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Original Paper

HPMA Copolymer Platinates as Novel Antitumour Agents: *In Vitro* Properties, Pharmacokinetics and Antitumour Activity *In Vivo*

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The aim of this study was to compare *in vitro* and *in vivo* HPMA copolymer platinates with cisplatin in terms of platinum release, toxicity and antitumour activity. N-(2-hydroxypropyl)methacrylamide (HPMA) conjugates containing peptidyl side-chains (Gly–Gly or Gly–Phe–Leu–Gly) terminating in either carboxylate or amino species were prepared. The carboxylate polymeric intermediate was reacted with cisplatin, and the polymeric diamine with potassium tetrachloroplatinate to produce HPMA copolymer platinates of Mw 25 000–31 000 Daltons with a platinum loading of 3–7 wt%. The diglycyl spacer was selected because it is non-biodegradable, whereas the tetrapeptide spacer is known to be cleaved by the lysosomal thiol-dependent proteases. *In vitro* the HPMA copolymer platinates displayed a range of platinum release rates at pH 7.4 and 5.5; from < 5%/24 h in the case of the diamino species which require enzymatic activation, to > 80%/24 h in the case of the carboxylate. Cisplatin and the fast releasing carboxylate species displayed IC_{50} values of 10 μg/ml Pt-equivalent against B16F10 cells *in vitro*, whereas the slow releasing conjugates were not cytotoxic over the dose range studied. Antitumour activity of HPMA copolymer platinates was first evaluated against L1210 and B16F10 tumours inoculated intraperitoneally (i.p.). When conjugates were administered i.p., the antitumour activity observed against L1210 tumours was within the range seen for free cisplatin (ratio of mean survival of treated animals to mean survival of controls, T/C, 1.20–1.70). Neither cisplatin nor HPMA copolymer platinates were active against intraperitoneal (i.p.) B16F10 tumours when administered i.p. However, when conjugates were administered intravenously (i.v.) to treat subcutaneous (s.c.) B16F10 tumours grown to palpable size, free cisplatin was still not active but the HPMA copolymer platinates bearing carboxylate and diamine platinates showed significant antitumour activity (T/C > 1.35). Throughout these studies, the polymer platinates were 5–15-fold less toxic than cisplatin *in vivo*. After i.v. administration, the blood clearance of HPMA copolymer platinate was considerably slower ($t_{1/2\alpha}$∼10 h) than seen for free cisplatin ($t_{1/2\alpha}$ < 5 min). HPMA copolymer platinates (15 mg/kg Pt-equivalent) gave rise to an approximately 60-fold increase in Pt AUC in B16F10 tumour tissue than was achieved after administration of cisplatin at its maximum tolerated dose (MTD) (1 mg/kg). © **1999 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

SINCE ITS discovery *cis*-diamminedichloroplatinum(II) (cisplatin) has had a major impact on cancer chemotherapy [1]. It is widely used in the treatment of solid tumours, including ovarian, testicular, and head and neck [2, 3], and is especially effective in combined chemotherapy against squamous cell carcinoma and small cell lung carcinoma [4]. Antitumour activity results from the ability of the diaqua species to cross-link the N-7 guanine residue of DNA producing intrastrand

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cross-links. The drug is therefore most active against proliferating cells entering G1 phase of the cell cycle [5]. Although numerous platinum analogues have undergone preclinical and clinical trials [reviewed in Refs 6–9] only cisplatin and carboplatin have been approved for routine clinical use. Many new analogues show little significant improvement in therapeutic index when compared with cisplatin. To display antitumour activity, platinum complexes must include two *cis* amine or ammine (in the case of cisplatin) functionalities that possess at least one hydrogen atom that will hydrogen-bond to the oxygen atoms of the DNA phosphate groups [10] and two strongly bound leaving groups, e.g. chloride. Cisplatin and its analogues have well recognised drawbacks. Many are inactive when administered orally, cisplatin has low solubility in water (2.4 mg/ml) [11], all induce severe toxic side-effects (importance differs for each analogue) including renal dysfunction [12] (this can be overcome clinically by administration of diuretics and prehydration), nausea and vomiting, myelosuppression and, not least, neurotoxicity. Furthermore, some tumours (colorectal and non-small cell lung cancers) have shown resistance to treatment by platinates [13–15]. In the longer term, secondary tumours may be induced.

Preparation of polymeric platinates is attractive as a means of increasing solubility, reducing systemic toxicity and localising more drug in the tumour via the enhanced permeability and retention effect (EPR effect) [16]. The concept of polymeric anticancer agents [reviewed in Refs. 17, 18] has been established clinically in the form of the N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates containing doxorubicin (PK1) [19]. This conjugate displays tumour selective deposition in animal tumours [20, 21], and at the cellular level is internalised via the endocytic route allowing lysosomal delivery of the active drug. The conjugate is inactive, hence non-toxic, and attachment of doxorubicin to the HPMA copolymer backbone via a peptidyl (Gly–Phe–Leu–Gly) spacer mediates release intratumorally by the lysosomal thiol-dependent proteases [22]; these enzymes are known to be elevated in human tumours [23]. PK1 antitumour activity is significantly higher than that seen for doxorubicin in many animal tumour models, particularly in solid tumours where the EPR effect is operative. The decreased doxorubicin myelo- and cardiotoxicity seen in animals [24, 25] has been confirmed clinically in phase I trials, where the maximum tolerated dose of PK1 was 320 mg/m^2 (in respect of doxorubicin) [19]. Anticancer activity was also seen and phase II trials are ongoing.

Design of effective polymer–platinates is more challenging. It is essential to bind the platinum irreversibly during transport in the circulation, but release a biologically active platinate intratumorally. Although several platinum–polymer systems have been reported including carboxymethyl–dextran [26], poly(aminoacids) [27, 28] divinylethermaleic anhydride (DIVEMA) [29], dextran [30], alginate [31] and a micelle-forming block copolymer [32], none have so far entered clinical investigation and few have displayed significant benefit *in vivo*. Failure has been due to toxicity of the proposed carrier, lack of antitumour activity (probably an inactive platinum conjugate is formed) or selection of an inappropriate polymer molecular weight.

Using the same rationale we employed to design HPMA copolymer–doxorubicin [19], HPMA copolymers containing pendant groups capable of coordinating platinum were prepared. To optimise the rate of platinum release, the rate of liberation was monitored *in vitro* under physiological conditions mimicking those found in plasma and intracellularly. As a prelude to studies in animals, *in vitro* cytotoxicity of conjugates was established *in vitro*, bearing in mind that polymer conjugates usually display low IC_{50} values due to their slow uptake by cells via the pinocytic route [17]. HPMA copolymer conjugates were tested for antitumour activity in mice bearing B16 melanoma or L1210 cells injected intraperitoneally (i.p.) or subcutaneously (s.c.). Further, the EPR effect was analysed in a preliminary study to determine the body distribution of the conjugates in mice bearing B16F10 melanoma.

MATERIALS AND METHODS

Materials

Cisplatin, dextran (Mw = 74 000), poly-L-lysine (Mw = 37 000), 5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), dimethylsulphoxide, Triton X-100 and *o*-phenylenediamine (*o*-PDA) were supplied by Sigma (Dorset, U.K.). Citric acid, sodium hydrogen orthophosphate dodecahydrate, nitric acid, hydrochloric acid and hydrogen peroxide were supplied by Merck (Lutterworth, U.K.).

Cell lines

The cell lines used (B16F10 and L1210) were obtained from ECACC (European Collection of Cell Cultures), Centre for Applied Biology, Microbiology and Research, Salisbury, Wiltshire, U.K.

Synthesis of HPMA copolymer conjugates

HPMA copolymer precursors containing Gly–Gly–p-nitrophenol (ONp) (approximately 5 mol% peptidyl side-chains and Mw 25 000–27 000 Da) or Gly–Phe–Leu–Gly–ONp (approximately 5 mol% peptidyl side-chains and Mw 29 000–31 000 Da) were prepared as previously reported [24] and supplied by Polymer Laboratories, Church Stretton, U.K. HPMA amine polymer precursors (HPMA–Gly–Gly–en or Gly–Phe–Leu–Gly–en) and HPMA carboxylate polymer precursors (HPMA–Gly–Gly–OH and or Gly–Phe–Leu–Gly–OH) were prepared, characterised and subsequently conjugated to platinum as described elsewhere [33]. HPMA copolymer platinates (Figure 1) were analysed in respect of total Pt content by atomic absorption spectroscopy and free Pt content by gel permeation chromatography (GPC) and their weight average molecular weight (Mw) and number average molecular weight (Mn) estimated by GPC (Table 1).

Table 1. *Characteristics of HPMA copolymer platinates*

Conjugate	Percentage peptidyl side-chain (mol%)	Platinum content (wt%)	Mw (kDa)
HPMA–Gly–Gly–en	2.7	none	26–28
HPMA–Gly–Gly–en–Pt	2.8	3.5	26–28
HPMA–Gly–Phe–Leu–Gly–en	3.5	none	29–31
HPMA–Gly–Phe–Leu–Gly–en–Pt	3.0	3.6	29–31
HPMA–Gly–Gly–COOH	3.5	none	26–28
HPMA–Gly–Gly–COO–Pt	4.0	3.1	26–28
HPMA–Gly–Phe–Leu–Gly–COOH	3.6	none	29–31
HPMA–Gly–Phe–Leu–Gly–COO-Pt	3.6	7.7	29–31

Evaluation of the cytotoxicity of the parent polymer and HPMA copolymer platinates in vitro

B16F10 cells were cultured using standard conditions in microtitre plates. After (24 h) seeding cells at a density of 1×10^6 cells/ml, the HPMA copolymers and their platinum derivatives, dextran and poly-L-lysine or cisplatin (as reference controls), were added (0–1 mg/ml). Cells were then incubated for 72 h prior to addition of 20 µl of MTT to the culture medium to assess cell viability [34]. After a further 5 h, the medium was removed and 100 µl of dimethylsulph-

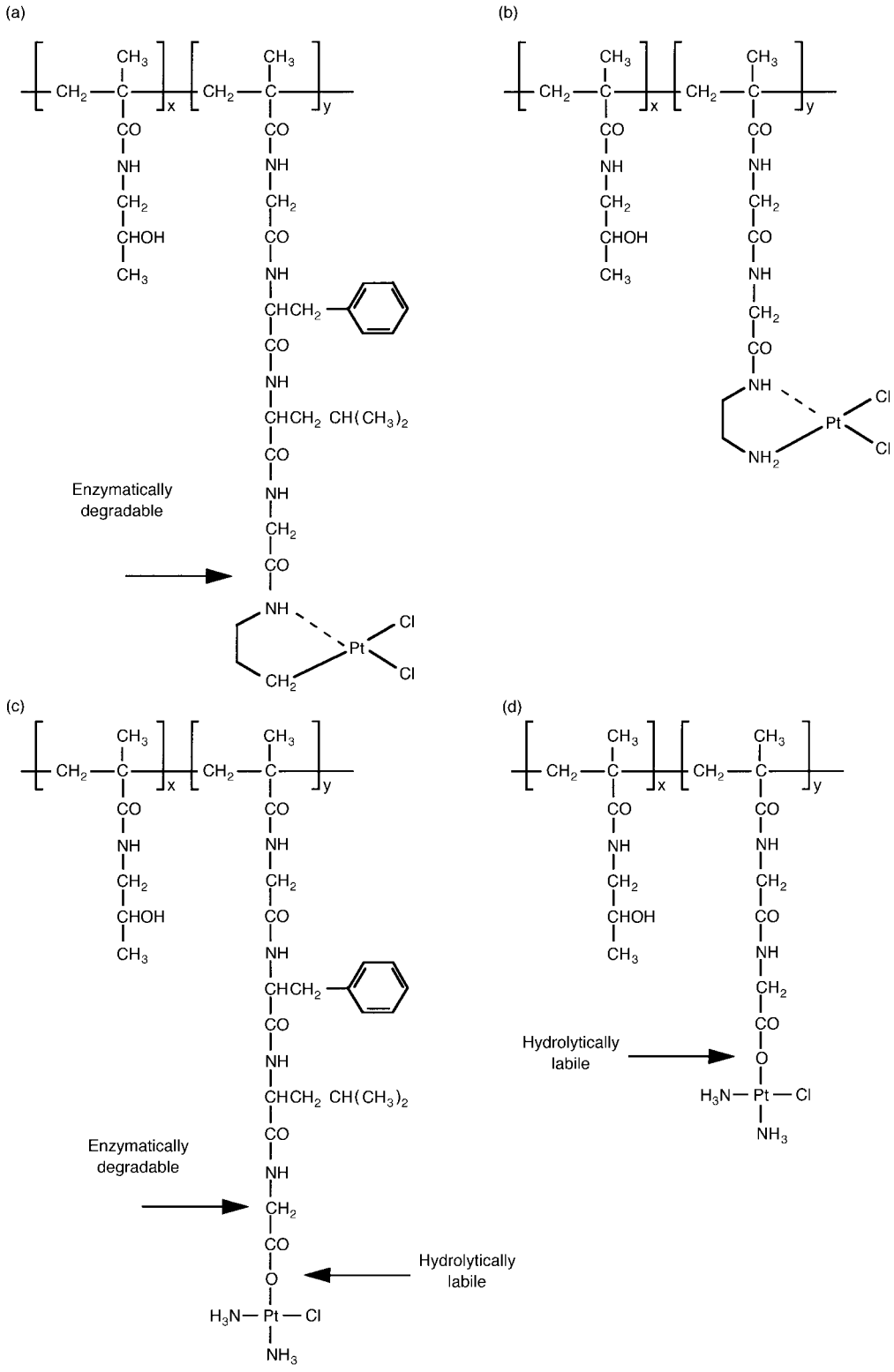

Figure 1. Structure of HPMA copolymer platinates. (a) HPMA–Gly–Phe–Leu–Gly–en–Pt; (b) HPMA–Gly–Gly–en–Pt; (c) HPMA–Gly–Phe–Leu–Gly–COO–Pt; (d) HPMA–Gly–Gly–COO–Pt. In all cases $x = 95$ mol% and $y = 5$ mol% and the conjugates have a weight average molecular weight (Mw) of 25 000–31 000 Da.

oxide (DMSO) added to dissolve the dark blue crystals. Absorbance at 550 nm was measured using a microtitre plate reader and the viability of the test cultures was expressed as a ratio of control incubation in the absence of any addition.

Release of Pt from HPMA copolymer platinates in vitro

HPMA copolymer platinate was dissolved in either phosphate buffered saline (PBS) (pH 7.4) or citrate–phosphate buffer (pH 5.5) and dialysed (seamless visking cellulose tubing with a pore size of 2.4 nm and molecular weight cut of approximately 10 000) against the respective solution at 37°C. Samples were taken regularly from the dialysate over 72 h and free Pt analysed using the *o*-phenylenediamine colorimetric assay (*o*-PDA) carried out according to a previously published method [35]: Samples with an unknown platinum content were added to 1 ml *o*-PDA solution in dimethylformamide (DMF) (1.2 mg/ml) and incubated for 10 min at 100°C. The amount of platinum present in the sample was determined by measuring the absorbance at 704 nm using cisplatin as a reference. The concentration of Pt released from the conjugate was expressed as a ratio of the total available.

Evaluation of antitumour activity of HPMA copolymer platinates in vivo

All animal experiments were conducted according to the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Guidelines.

L1210 i.p. tumour model. 10^6 viable cells were administered to DBA_2 mice (male 9–12 weeks, 20–30 g) i.p. on day 0. Animals were subsequently treated with multiple doses (day 1, 2, 3) of cisplatin and polymer–cisplatin. Animals were weighed daily and observed twice a day for signs of tumour progression and sacrificed if their body weight decreased below 80% of the starting weight or if other severe toxicological problems were seen.

B16 Melanoma i.p. and s.c. models. Male C57BL/6J mice (male 9–12 weeks, 20–30 g) were inoculated with either 10^5 viable B16F10 cells s.c. or 10^6 cells i.p. In the case of the s.c. tumour, the tumour was allowed to establish until the area was approximately 50–70 mm^2 as measured by the product of two orthogonal diameters. Animals bearing s.c. tumours were treated by either i.p. or i.v. injection of free cisplatin or polymer platinate and the tumour area monitored on subsequent days. In the case of the i.p. tumour model, cells were injected on day 0. Free cisplatin or polymer platinate were injected as single doses i.p. on day 1. Animals were monitored as described above.

Experimental data were expressed as the mean survival time, T/C defined as the ratio of the mean survival time of the treated animals (T) divided by the mean survival of the untreated control group (C). Deaths attributed to toxicity and animals with prolonged survival were also noted.

Evaluation of the body distribution of HPMA copolymer platinates and cisplatin in mice bearing B16 melanoma s.c.

Male C57BL/6J mice were inoculated with 10^5 viable B16F10 cells s.c. and the tumour was allowed to establish until the area was approximately 50–70 mm^2 as measured by the product of two orthogonal diameters. Animals were injected i.v. with free cisplatin (1 mg/kg) or HPMA–Gly–Gly–en–Pt (15 mg/kg) or HPMA–Gly–Phe–Leu–Gly–en–Pt (15 mg/kg) and animals sacrificed at times up to 72 h. The main organs were dissected and the blood collected. The tumour and blood samples were dissolved in nitric acid followed by hydrogen peroxide (30%) to a give colourless solution and subsequently made up to a known volume with water. Samples were analysed using an atomic absorption (flameless graphite furnace) spectrometer. Cisplatin was used as a standard with matrix matching.

Statistical methods

All the *in vitro* data are expressed as the mean ± standard deviation of the mean (S.D.). All the *in vivo* data are expressed as the mean ± standard error of the mean (S.E.) and statistical analysis of the mice survival time data and tumour and blood data were performed using the Student's *t*-test. *P* values of 0.05 or less were considered statistically significant.

RESULTS

The HPMA copolymer platinates used in this study (Figure 1) had a molecular weight of 25 000–31 000 Da and platinum content of 3–7 wt% (Table 1). Platinum loading was dependent on the linker used to bind platinum. The HPMA copolymer carrier was not cytotoxic for B16F10 cells (Figure 2a) and the polymeric platinates (Figure 2b) were also not cytotoxic over the concentration range used, with the exception of the HPMA copolymer–Gly–Phe–Leu–Gly–COO–Pt, which was equi-active compared with cisplatin; IC_{50} = 10–20 μg/ml.

Platinum release from the conjugates was similar at pH 7.4 and pH 5.5 (Figure 3). HPMA copolymer–Gly–Phe–Leu–Gly–COO–Pt (closed triangles in Figure 4) released platinum very quickly, with > 80% release in 1 h. HPMA–Gly–Gly–COO–Pt (closed circles) also released platinum relatively fast; 20–30% in the first hour leading to 40% release at 72 h at pH 7.4. At pH 5.5 80% platinum was released at 48 h. This might be expected as the carboxylate is a relatively poor ligand for chelation of platinum species. The HPMA copolymer–peptidyl–en–Pt (open symbols) did not release platinum species over 72 h at either pH, and the free platinum remained < 10% total throughout. This observation was not surprising as the Gly–Phe–Leu–Gly–en–Pt spacer requires cleavage by the lysosomal thiol-dependent proteases to liberate a low molecular weight species [17] and the Gly–Gly–en–Pt side-chain was prepared as a control: this spacer should be non-biodegradable even in the presence of proteolytic enzymes.

Tumour models inoculated i.p. were first used to verify the antitumour activity of HPMA copolymer platinates with cisplatin as a reference control throughout. When cisplatin was administered i.p. daily for 3 days (1, 2, 3) to treat an i.p. L1210 tumour, the maximum tolerated dose (MTD) observed was 2 mg/kg (Table 2). At this dose the T/C was 1.57. HPMA–Gly–Phe–Leu–Gly–en–Pt showed similar antitumour activity (maximum T/C = 1.49) against L1210 i.p., but the conjugate was considerably less toxic (Figure 4). The MTD observed without toxic deaths was 15 mg/kg (Pt-equivalent) and at the next dose level of 30 mg/kg there were only 3/5 toxic deaths (Table 2). This study confirmed that the polymer platinate could be degraded *in vivo* to release biologically active platinum species, and that the HPMA–Gly–Phe–Leu–Gly–en–Pt displayed a > 7-fold reduction in platinum toxicity after repeated i.p. administration. The HPMA–Gly–Gly–en–Pt did not show significant antitumour activity in the i.p. L1210 model over a wide range of doses (6–57 mg/kg) (Table 2).

Cisplatin was poorly active against B16F10 *in vitro* (Figure 2b) and was poorly active after i.p. administration to treat B16F10 tumour implanted i.p. (Table 3). When given as a single dose, the MTD of cisplatin was 5 mg/kg (2/5 toxic deaths). HPMA–Gly–Phe–Leu–Gly–en–Pt was likewise poorly active when used to treat the i.p. B16F10 model. Cisplatin also did not show antitumour activity when given as a single i.v. dose to treat B16F10 tumours implanted s.c. (Table 4), but the HPMA copolymer platinates were active. The maximum T/C value was 1.35 for the HPMA–Gly–Phe–Leu–Gly–en–Pt and 1.37 for the HPMA–Gly–Phe–Leu–Gly–COO–Pt, respectively. The MTD of cisplatin in this model was 1 mg/kg. The polymer platinates were much less toxic than cisplatin (Table 4) and i.v. HPMA–Gly–Phe–Leu–Gly–en–Pt had a MTD of > 15 mg/kg Pt-equivalent. The faster releasing HPMA–Gly–Phe–Leu–Gly–COO–Pt conjugate had an MTD of 5–10 mg/kg Pt-equivalent. Throughout these *in vivo* it was evident that the polymer platinates were considerably less toxic than cisplatin (5–> 15-fold less toxic) (Tables 2–4).

Polymer platinum conjugation altered dramatically its body distribution. Blood clearance of cisplatin (1 mg/kg) had a $t_{1/2\alpha}$ of < 5 min whereas both the HPMA copolymer platinates (15 mg/kg Pt-equiv) had a $t_{1/2\alpha}$ of approximately 10 h (Figure 5). The maximum tumour platinum concentration observed after administration of cisplatin (1 mg/kg) was seen at 5 min. In contrast, the polymeric platinates continued to accumulate in tumour tissue over the first 24 h. HPMA–Gly–Phe–Leu–Gly–en–Pt and HPMA–Gly–Gly–en–Pt (15 mg/kg) produced significantly higher tumour platinum levels than seen using cisplatin at its MTD and the area under the curve

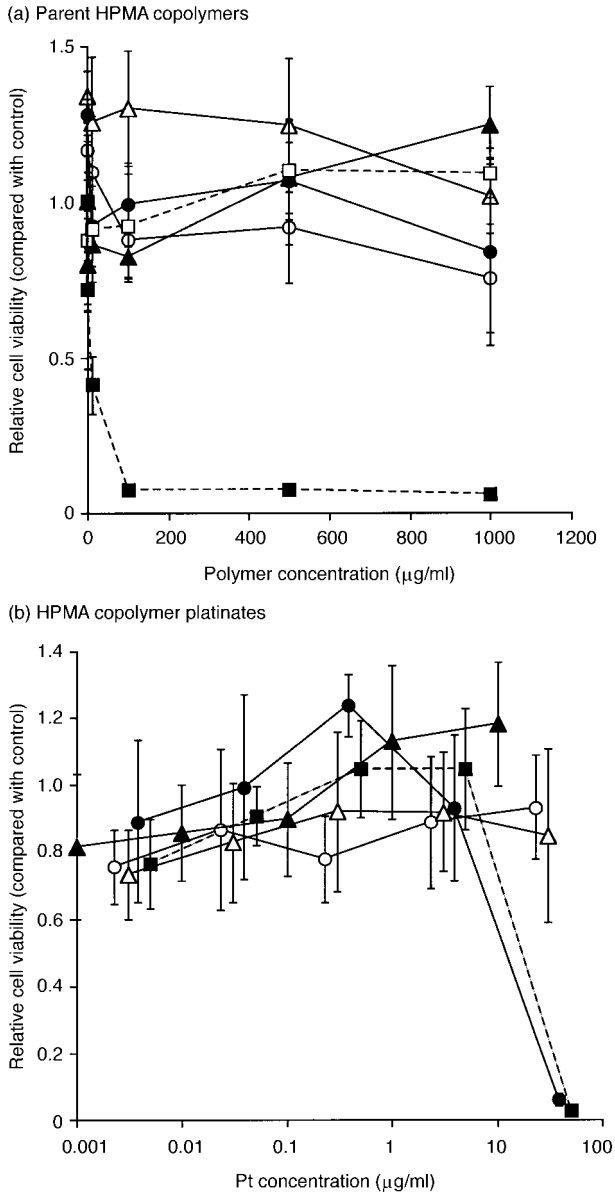

Figure 2. (a) Cytotoxicity of the parent HPMA copolymers towards B16F10 cell *in vitro*; HPMA–Gly–Phe–Leu–Gly–en (△—△); HPMA–Gly–Gly–en (○—○); HPMA–Gly–Phe–Leu–Gly–COO (▲—▲); HPMA–Gly–Gly–COO (●—●) and the reference controls dextran (□– – –□) and poly-L-lysine (■– – –■). (b) Cytotoxicity of HPMA copolymer platinates towards B16F10 cell *in vitro*. HPMA–Gly–Phe–Leu–Gly–en–Pt (△—△); HPMA–Gly–Gly–en–Pt (○—○); HPMA–Gly–Gly–COO–Pt (▲–▲); HPMA–Gly–Phe–Leu–Gly–COO–Pt (●—●) and cisplatin as a reference control (■– – –■).

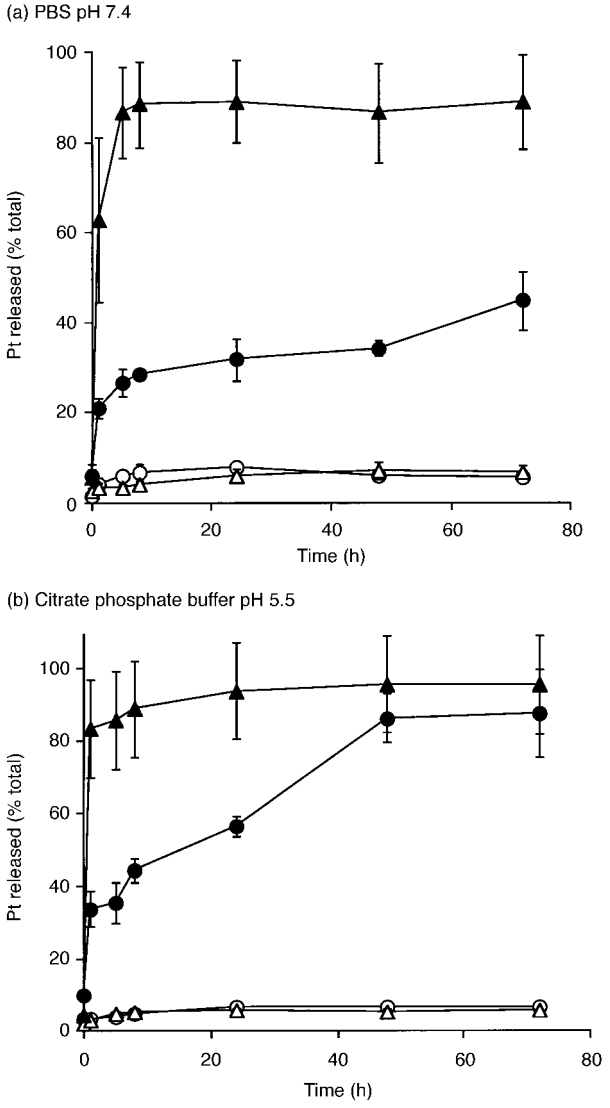

Figure 3. Release of Pt from HPMA copolymer platinates *in vitro*. (a) Release in phosphate buffered saline (pH 7.4). (b) Release in citrate phosphate buffer (pH 5.5). HPMA–Gly–Phe–Leu–Gly–en–Pt (△—△); HPMA–Gly–Gly–en–Pt (○—○); HPMA–Gly–Phe–Leu–Gly–COO–Pt (▲—▲); HPMA–Gly–Gly–COO–Pt (●–●).

(AUC) for platinum was increased 30 or 63-fold, respectively (Figure 5).

DISCUSSION

Due to the lack of significant advances in cancer chemotherapy over the last two decades, common solid tumours such as colon, lung, breast and prostate cancer are still very difficult to treat [36]. Polymer therapeutics afford the opportunity to inactivate the bound cytotoxic drug during transport to the tumour and thus reduce non-specific toxicity, restrict cellular uptake to the endocytic route and therefore improve tumour targeting by the EPR effect [20, 21]. HPMA copolymer–doxorubicin (PK1) has verified this concept in animals and man [37, 38]. In a phase I clinical trial, PK1 displayed the same pharmacokinetic profile as seen in mice when monitored by HPLC (plasma and urine) and gamma camera imaging. Polymer conjugation decreased anthracycline toxicity approximately 5-fold and antitumour activity was

Table 2. Antitumour activity of cisplatin and HPMA copolymer platinate administered i.p. against an i.p. L1210 model

Treatment	Dose (mg/kg)	Day of treatment	Survival (days mean ± S.D.)	T/C	No. of toxic deaths
Control	–	–	10.5 ± 0.7	1.00	0/15
Cisplatin	2	1, 2, 3	16.5 ± 3.6★	1.57	0/15
Cisplatin	3	1, 2, 3	6.4 ± 2.9★	0.64	9/10
HPMA–GPLG–en–Pt	3	1, 2, 3	15.0★	1.43	0/5
HPMA–GPLG–en–Pt	5	1, 2, 3	15.6 ± 2.9★	1.49	0/5
HPMA–GPLG–en–Pt	10	1, 2, 3	14.4 ± 0.8★	1.37	0/5
HPMA–GPLG–en–Pt	15	1, 2, 3	14 ± 1.3★	1.33	0/10
HPMA–GPLG–en–Pt	30	1, 2, 3	7 ± 2.4★	0.67	3/5
HPMA–GPLG–en–Pt	45	1, 2, 3	3★	0.29	5/5
HPMA–GG–en–Pt	6	1, 2, 3	11.4 ± 2.0^{ns}	1.09	0/5
HPMA–GG–en–Pt	19	1, 2, 3	3.8±0.4★	0.36	5/5
HPMA–GG–en–Pt	38	1†	2★	0.19	5/5
HPMA–GG–en–Pt	57	1†	2★	0.19	5/5

Level of significance ★$P \leq 0.01$; ns, non-significant; †Only one treatment given due to toxicity. GG, Gly–Gly; GPLG, Gly–Phe–Leu–Gly; Pt, platinum; T/C, ratio of mean survival of treated animals to that of controls.

Table 3. Antitumour activity of cisplatin and HPMA copolymer platinate administered i.p. against an i.p. B16F10 model

Treatment	Dose (mg/kg)	Day of treatment	Survival (days mean ± S.D.)	T/C	No. of toxic deaths
Control	–	–	17	1.00	0/4
Cisplatin	5	1	15.6 ± 6.2^{ns}	0.92	2/5
HPMA–GPLG–en–Pt	5	1	16.8 ± 0.4	0.99	0/5
HPMA–GPLG–en–Pt	10	1	17.6 ± 0.8^{ns}	1.04	0/5
HPMA–GPLG–en–Pt	15	1	17.8 ± 0.4★	1.05	0/5
HPMA–GPLG–en–Pt	20	1	17.0 ± 1.7^{ns}	1.00	0/5

★Level of significance $P \leq 0.01$. GG, Gly–Gly; GPLG, Gly–Phe–Leu–Gly; Pt, platinum; ns, non-significant; T/C, ratio of mean survival of treated animals to that of controls.

Table 4. Antitumour activity of cisplatin and HPMA copolymer platinate administered i.v. against a s.c. B16F10 model

Polymer–cisplatin	Dose (mg/kg)	Days survived after treatment (mean ± S.D.)	T/C	No. of toxic deaths
Experiment 1				
Control	–	5.1 ± 1.5	1.00	0/20
Cisplatin	1	5.7 ± 2.3^{ns}	1.12	2/19
HPMA–GPLG–en–Pt	5	6.0 ± 1.9^{ns}	1.18	0/10
HPMA–GPLG–en–Pt	10	6.9 ± 2.3★	1.35	0/15
HPMA–GPLG–en–Pt	15	6.3 ± 1.7★	1.24	0/10
Experiment 2				
Control	–	5.4 ± 1.0^{ns}	1.00	0/5
Cisplatin	0.5	5.4 ± 0.6^{ns}	1.00	0/5
Cisplatin	1	7.2 ± 0.8^{ns}	1.20	0/5
HPMA–GPLG–COO–Pt	5	7.2 ± 0.7	1.20	0/5
HPMA–GPLG–COO–Pt	10	7.4 ± 5.3^{ns}	1.37	2/5
HPMA–GPLG–COO–Pt	15	4.0 ± 1.9^{ns}	0.74	2/4

★Significant difference $P < 0.05$; GG, Gly–Gly; GPLG, Gly–Phe–Leu–Gly; Pt, platinum; ns, non-significant; T/C, ratio of mean survival of treated animals to that of controls.

observed in several chemotherapy refractory patients [38]. PK1 is currently undergoing phase II evaluation in colon, lung and breast cancer. HPMA copolymer–doxorubicin–galactose (PK2; designed to exhibit liver targeting) [39] and HPMA copolymer–paclitaxel [40] have also entered phase I studies.

It is therefore timely to consider HPMA copolymers as a platform for delivery of other antitumour agents and here we describe polymer platinates. HPMA copolymers were synthesised to contain amine or carboxylate substitutents and the resultant carriers were not toxic *in vitro* (Figure 2). Unlike natural polymer platinates based on carboxymethyl dextran, poly-L-glutamic acid and alginates [41] which were found to be poorly water soluble when platinated, HPMA copolymer platinates were highly water soluble, indeed more than 10 fold more soluble than cisplatin [33]. All the polymer platinates prepared using natural polymers as a carrier became almost totally insoluble on storage for 2–4 weeks. In contrast, the HPMA copolymer platinates retained good water solubility for many months (>12 months), indicating their improved formulation properties.

The library of monodenate HPMA copolymer constructs described allows comparison of linkages that rely solely on enzymatic cleavage to release a biologically active species (HPMA copolymer–en–Pt), display hydrolytic release (HPMA copolymer–Gly–Gly–COO–Pt) or potentially that combine both mechanisms of platinum release (HPMA copolymer–Gly–Phe–Leu–Gly–COO–Pt). The HPMA copolymer–en–platinates were stable in simple buffers chosen to mimic plasma and the lysosomal compartment (Figure 3), confirming the requirement for enzymatic activation. Indeed, the poor activity of the non-biodegradable HPMA copolymer–Gly–Gly–en–Pt conjugate against L1210 *in vivo* (Table 2) confirmed that, like HPMA copolymer–Gly–Gly–doxorubicin [42], this conjugate cannot liberate an active antitumour drug. HPMA copolymer–Gly–Gly–COO–Pt released platinum more slowly than the corresponding HPMA copolymer–Gly–Phe–Leu–Gly–COO–Pt (Figure 3) and this is indicative of platinum shielding within the interior of the polymer coil, as has been reported previously in the case of HPMA copolymer–melphalan conjugates [43].

In vitro IC_{50} values are frequently used to predict the therapeutic potential of novel antitumour agents. However, due to the difference in cellular pharmacokinetics of low molecular weight drugs (they usually penetrate cells readily) and their related macromolecular conjugates (which are

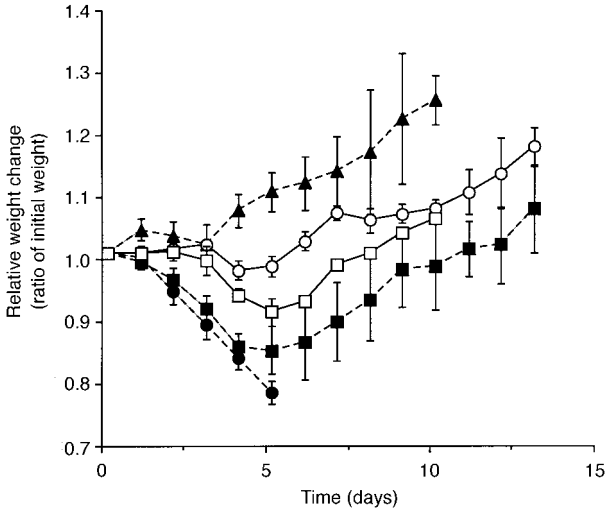

Figure 4. Effect of HPMA copolymer platinates and cisplatin on animal weight after repeated i.p. administration (days 1, 2, 3). Weight change is expressed as a ratio of the initial weight after administration of phosphate buffered saline (▲– – –▲); cisplatin 2 mg/kg (■– – –■); cisplatin 3 mg/kg (●– – –●); HPMA–Gly–Phe–Leu–Gly–en–Pt 15 mg/kg Pt-equivalent (○–○) and HPMA–Gly–Phe–Leu–Gly–en–Pt 30 mg/kg Pt-equivalent (□–□).

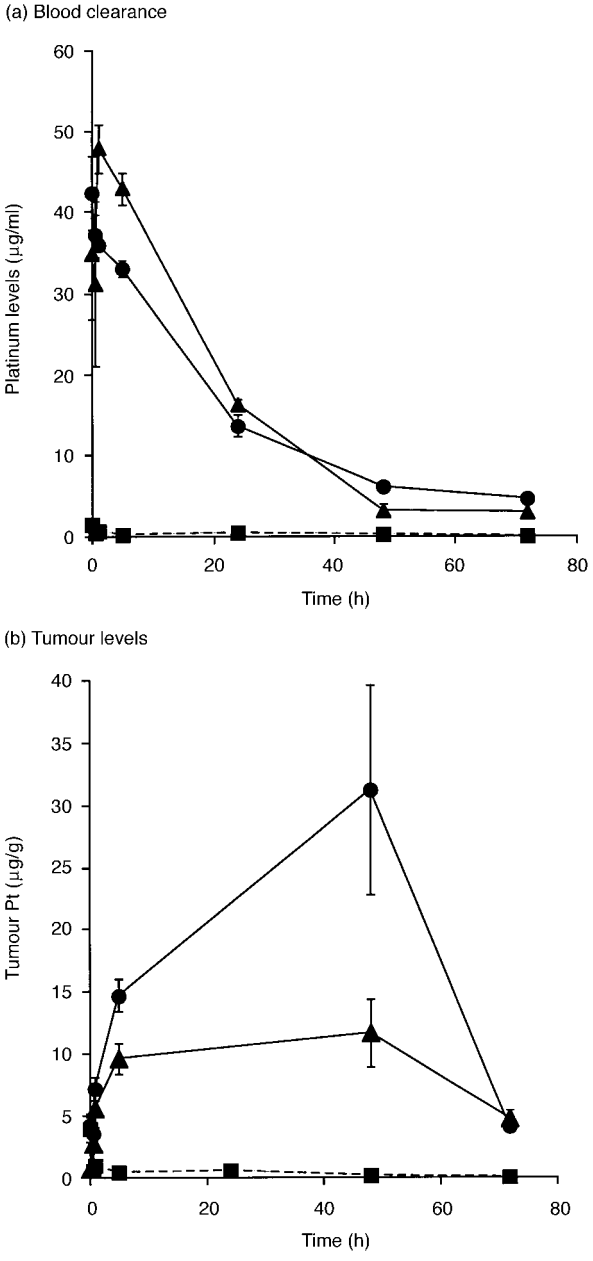

Figure 5. Blood clearance and tumour uptake of cisplatin and HPMA copolymer–en–platinates. (a) Blood clearance of cisplatin (1 mg/kg) (■– – –■); and HPMA–Gly–Phe–Leu–Gly–en–Pt (15 mg/kg Pt-equivalent) (●–●) and HPMA–Gly–Gly–en–Pt (15 mg/kg Pt-equivalent) (▲–▲); after i.v. administration to mice bearing a palpable B16F10 s.c. tumour. The level of statistical significance was $P \leq 0.01$ for all HPMA copolymer platinum values versus cisplatin values. (b) Tumour platinum levels detected against time after cisplatin or conjugate administration. The level of statistical significance was $P \leq 0.05$ for all HPMA copolymer platinum values versus cisplatin values.

internalised very slowly by endocytosis) the predictive value of IC_{50} values for polymer conjugates has little value. In this study the *in vitro* cytotoxicity experiments only verified that the fast releasing HPMA copolymer–Gly–Phe–Leu–Gly–COO–Pt liberated a biologically active platinum species (Figure 2). As could be anticipated from earlier studies with PK1, the slower releasing conjugates were all inactive *in vitro*.

Observation that HPMA copolymer–Gly–Phe–Leu–Gly–en–Pt was equi-active compared with cisplatin in the i.p. L1210 model confirms its ability to liberate an active platinate *in vivo* (Table 2). However, like PK1, the HPMA copolymer platinates displayed the most interesting antitumour activity when administered i.v. to treat the solid B16F10 tumour model (Table 4). Subsequent biodistribution studies revealed that the polymer platinates display substantial tumour targeting by the EPR effect (Figure 5). At their maximum tolerated doses, HPMA copolymer–Gly–Phe–Leu–Gly–en–Pt increased the platinum area under the curve approximately 60-fold in comparison with cisplatin. Further studies are currently ongoing to determine the dose dependency of pharmacokinetics of HPMA copolymer platinates.

Human cancers often display inherent or acquired resistance to the platinates at, or soon after, the onset of therapy and many mechanisms of resistance have been described including increased ability to repair the DNA, high levels of glutathione and glutathione-*S*-transferase, and increased metallothioneins [44, 45]. Typically, tumour cells retrieved from patients have a 3–5-fold decrease in sensitivity towards platinum anticancer agents. Therefore, if polymer conjugation has the ability to increase the concentration of biologically active platinates in human tumours more than 10-fold this could provide an opportunity to overcome platinum resistance in all its forms. In addition, commonly patients responding during a course of cisplatin or carboplatin treatment cannot be retreated on schedule due to insufficient recovery from continued myelotoxicity. Thus planned retreatment has to be postponed. As the polymeric platinates described here were consistently less toxic (5–15-fold) than cisplatin and they showed improved antitumour activity in the B16F10 tumour model, further development is warranted leading to clinical evaluation. Bidentate, malonate- and aspartate-containing HPMA copolymer platinates have also recently been synthesised and are currently under investigation [46].

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