European Journal of Pharmaceutics and Biopharmaceutics 70 (2008) 770–776

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

In vitro cytotoxicity, *in vivo* biodistribution and antitumor activity of HPMA copolymer–5-fluorouracil conjugates

Fang Yuan, Xuan Qin, Dan Zhou, Qing-Yu Xiang, Min-Ting Wang, Zhi-Rong Zhang, Yuan Huang *

Key Laboratory of Drug Targeting and Drug Delivery, Ministry of Education, West China School of Pharmacy, Sichuan University, Chengdu, PR China

ARTICLE INFO

Article history:
Received 13 April 2008
Accepted in revised form 23 June 2008
Available online 8 July 2008

Keywords:
N-(2-hydroxypropyl) methacrylamide copolymer
5-Fluorouracil
Antitumor drug
Cytotoxicity
Hepatoma 22

ABSTRACT

5-Fluorouracil (5-FU) is an antimetabolite with a broad-spectrum activity against solid tumors. However, its very short half-life in plasma circulation greatly limited the *in vivo* antitumor efficacy and clinical application. The current work aimed to solve this problem as well as to increase 5-FU biodistribution to tumor by covalently conjugating 5-FU to a biocompatible, non-toxic and non-immunogenic drug carrier – *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer. The *in vitro* cytotoxicity, *in vivo* biodistribution and therapeutic efficacy of HPMA copolymer–5-FU conjugates (P-FU) were reported. Cytotoxicity was evaluated by using a serial of tumor cells (A549, CT-26, Hela, $HepG_2$ cells and 5-FU resistant $HepG_2$ cells). *In vivo* biodistribution and therapeutic efficacy were investigated in Kunming mice-bearing hepatoma 22 (H_{22}). Results indicated that P-FU could increase the cytotoxicity of 5-FU in Hela, $HepG_2$ and 5-FU resistant $HepG_2$ cells, while it decreases the cytotoxicity of 5-FU in A549 and CT-26. Both in vitro release profile in plasma and biodistribution study showed that P-FU significantly prolonged the drug plasma circulation time. P-FU also showed an over 3-fold larger area under the concentration–time curve (AUC) in tumor when compared with free drug. Therapeutic evaluation also demonstrated that the treatment with P-FU displayed stronger inhibition of the tumor growth when compared with that of control group (physiologic saline) or 5-FU group at the same dose. All the results suggested that P-FU could increase cytotoxicity of 5-FU in certain cancer cell lines, prolong 5-FU circulation time *in vivo*, enhance 5-FU distribution to tumor and improve therapeutic efficacy. Therefore, HPMA copolymer is a potential carrier for 5-FU for the effective treatment of cancer.

1. Introduction

5-Fluorouracil (5-FU), a commonly used antitumor agent, acts mainly through the inhibition of synthesis of DNA and RNA during the S-phase of the cell cycle [1]. Nevertheless, more than 85% of administered 5-FU is quickly catabolized to inactive metabolites [2,3], which leads to a short circulation time *in vivo* ($t_{1/2}$ is about 10–20 min), also low drug accumulation in tumor. Considering the limited number of cells in the S-phase of the cell cycle during the short circulation period of 5-FU, the quick metabolism may also affect the therapeutic response of the drug. Therefore, a novel tumor-targeted, long-circulation drug delivery system needs to be developed for the tumor-targeting of 5-FU.

Polymer–drug conjugates have been developed for more than three decades to promote tumor-targeting efficacy and prolong drug circulation time [4]. They can provide the following two merits to small molecular drugs. First, the large molecular weight of polymer–drug conjugates could lead to a decreased glomerular filtration rate in the kidney, which increases the retention of drug in the blood circulation. Second, the conjugates are able to preferentially accumulate in tumor tissue due to the enhanced permeability and retention (EPR) effect [5]. The EPR effect utilizes increased permeability of tumor vessels, high vascular density and suppressed lymphatic drainage in tumor tissues (which retain the conjugates in the tumor) to increase the accumulation of the conjugates in tumors.

Among those polymeric drug delivery systems, one class of copolymers based on *N*-(2-hydroxypropyl) methacrylamide (HPMA) was extensively examined as biocompatible, non-toxic and non-immunogenic drug carriers [6–9]. The conjugation of low-molecular weight drugs to HPMA copolymers exhibits more desirable biodistribution, elimination and metabolism properties than free drugs [10–12]. In addition, several HPMA copolymer–drug conjugates have progressed into clinical trials [13–16]. Therefore, HPMA copolymer could be a perfect carrier for 5-FU to prolong drug circulation time and increase drug distribution to tumor. In the previous study, HPMA copolymer–5-fluorouracil conjugates were synthesized based on the α-substituted glycine derivatives of 5-FU [17]. However, the content of 5-FU in the

* Corresponding author. Tel./fax: +86 28 85501617.
E-mail address: huangyuan0@yahoo.com.cn (Y. Huang).

0939-6411/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2008.06.028

conjugates was relatively low (only 1.38 wt%), and the cytotoxicity, biodistribution and antitumor activity of the conjugates have not been reported.

Recently, we developed a new, improved synthetic method for HPMA copolymer–5-FU conjugates (P-FU) for tumor-targeting delivery of 5-FU. 5-FU was successfully conjugated to HPMA copolymers [18]. The objective of this study was to further evaluate whether the enhancement of the circulation longevity and antitumor activity of 5-FU could be achieved by conjugating the drug to HPMA copolymers. Therefore, the *in vitro* release behavior of the conjugates in plasma was characterized. *In vitro* cytotoxicity was examined by using a number of tumor cell lines, including A549, CT-26, Hela, $HepG_2$ cells and 5-FU resistant $HepG_2$ cells ($HepG_2$/5-FU), and the biodistribution, pharmacokinetics and therapeutic efficacy of P-FU were investigated in hepatoma 22 (H_{22})-bearing mice for the first time. The results showed that P-FU significantly enhanced the circulation longevity and antitumor activity of 5-FU *in vivo*. We also found that the conjugates seem to enhance the *in vitro* antitumor activity of 5-FU to certain tumor cells that are not very sensitive to free 5-FU.

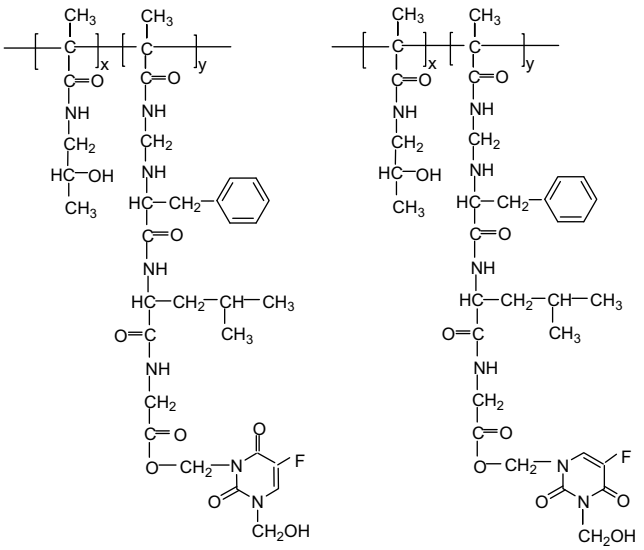

Fig. 1. Chemical structures of HPMA copolymer–5-FU conjugates.

2. Materials and methods

2.1. Materials

5-Fluorouracil was supplied by Nantong pharmaceutical Co. Ltd. (Jiangsu, China); *N*,*N*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) and 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma (USA); Cell culture medium RPMI-1640 and dimethyl sulphoxide (DMSO) were purchased from Gibco Co. (USA).

P-FU were synthesized as previously described [18]. Briefly, the conjugates were obtained by the ester condensation of HPMA carboxylate copolymer precursor P-Gly-Phe-Leu-Gly-OH (approximately 15 mol% peptidyl side-chains, M_w = 134 kDa, M_w/M_n = 1.5) (5.7 nmol) and 1,3-dimethylol-5-FU (12 mmol), in the presence of DCC (0.66 mmol) and a catalytic amount of DMAP at room temperature for 18 h. The 5-FU content in the conjugates was analyzed by high-performance liquid chromatography (HPLC) system equipped with a Dikma Diamonsil® C_{18} (250 × 4.6 mm, 5 μm) column (USA), an Alltech UVIS-201 Absorbance Detector and an Allchrom plus Client/Sever data operator (Multilink Services Co. Ltd., USA) at 266 nm. The mobile phase was distilled water. The conjugated 5-FU content in P-FU was 3.93 wt% and the free 5-FU content was less than 0.03 wt%. The chemical structures of the conjugates are shown in Fig. 1. All other reagents were of high-performance liquid chromatography (HPLC) grade.

2.2. In vitro stability assay of P-FU

Fifty milligrams of P-FU was dissolved in 10 mL of 30% (v/v) mice plasma in PBS (pH 7.4) and kept at 37 °C with mild stirring. Samples (100 μL) were taken at different time intervals. 400 μL of methanol was added to deproteinize each sample then centrifuged for 10 min at 14,000*g*. Twenty microliters of the clear supernatant was analyzed by HPLC to determine the amount of the released 5-FU, using HPLC conditions as described above.

2.3. In vitro cytotoxicity assay

2.3.1. Cells culture

Five tumor cell lines were used in this study, including A549, CT-26, Hela, $HepG_2$, and 5-FU resistant $HepG_2$ ($HepG_2$/5-FU) cells. A549, CT-26, Hela, and $HepG_2$ were purchased from the Institute of Biochemistry and Cell Biology. $HepG_2$/5-FU cells were obtained by continuously exposing $HepG_2$ cells to a media with 5-FU at 10 μg/mL for 2 weeks [19]. All cells were maintained in RPMI-1640 media supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C under a humidified atmosphere of 5% CO_2.

2.3.2. IC_{50} values evaluation

The 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) enzyme assay was employed to determine the number of surviving cells. Briefly, cells were plated into 96-well plate at a density of 10^4 cells per well. After 24 h, the growing cells were treated with 100 μL of 5-FU or P-FU (containing the same amount of 5-FU) at various concentrations (ranging from 0.048–619.0 μg/mL of 5-FU) for 24 h at 37 °C. Then, 20 μL MTT (5 mg/mL) was added to each well. Four hours later, the solution was removed and 150 μL of DMSO was added to each well to dissolve the formazane of MTT. The absorption at 570 nm was read using an ELISA plate reader (Bio-Rad, Microplate Reader 550). The growth inhibition rate (GI) was calculated as Eq. (1):

$$GI(\%) = 100 - [(T - B)/(C - B)] \times 100 \quad (1)$$

where T is the absorption value of the treatment group; C is the absorption value of the control (untreated) group; and B refers to the absorption value of the culture medium. IC_{50} values (μg/mL) were calculated by SPSS software.

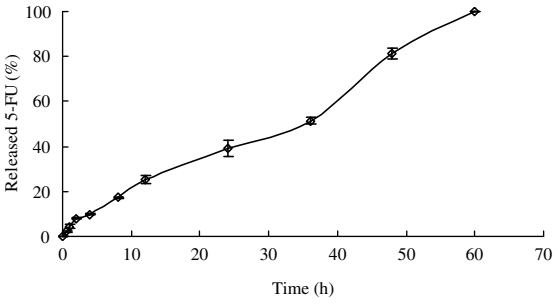

Fig. 2. Release profile of 5-FU from P-FU in plasma (concentration of 5-FU was measured). All data are means ± SD (n = 3).

Table 1
The IC_{50} values of P-FU and 5-FU in different cell lines

Sample	IC_{50}[a](μg/mL)					Ratio[c]
	A549	CT-26	$HepG_2$	Hela	$HepG_2$/5-FU	
5-FU	1.70 ± 0.86	4.30 ± 1.42	33.60 ± 0.95	80.48 ± 13.92	336.49 ± 41.96	10.0
P-FU	9.65 ± 3.64	7.19 ± 2.84	22.35 ± 7.08	17.21 ± 4.96	142.13 ± 14.20	6.4
Ratio[b]	0.18	0.60	1.50	4.68	2.37	/

All data are means ± SD ($n = 4$).
[a] IC_{50}, Concentration of 5-FU (or equivalent) (μg/mL) required to inhibit the cellular growth by 50% after 24 h of drug exposure, as determined by the MTT assay.
[b] Ratio, IC_{50} for 5-FU vs. IC_{50} for P-FU.
[c] Ratio, IC_{50} for $HepG_2$/5-FU vs. IC_{50} for $HepG_2$.

2.4. Animal model

The Sichuan University Animal Ethical Experimentation Committee, according to the requirements of the National Act on the Use of Experimental Animals (PR China), approved all procedures of the *in vivo* studies. Female Kunming mice, aged 6–8 weeks (20 ± 2 g), were purchased from Sichuan Industrial Institute of Antibiotics (PR China). H_{22} murine hepatoma cells were provided by Sichuan Industrial Institute of Antibiotics (PR China). Hepatoma model was established by subcutaneous injection of H_{22} tumor cells (1×10^6 cells/0.2 mL) into the right flank of each mouse [20,21]. Experiments were not initiated until a consistent growth rate and a minimum tumor volume of 700 mm^3 were achieved.

2.5. Biodistribution studies

Animals received a single i.v. injection of 5-FU or P-FU solution at a 5-FU equivalent dose of 24 mg/kg in 0.9% NaCl solution. The mice were sacrificed at 5, 15, 30 min, 1, 2, 4, 8, 12, and 18 h (for 5-FU) and at 5, 15, 30 min, 1, 2, 4, 8, 12, 24, 36, 48, and 72 h (for P-FU) after i.v. administration. Blood samples were collected from orbit venous plexus, and plasma samples were obtained by centrifugation at 5000*g* for 10 min. The tissues, including heart, lung, liver, spleen, kidney and tumor, were harvested and accurately weighed and homogenized. Plasma and tissue samples within the same group were pooled together for further analysis ($n = 5$).

The 5-FU concentrations in the plasma or tissues were determined by an HPLC assay. The optimal hydrolysis condition for P-FU was investigated at first. The samples containing P-FU were hydrolyzed by 3 M NaOH [22] or 6 M HCl [23] at different temperatures (from room temperature to 85 °C) for different time periods (10–20 min) until no change in 5-FU concentration to make sure that all conjugated 5-FU was released. Results showed that the conjugated 5-FU could be fully released by hydrolysis with 3 M NaOH at room temperature for 15 min. The experiments were performed as follows: Plasma or homogenized tissue (0.5 g) was mixed with 50 μL of 5-bromouracil solution (100 μg/mL) as the internal standard, followed by the addition of 40 μL of 3 M NaOH and hydrolyzed at room temperature for 15 min to fully release 5-FU (for free 5-FU groups, NaOH was still added). Then, appropriate amount of HCl and PBS (pH 3.0) was added to neutralize the solution. Extraction was performed after adding acetoacetate (6 mL) and vigorous vortex. The organic layers were collected after centrifugation at 3000*g* for 5 min and evaporated to dryness. Finally, the residue was redissolved in 250 μL mobile phase (distilled water), following centrifugation at 14,000*g* for 10 min. 20 μL of the clear supernatant was injected into the HPLC system. The same HPLC condition was used as described above.

2.6. In vivo antitumor activity evaluation

Mice, randomly divided into five groups ($n = 5$), were injected subcutaneously with H_{22} tumor cells (1×10^6 cells/0.2 mL) into the right flanks. Four days after inoculation, drugs (dissolved in saline) were continuously administrated via tail veins for 4 days: groups 1 and 2 were injected with P-FU (5-FU equivalent dose of 6 mg/kg or 18 mg/kg per day), groups 3 and 4 received 5-FU (6 mg/kg or 18 mg/kg per day), and group 5 (control) received saline. The first day mice received treatment was set as day 0. Tumor volumes were monitored everyday from day 0 to day 8. On day 8, the mice were sacrificed and the tumors were excised and weighted. The tumor volumes and the inhibition rate of tumor growth (IR) were calculated as follows:

$$\text{Volume} = L \times W^2/2 \quad (2)$$

where L is the longest dimension parallel to the skin surface and W is the dimension perpendicular to L and parallel to the surface [24].

$$IR(\%) = [(A - B)/A] \times 100 \quad (3)$$

where A is average tumor weight of the control group, and B is that of the treatment group.

2.7. Histopathology observations

The tumors were fixed with polyoxymethylene for 48 h and embedded in paraffin. Then each section was cut to 5 μm, processed for routine hematoxylin and eosin (H&E) staining, and photographed under an OLYMPUS microscope.

3. Results and discussion

3.1. In vitro stability assay of P-FU

The stability of P-FU in plasma, shown in Fig. 2, indicated that the conjugates were relatively stable with only 25% drug released in the initial 12 h and the release continued to 60 h. Data were fitted into four models (first-order model, zero-order model, Niebergull equation and Peppas equation), and the release profile could be best modeled to zero-order kinetics and the half-life (32.4 h) was obtained. Since 5-FU has a very short half-life (10–30 min) in plasma circulation and about 85% of the drugs administrated

Table 2
Percentage of TAD of free 5-FU in plasma and tissues

	Heart	Liver	Spleen	Lung	Kidney	Tumor	plasma	Total
5 min	0.051	0.099	0.022	0.100	0.011	0.150	4.94	5.373
15 min	0.026	0.082	0.006	0.029	0.057	0.179	2.11	2.489
30 min	0.004	0.066	0.005	0.005	0.022	0.096	0.71	0.908
1 h	0.002	0.061	0.003	0.005	0.022	0.039	0.063	0.195
2 h	0.001	0.034	0.003	0.002	0.019	0.055	0.019	0.134
4 h	Nd	0.065	0.002	0.001	0.010	0.035	0.018	0.132
8 h	Nd	0.054	0.002	Nd	0.009	0.044	Nd	0.109
12 h	Nd	0.030	0.001	Nd	0.004	0.046	Nd	0.081
18 h	Nd	0.015	0.001	Nd	Nd	0.008	Nd	0.024

Nd, concentration of 5-FU was not detectable.

Table 3
Percentage of TAD of P-FU in plasma and tissues (concentrations of 5-FU were measured after complete hydrolysis of the samples)

	Heart	Liver	Spleen	Lung	Kidney	Tumor	plasma	Total
5 min	0.119	1.47	0.191	1.26	0.619	0.031	95.75	99.44
15 min	0.101	1.57	0.206	0.373	0.541	0.136	94.46	97.39
30 min	0.112	2.35	0.130	0.284	0.522	0.336	88.03	91.76
1 h	0.056	1.12	0.129	0.244	0.401	0.641	83.81	86.40
2 h	0.041	0.561	0.016	0.107	0.264	0.240	38.30	39.52
4 h	0.042	0.738	0.021	0.086	0.142	0.115	22.16	23.30
8 h	0.020	0.0439	0.015	0.050	0.105	0.075	14.11	14.81
12 h	0.014	0.261	0.010	0.027	0.061	0.089	9.49	9.95
24 h	0.005	0.168	0.005	0.008	0.024	0.026	1.56	1.80
36 h	0.002	0.114	0.002	Nd	0.008	0.018	0.55	0.694
48 h	Nd	0.064	Nd	Nd	Nd	0.015	0.35	0.429
72 h	Nd	0.033	Nd	Nd	Nd	0.008	0.22	0.261

Nd, concentration of 5-FU was not detectable.

are degraded to inactive metabolites in most tissues [2,3], a prolonged half-life is vital for the success of 5-FU treatment. The half-life of P-FU in plasma was calculated to be 32.4 h. The result was very close to the half-life measured in *in vivo* study (26.657 h), which strongly supported the relevance of the *in vitro* and *in vivo* results, indicating that the circulation time can be significantly prolonged by attaching the drug to a macromolecular polymeric chain.

3.2. Cytotoxicity evaluation

The cytotoxicities of 5-FU and P-FU in four different cancer cell lines were examined (Table 1). The IC_{50} values for 5-FU increased in the following order: A549, CT-26, $HepG_2$, and Hela, which means A549 was most sensitive to 5-FU, whereas Hela was least sensitive. Here, we had a very interesting observation that A549 and CT-26 were more sensitive to 5-FU than to P-FU; but in the case of $HepG_2$, Hela, and $HepG_2$/5-FU, which were more resistant to 5-FU, the cells were more sensitive to the conjugate. These results suggested that P-FU might have an increased cytotoxicity toward tumor cells that are less sensitive to 5- FU.

It is known that different gene expression levels for a serial of rate-limiting enzymes in 5-FU catabolism and anabolism in different cells are responsible for their different sensitivities to 5-FU [1,25]. HPMA copolymer conjugates with peptide spacers remain intact before releasing free drugs by the hydrolysis of lysosomal enzymes in lysosome compartment [26], while the rate-limiting enzymes in 5-FU catabolism mainly exist in cytoplasm, therefore, these enzymes may have less impact on the activity of the conjugates. However, the endocytosis of HPMA conjugates was much slower than the diffusion of free drug through cellular membrane [27], which might explain the results we observed that P-FU was less effective than 5-FU for the treatment of tumor cells that are more sensitive to 5-FU (A549 and CT-26) but more effective for the treatment of tumor cells that are less sensitive to free drug ($HepG_2$ and Hela) (Table 1).

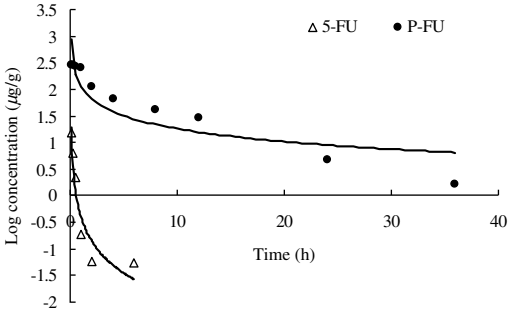

Fig. 3. Log concentration of 5-FU in plasma of H_{22}-bearing mice after i.v. administration of 5-FU or P-FU (concentrations of 5-FU were measured after complete hydrolysis of the samples).

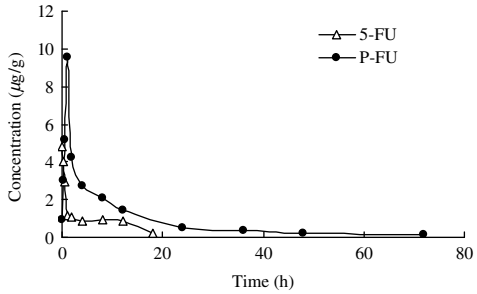

Fig. 4. Concentration of 5-FU in tumors of H_{22}-bearing mice after i.v. administration of 5-FU or P-FU (concentrations of 5-FU were measured after complete hydrolysis of the samples).

Table 4
Pharmacokinetic parameters of P-FU and free 5-FU in plasma and tumor

Tissue	$AUC_{0\to\infty}$ (mg/L * h)		$t_{1/2}$ (h)		CL (L/h/kg)	
	P-FU	5-FU	P-FU	5-FU	P-FU	5-FU
Plasma	1302.161	5.103	26.657	0.16	0.018	3.420
Tumor	59.881	18.296	17.239	7.438	0.615	1.488

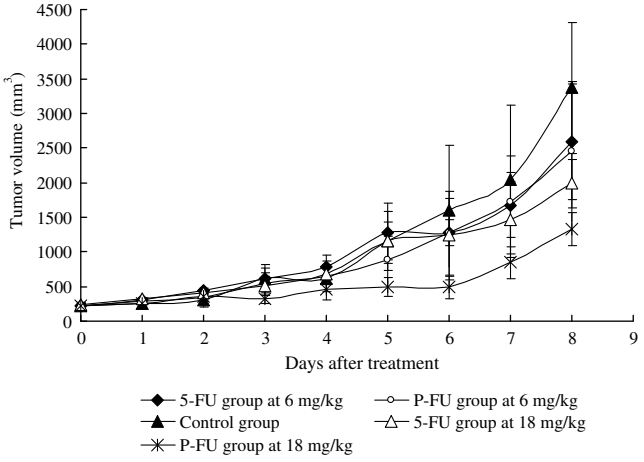

Fig. 5. Growth inhibition of hepatoma 22 in mice by i.v. administration of P-FU and 5-FU. All data are means ± SD (n = 5).

Table 5
Antitumor activity of P-FU and 5-FU in H_{22}-bearing mice

Group	Tumor weight (g) mean ± SD (n = 5)	IR (%)
5-FU group (at 6 mg/kg)	0.946 ± 0.364	6.89
P-FU group (at 6 mg/kg)	0.894 ± 0.438	12.00
5-FU group (at 18 mg/kg)	0.442 ± 0.082*	56.49
P-FU group (at 18 mg/kg)	0.314 ± 0.044*,**	69.09
Control group	1.016 ± 0.155	–

* Significant statistical differences from control group: $P < 0.005$.
** Significant statistical differences from 5-FU group (at 18 mg/kg): $P < 0.03$.

We further tested the cytotoxicities of 5-FU and P-FU in 5-FU resistant $HepG_2$ cells ($HepG_2$/5-FU). Results showed that although P-FU had a lower cytotoxicity on 5-FU resistant $HepG_2$ cells than on normal $HepG_2$ cells, it was still more effective than free 5-FU for the treatment of $HepG_2$/5-FU cells (Table 1).

3.3. Biodistribution of P-FU in H22-bearing mice

The biodistribution of free 5-FU and the conjugates was investigated in mice-bearing H_{22} tumor. The percentage of the total administered dose (TAD) of 5-FU and P-FU in plasma and tissues at different time intervals after i.v. administration are summarized in Tables 2 and 3. The 5-FU levels in plasma and tumors after administration of free 5-FU and P-FU are illustrated in Figs. 3 and 4. Different compartment models were used to describe the pharmacokinetics of free 5-FU and P-FU in plasma and tumor. The calculated data were closer to the observed data when two-compartment model was applied. The area under the concentration–time curve (AUC) was calculated using the linear trapezoidal rule and extrapolated to infinity by dividing the last measurable concentration by the elimination rate constant. The main pharmacokinetic parameters in plasma and tumor are shown in Table 4.

Both circulation time and tumor distribution of 5-FU were significantly increased by using HPMA copolymer conjugates. As shown in Table 4, in plasma, P-FU had a 166 times longer half-life and a 190 times slower clearance rate than 5-FU. As for in tumor, the AUC of P-FU was 3.27 times higher than that of 5-FU. Meanwhile, P-FU has an increased $t_{1/2}$ and decreased CL when compared to 5-FU, indicating that P-FU was better retained in tumor than 5-FU.

5-FU could be immediately distributed and metabolized in tissues such as liver, kidney and intestine after i.v. injection [28], which is the main reason for its low *in vivo* antitumor efficacy. Our data suggested that the circulation time of 5-FU was greatly increased by using HPMA copolymer as carrier, which might con-

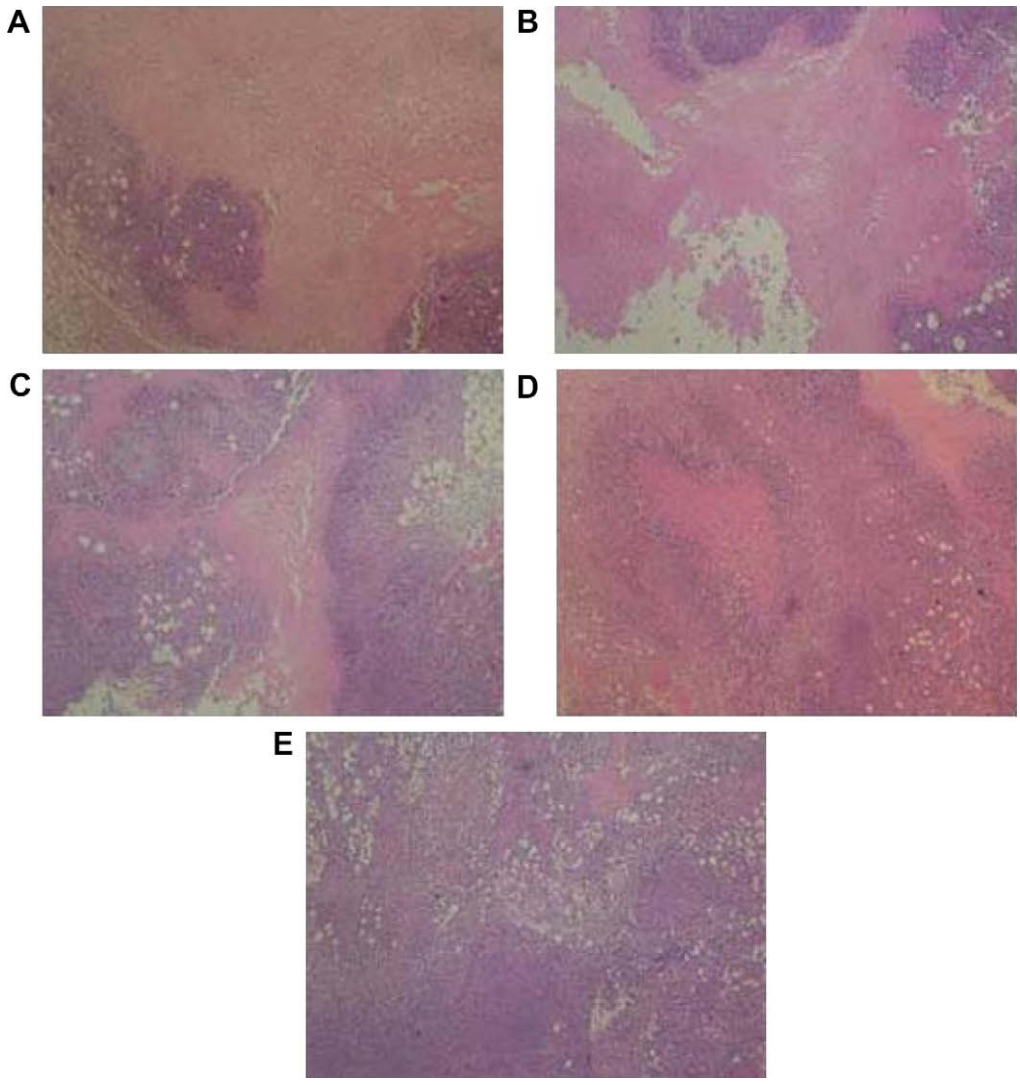

Fig. 6. Histopathology of tumors from H_{22}-bearing mice: P-FU group at 18 mg/kg (A), 5-FU group at 18 mg/kg (B), P-FU group at 6 mg/kg (C), 5-FU group at 6 mg/kg (D) and control group (E), magnification, 40×.

sequently improve the antitumor efficacy of 5-FU. The increase of retention time in plasma may be attributed to the large molecular weight of the conjugates, resulting in a decreased glomerular filtration rate in the kidney. Also, by conjugation to HPMA copolymer, 5-FU can be protected from rapid metabolism in the body.

The accumulation of 5-FU in tumor is also very important for its antitumor efficacy. An over 3-fold larger AUC for P-FU was achieved when compared with 5-FU, suggesting that the conjugates are preferentially accumulated in tumor, which can be explained by the EPR effect, and a better therapeutic efficacy might be achieved for the HPMA copolymer conjugated 5-FU than the free drug. However, we also observed that the accumulation of P-FU in tumor (increase in detected 5-FU concentration) only appeared within the first hour. After 1 h, the drug concentration in tumor decreased.

3.4. Therapeutic effects and histopathology observations

The therapeutic efficacy of P-FU was investigated in H_{22}-bearing mice. Fig. 5 shows the growth of tumor volumes and Table 5 shows the tumor weights of the five groups. They all indicated that neither of the lower-dose groups (6 mg/kg) had a statistical difference in tumors growth when compared to the control group (both $P > 0.1$). However, both higher-dose groups (18 mg/kg) significantly inhibited tumor growth (both $P < 0.005$). Furthermore, P-FU group at higher dose exhibited more significant responses than the 5-FU group at the same dose ($P < 0.03$). The low antitumor efficacy of low P-FU dose group (6 mg/kg) might be due to an insufficient drug dosage. As for the high-dose groups, the contributions from angiogenesis, vascular permeability factors, the absence of lymphatic, and slower diffusion rates in the tumor resulting in higher concentration and accumulation of the HPMA copolymer-bound drugs in the interstitial space of the tumor [29] may be responsible for the higher activity of the conjugates than free drug.

H&E stain involves application of the basic dye hematoxylin, which colors basophilic structures (such as ribosomes and cell nucleus) with blue-purple hue, and alcohol-based acidic eosin Y, which colors eosinophilic structures (such as cytoplasmatic regions) bright pink. Necrosis areas after treatment were colored pink which indicated the effectiveness of the drug. Fig. 6 shows the histopathology characteristics of the experimental groups. Different degrees of necrosis areas were observed in these sections, in the following order: P-FU group (18 mg/kg) > 5-FU group (18 mg/kg) >> P-FU group (6 mg/kg) > 5-FU group (6 mg/kg) > control group. The results further confirmed that the conjugates possessed a greater therapeutic index than 5-FU. This is also in accordance with the increased tumor accumulation and the extended residence time of the conjugates in our biodistribution study.

4. Conclusion

This study indicated that HPMA copolymer–5-FU conjugates significantly improved the cytotoxicity, biodistribution, pharmacokinetic and therapeutic properties of 5-FU. The conjugates might be able to sensitize cell lines with less sensitivity to 5-FU. In accordance with the *in vitro* release profile, the conjugates remarkably prolonged drug circulation time in plasma and increased drug accumulation in tumor. *In vivo* antitumor study showed that P-FU more significantly inhibited tumor growth when compared to free 5-FU indicating an enhanced therapeutic efficacy and a promising potential for clinical applications.

Acknowledgements

The research described above was supported by the National Natural Science Foundation (30500636), Ministry of Education (NCET-06-0786) of the People's Republic of China and the National Basic Research Program of China (973 Program, No. 2007CB935801).

References

[1] U. Scherf, D.T. Ross, M. Waltham, L.H. Smith, J.K. Lee, L. Tanabe, K.W. Kohn, W.C. Reinhold, T.G. Myers, D.T. Andrews, D.A. Scudiero, M.B. Eisen, E.A. Sausville, Y. Pommier, D. Botstein, P.O. Brown, J.N. Weinstein, A gene expression database for the molecular pharmacology of cancer, Nat. Genet. 24 (2000) 236–244.
[2] J.L. Grem, 5-Fluoropyrimidines, Cancer Chemotherapy and Biotherapy Principles and Practice, in: B.A. Chabner, D.L. Longo (Eds.), second ed., Lippincott, Philadelphia, PA, 1996, pp. 149–211.
[3] G.C. Daher, B.E. Harris, R.B. Diasio, Metabolism of pyrimidine analogues and their nucleosides, Pharmacol. Ther. 48 (1990) 189–222.
[4] H. Ringsdorf, Structure and properties of pharmacologically active polymers, J. Polym. Sci. Polym. Symp. 51 (1975) 135–153.
[5] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumour agent SMANCS, Cancer Res. 46 (1986) 6387–6392.
[6] J. Kopeček, Soluble biomedical polymers, Polim. Med. 7 (1977) 191–221.
[7] V. ChytryÂ, A. Vrána, J. Kopeček, Synthesis and activity of a polymer which contains insulin covalently bound on a copolymer of *N*-(2-hydroxypropyl) methacrylamide and *N*-methacryloylglycylglycine 4-nitrophenyl ester, Makromol Chem. 179 (1978) 329–336.
[8] B. Obereigner, M. Burešová, A. Vrána, J. Kopeček, Preparation of polymerizable derivatives of *N*-(4-aminobenzenesulfonyl)-*N'*-butylurea, J. Polym. Sci. Polym. Symp. 66 (1979) 41–52.
[9] J. Kopeček, P. Kopečková, T. Minko, Z.R. Lu, HPMA copolymer–anticancer drug conjugates: design, activity, and mechanism of action, Eur. J. Pharm. Biopharm. 50 (2000) 61–81.
[10] J.G. Shiah, Y. Sun, C.M. Peterson, J. Kopeček, Biodistribution of free and *N*-(2-hydroxypropyl) methacrylamide copolymer-bound mesochlorin e_6 and adriamycin in nude mice bearing human ovarian carcinoma OVCAR-3 xenografts, J. Control. Release 61 (1999) 145–157.
[11] J.G. Shiah, M. Dvorak, P. Kopečková, Y. Sun, C.M. Peterson, J. Kopeček, Biodistribution and antitumor efficacy of long-circulation *N*-(2-hydroxypropyl) methacrylamide copolymer–doxorubicin conjugates in nude mice, Eur. J. Cancer 37 (2001) 131–139.
[12] Y. Huang, H. Ghandehari, Y.R. Duan, A. Nan, Z.R. Zhang, HPMA copolymer–mitoxantrone conjugates for targeted cancer chemotherapy, J. Drug Deliv. Sci. Technol. 14 (2004) 187–191.
[13] W.W. Ten Bokkel Huinink, J. Meerum Terwogt, R. Dubbelman, L. Valkenet, M.G. Zurlo, J.H.M. Schellens, J.H. Beijnen, Phase I and pharmacokinetics study of PNU166945, a polymer formulated placlitaxel, Proceedings of the Third International Symposium on Polymer Therapeutics, London, U.K. (1998) p. 12.
[14] D.J. Kerr, L.W. Seymour, C. Boivin, P. Julyan, J. Doran, M. David, D. Anderson, C. Christodolou, S. Daryani, A.M. Young, S. Hesslewood, D.R. Ferry, Phase I clinical trial of HPMA copolymers bearing doxorubicin and galactosamine. Proceedings of the Third International Symposium on Polymer Therapeutics, London, U.K. (1998) p. 23.
[15] P.A. Vasey, S.B. Kaye, R. Morrison, C. Twelves, P. Wilson, R. Duncan, A.H. Thomson, L.S. Murray, T.E. Hilditch, T. Murray, S. Burtles, D. Fraier, E. Frigerio, J. Cassidy, Phase I clinical and pharmacokinetic study of PK1 [*N*-(2-hydroxypropyl)-methacrylamide copolymer doxorubicin]: first member of a new class of therapeutic agents-drug–polymer conjugates, Clin. Cancer Res. 5 (1999) 83–94.
[16] V.R. Caiolfa, M. Zamai, A. Fiorino, E. Frigerio, C. Pellizzoni, R. d'Argy, A. Ghiglieri, M.G. Castelli, M. Farao, E. Pesenti, M. Gigli, F. Angelucci, A. Suarato, Polymer-bound camptothecin: initial biodistribution and antitumour activity studies, J. Control. Release 65 (2000) 105–119.
[17] D. Putnam, J. Kopeček, Enantioselective release of 5-fluorouracil from *N*-(2-hydroxy-propyl)-methacrylamide based copolymers via lysosomal enzymes, Bioconjugate Chem. 6 (1995) 483–492.
[18] F. Yuan, F. Chen, Q.Y. Xiang, X. Qin, Z.R. Zhang, Y. Huang, Synthesis and characterization of HPMA copolymer–5-FU conjugates, Chinese Chem. Lett. 19 (2008) 137–140.
[19] H. Xu, S.M. Choi, C.S. An, Y.D. Min, K.C. Kim, K.J. Kim, C.H. Choi, Concentration-dependent collateral sensitivity of cisplatin-resistant gastric cancer cell sublines, Biochem. Bioph. Res. Co. 328 (2005) 618–622.
[20] Y.Q. Wei, M.J. Huang, L. Yang, X. Zhao, L. Tian, Y. Lu, J.M. Shu, C.J. Lu, T. Niu, B. Kang, Y.Q. Mao, F. Liu, Y.J. Wen, S. Lei, F. Luo, L.Q. Zhou, F. Peng, Y. Jiang, J.Y. Liu, H. Zhou, Q.R. Wang, Q.M. He, F. Xiao, Y.Y. Lou, X.J. Xie, Q. Li, Y. Wu, Z.Y. Ding, B. Hu, M. Hu, W. Zhang, Immunogene therapy of tumors with vaccine based on Xenopus homologous vascular endothelial growth factor as a model antigen, Proc. Natl. Acad. Sci. USA 98 (2001) 11545–11550.
[21] J. Wang, C. Chen, B. Li, H. Yu, Y. Zhao, J. Sun, Y. Li, G. Xing, H. Yuan, J. Tang, Z. Chen, H. Meng, Y. Gao, C. Ye, Z. Chai, C. Zhu, B. Ma, X. Fang, L. Wan, Antioxidative function and biodistribution of $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles in tumor-bearing mice, Biochem. Pharmacol. 71 (2006) 872–881.
[22] T. Ouchi, M. Tada, M. Matsumoto, Y. Ohya, K. Hasegawa, Y. Arai, K. Kadowaki, S. Akao, T. Matsumoto, S. Suzuki, Design of macromolecular prodrug of *S*-

fluorouracil using *N*-acetylpolygalactosamine as a targeting carrier to hepatoma, React. Funct. Polym. 37 (1998) 235–244.
[23] J. Huang, J.W. Wang, T. Gong, Z.R. Zhang, Synthesis and characterization of insulin–5-FU conjugate, enabling insulin as multi-drug carrier *via* dendritic approach, Chin. Chem. Lett. 18 (2007) 247–250.
[24] T.H. Corbett, D.P. Griswold Jr., B.J. Roberts, J.C. Peckham, F.M. Schabel Jr., Biology and therapeutic response of a mouse mammary adenocarcinoma (16/C) and its potential as a model for surgical adjuvant chemotherapy, Cancer Treat. Rep. 60 (1978) 1471–1488.
[25] D. Salonga, K.D. Danenberg, M. Johnson, R. Metzger, S. Groshen, D.D. Tsao-Wei, H.J. Lenz, C.G. Leichman, L. Leichman, R.B. Diasio, P.V. Danenberg, Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase, Clin. Cancer Res. 6 (2000) 1322–1327.
[26] K. Ulbrich, V. Šubr, J. Strohalm, D. Plocová, M. Jelínková, B. Říhová, Polymeric drugs based on conjugates of synthetic and natural macromolecules I. Synthesis and physico-chemical characterization, J. Control. Release 64 (2000) 63–79.
[27] C. de Duve, T. de Barsy, B. Poole, A. Trouet, P. Tulkens, F. Van Hoof, Lysosomotropic agents, Biochem. Pharmacol. 23 (1974) 2495–2531.
[28] D.B. Longley, D.P. Harkin, P.G. Johnston, 5-Fluorouracil: mechanisms of action and clinical strategies, Nat. Rev. Cancer 3 (2003) 330–338.
[29] T. Minko, P. Kopečková, J. Kopeček, Efficacy of the chemotherapeutic action of HPMA copolymer-bound doxorubicine in a solid tumor model of ovarian carcinoma, Int. J. Cancer 86 (2000) 108–117.