are for reasons of limited dose points, would suggest that the Pt polymer conjugate is indeed more toxic than cisplatin to tumor cells and this may be due to better bioavailability.

Lower clearance and better bioavailability have recently been demonstrated for a methacrylate Pt polymer of similar structure where a bidentate ligand replaces the Cl groups in cisplatin [13]. The high Pt concentration in liver and kidney generated by our Pt polymer conjugate (Table 1) thus may be the result of greater stability in plasma and delayed processing. We have no data on the biodegradation of the Pt polymer conjugate, but expect it to be stable in neutral aqueous solutions and to degrade at a lower pH as is the case in the lysosomal compartments. Furthermore, it has been shown that the polymeric ligand alone in the absence of Pt is also not noticeably toxic (not shown). A limitation of the present study is that there are no measurements as yet for tumor control in transplanted mouse tumors, but such experiments are highly desirable and are planned. The results presented here are encouraging in the sense that they

Fig. 4



Cytotoxicity of cisplatin (solid squares), Pt-6 (diamonds) and Pt-7 (open squares) in CoLo 320DM colon carcinoma cells measured by the MTT assay.

demonstrate interesting pharmacokinetic properties of a new Pt-containing polymer conjugate which appears to have a higher residence time in peripheral tissues and better cytotoxic properties, hence suggesting a clinical potential. The principal advantages of polymeric drugs have been reviewed, *inter alia*, by Maeda *et al.* [11] and Putnam and Kopeček [14]. Very recent data on a methacrylate Pt polymer [13] are fully consistent with these concepts. The critical factors such as ligand inertness, molecular size, solubility requirements, peptide copolymer design and linker stability have been extensively researched [15,16]. The rationale for the asparagine polymer ligands employed here has been discussed elsewhere [17–19].

The satisfactory performance of MTT assay is evident from the fact that the IC_{50} for cisplatin, 0.14 μg Pt/ml for DU145 cells (Table 2), is in close agreement with IC_{50} measurements by colony assay in the same cell line, which produced an IC_{50} of 0.20 μg Pt/ml [20].

Results of our *in vitro* toxicity study in HeLa, CoLo 320DM and DU145 cells demonstrate that Pt given as Pt polymer conjugates is up to 150 times more effective to kill tumor cells than normal cells, whereas cisplatin only displays a preferential toxicity which is 6 times higher in tumor cells than in normal cells (Table 2). These results are fully consistent with a better conjugate bioavailability, and the *in vitro* Pt uptake measurements in HeLa and other tumor cells (Fig. 4). In this interpretation we reiterate that the very low IC_{50} of 0.0002 for Pt-7 in HeLa cells (Table 2) may have to be interpreted with caution. The overall trend suggests that the Pt polymer conjugates are more cytotoxic and more tumor specific (in accordance with the EPR effect [11]) than cisplatin (Table 2). Some aspects of the responses of normal cells to cisplatin have been unraveled in studies on PMN leucocytes, where it has been shown that free Pt^{2+} enhances the reactivity of neutrophil-derived oxidants [21], while cisplatin given at pH 8.3–8.8 when the complex is stable does not influence the generation of active oxygen species [22]. The greater cytotoxicities of the Pt polymers, in summary, thus may rest in their greater stability in the serum, delayed ligand exchange, (very importantly) facilitated (pinocytic) cell entry and increased accumulation in the tumor tissue as a consequence of the EPR effect that is operative with macromolecular compounds.

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