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Research paper

Synthesis and *in vitro* anti-tumor activity of novel HPMA copolymer–drug conjugates with potential cell surface targeting property for carcinoma cells

Qingyu Xiang, Yang Yang, Zhou Zhou, Dan Zhou, Yun Jin, Zhirong Zhang, Yuan Huang *

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

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ABSTRACT

In several groups of malignant tumors including head and neck tumors, a protein named Hsp47/CBP2 leaked from the cell was expressed on the tumor cell surface. Several synthetic peptides have been identified as effective ligands for binding to Hsp47/CBP2. This study has focused on the synthesis and *in vitro* characterization of a targeting delivery system of 5-fluorouracil (5-FU) to human head and neck squamous cell carcinoma (HNSCC) in order to improve anti-cancer efficacy and reduce dose-limiting toxicity of 5-FU. An N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer, with Hsp47/CBP2 binding peptide sequence (namely WHYPWFQNWAMA) as a targeting ligand, was synthesized by a novel and simplified synthetic route. Under the controlled synthetic conditions, 1,3-dimethylol-5-FU, derived from 5-FU, was attached to the HPMA copolymer backbone via the lysosomally degradable GFLG linker, while the WHYPWFQNWAMA was conjugated via a non-degradable Gly-Gly (GG) linker. A control polymer without targeting moiety was also synthesized (P-FU). The *in vitro* cytotoxicity, internalization and apoptosis assays of the polymeric conjugates were evaluated. The characteristic apoptotic morphological changes were also assessed. Compared to 5-FU and P-FU, the HPMA copolymer containing the Hsp47/CBP2 binding peptide (P-FU-peptide) exhibited the highest cytotoxic efficacy to cell line of human head and neck squamous cell carcinoma ($p < 0.05$) and was internalized much faster than P-FU, especially after being incubated for 30 min. Both of the morphology and apoptosis analyses demonstrated that the treatment of P-FU-peptide resulted in more apoptotic and necrotic induction of tumor cells than P-FU. Meanwhile, the rate of apoptosis induced by P-FU-peptide was higher than that of necrosis. In summary, the HPMA copolymer–Hsp47/CBP2 binding peptide conjugates showed a promising future for the treatment of HNSCC with improved efficacy.

1. Introduction

5-Fluorouracil (5-FU) is widely used in the treatment of a range of cancers, including human head and neck squamous cell carcinoma (HNSCC). The mechanism of cytotoxicity of 5-FU has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and the inhibition of activity of the nucleotide synthetic enzyme-thymidylate synthase (TS) [1]. Nevertheless, the administration of this drug is always accompanied with systemic toxicities [2,3]. Although 5-FU in combination with other chemotherapeutic agents can enhance the response and survival rates in some kinds of cancers, it is still a challenge to improve the therapeutic response of the drug due to the short circulation time *in vivo* ($t_{1/2}$ is about 10–20 min) [4] and low drug accumulation in tumors [5,6]. To overcome this problem, new therapeutic strategies are urgently expectant.

In the last three decades, the use of polymeric drug delivery system has emerged as an established approach for improving cancer chemotherapy [7–10]. To achieve a tumor-specific or a tumor cell-specific drug delivery [11] and increase the anti-tumor activity, proper polymer carrier, type of linkage between the drug and carrier as well as a targeting moiety and its binding procedure are necessarily required [12–15]. Among those polymeric drug delivery systems, a hydrophilic, biocompatible polymer, based on N-(2-hydroxypropyl) methacrylamide (HPMA), is extensively examined as a non-toxic and non-immunogenic drug carrier [16–19]. Drug molecules can be attached to the polymer backbone via the lysosomally degradable Gly-Phe-Leu-Gly (GFLG) peptide allowing intracellular release by lysosomal proteases [20] while remaining stable during systemic circulation [21]. Also, the use of the HPMA polymeric conjugates was reported to significantly alter the pharmacokinetics and biodistribution of drugs [22,23]

* Corresponding author. Key Laboratory of Drug Targeting and Drug Delivery System, Ministry of Education, West China School of Pharmacy, Sichuan University, No. 17, Block 3, Southern Renmin Road, Chengdu 610041, PR China. Tel./fax: +86 28 85501617.
E-mail address: huangyuan0@yahoo.com.cn (Y. Huang).

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so as to prolong their half-life in circulation and increase the total drug exposure. In addition, several HPMA copolymer–drug conjugates have already progressed into clinical trials [24–27].

However, the studies about the synthesis and anti-tumor potential of HPMA copolymer–5-FU conjugates (P-FU) were unsatisfied [28–33]. The synthetic method was complicated, thus resulting in a low 5-FU content of the conjugates (only 1.38 wt%) [33]. Previous work in our laboratory has described a new, improved synthetic method for P-FU [34,35]. Though the content of 5-FU was improved (3.41 wt%), the *in vitro* anti-tumor activity was not satisfied, and only the group of higher-dosage showed some effects. Therefore, it was supposed that, besides increase of the drug content, the inclusion of targeting moieties bound to the polymer backbone may further enhance the accumulation of drug in the target site, improve its therapeutic efficacy and minimize systemic exposure.

Previous studies have demonstrated that in several groups of malignant tumors including head and neck tumors, a protein named Hsp47/CBP2 leaked from the cell was expressed on the tumor cell surface [36]. This makes it a favorable candidate for targeted delivery of anti-cancer drugs. A combinatorial approach utilized a repertoire of bacteriophage-peptides has identified a number of non-natural Hsp47/CBP2 binding peptides. Among these sequences, WHYPWFQNWAMA was the most effective Hsp47/CBP2 binding peptide [36,37].

In the present study, therefore, a novel HPMA copolymer containing the Hsp47/CBP2 binding peptide (P-FU-peptide) was developed with 5-fluorouraci as a model drug. In particular, several issues were considered simultaneously. Firstly, a proper synthetic route was studied to increase the drug content of the polymer conjugates. Secondly, in the present study, the drug was attached to the polymer before the conjugation of the targeting peptide. However, the ester bond between 5-FU and spacer GFLG was easier to be degraded during the targeting peptide attachment in basic conditions, which also led to low drug content. Therefore, a new method was designed to successfully attach the ligand to HPMA copolymer while mostly avoiding the degradation of ester bond. The characterization, *in vitro* cytotoxicity, internalization and apoptosis assays of P-FU-peptide were examined compared to the synthesized control polymer without targeting moiety (P-FU) and the free drug.

2. Materials and methods

2.1. Chemicals

5-Fluorouracil was purchased from 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 obtained from Sigma (St. Louis, MO, USA). The Hsp47/CBP2 binding peptide (WHYPWFQNWAMA) was synthesized according to our design by CP Biochem Co., Ltd. (Sichuan, China). All other chemicals and reagents were gained from Sigma Chemical Co. (St. Louis, MO, USA) and used as received.

2.2. Synthesis of monomers

The monomers of HPMA [38], methacryloylglycylglycine-p-nitrophenyl ester (MAGGONp) [39], methacryloylglycyl phenylalanyllecuyl glycyl acid (MAGFLGOH) [40], 1,3-dimethylol-5-Fu [34] and 5-[3-methacryloylaminopropyl)thioureidyl] fluorescein (MA-AP-FITC) [41] were synthesized according to previously published procedures.

2.3. Synthesis of polymeric conjugates

Four polymeric conjugates were synthesized, namely HPMA copolymer–(Hsp47/CBP2 binding peptide)-5-FU (P-FU-peptide), HPMA copolymer–5-fluorouracil conjugates (P-FU), FITC-labeled P-FU-peptide (FITC-P-FU-peptide) and FITC-labeled P-FU (FITC-P-FU). P-FU-peptide was synthesized in a three-step procedure (Fig. 1). Firstly, a polymeric precursor containing reactive ONp ester groups (P′) was synthesized by free radical precipitation copolymerization of the monomers of HPMA, MAGFLGOH and MAGGONp in acetone/DMSO mixture at 50 °C for 24 h with 2,2′-azobisisobutyronitrile (AIBN) as the initiator by established procedure described previously [42]. The ONp content of the precursor was determined spectrophotometrically (λ_{max} = 400 nm). Subsequently, the polymer–drug conjugates were prepared by the reaction of 1,3-dimethylol-5-Fu with P′ in anhydrous acetonitrile/dimethylformamide (DMF, 5:12, v/v). N,N-dicyclohexyl-carbodiimide (DCC) was added under stirring. The reaction mixture was stirred at room temperature for 18 h. Then, the peptide was conjugated to the polymer by aminolysis of the reactive ONp groups. Briefly, to a solution of the polymer precursor in DMF, a 1.0 M peptide solution was added. The reaction mixture was

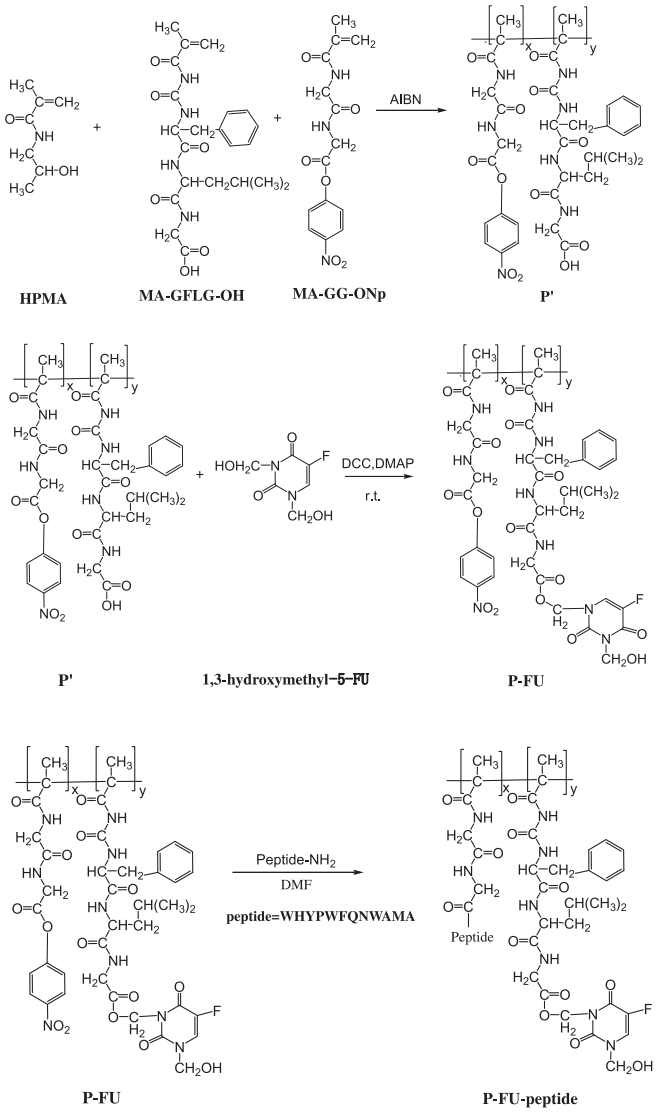

Fig. 1. Synthesis of HPMA copolymer–5-FU conjugates (P-FU-peptide and P-FU).

stirred in the dark at room temperature for 20 h. P-FU-peptide was isolated and purified by methods described previously [43]. Briefly, the polymer–peptide–drug conjugates were rotary evaporated to remove the DMF, dissolved in methanol and precipitated in ether. The precipitated polymer was dissolved in distilled water and dialyzed for 2 days followed by lyophilization to obtain the pure products. The control conjugates P-FU were synthesized by the same copolymerization of the comonomers of HPMA, MAGFLGOH and MAGGONp and then reacted with 1,3-dimethylol-5-Fu as described above. The detecting conjugates, FITC-P-FU-peptide and FITC-P-FU, were prepared with 2 mol% MA-AP-FITC as the additional monomers in the similar methods mentioned above. The 5-FU content in the conjugates was analyzed by high-performance liquid chromatography (HPLC) system equipped with a Dikma Diamonsil® C18 column (250 × 4.6 mm, 5 μm, 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 peptide content of P-FU-peptide was measured by amino acid analysis (Commonwealth Biotech. Inc., Richmond, VA). The molecular weight and molecular weight distribution of the conjugates were estimated by size-exclusion chromatography (SEC) on a Superose 200 10/300GL analytical column (Amersham Biosciences, NJ) calibrated with poly(HPMA) fractions using a Fast Protein Liquid Chromatography (AKTA FPLC) system (Amersham Biosciences, NJ). The chemical structures of the conjugates were shown in Fig. 1.

2.4. In vitro drug release studies

Fifty milligrams of P-FU-peptide and 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 and then centrifuged for 10 min at 14,000 g; 20 μL of the clear supernatant was analyzed by HPLC to determine the amount of the released 5-FU, using HPLC conditions as described above.

2.5. Cell lines

SCC9 cells were obtained from American Type Culture Collection (ATCC). Cells were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units of penicillin, 100 mg/mL streptomycin and 0.4 mg/mL hydrocortisone at 37 °C in a 5% CO_2/95% air atmosphere. Cells were subcultured with Trypsin (0.25%)–EDTA (0.03%) in phosphate-buffered saline (PBS) at pH 7.5. All experiments were performed on cells in the exponential growth phase.

2.6. Cytotoxicity assays

All concentrations of P-FU-peptide and P-FU were expressed in 5-FU equivalents. All solutions were sterilized by filtering through a 0.22 μm membrane filter.

The cytotoxicity of free 5-FU, P-FU-peptide and P-FU conjugates toward SCC9 was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide (MTT) assay [44]. The results were used to calculate the IC_{50} dose (drug concentration causing 50% growth inhibition) relative to a control of non-treated cells [45].

Briefly, cells were seeded onto 96-well plate at a density of 1×10^4 cells per well for 24 h. Then, the growing cells were treated with 100 μL of 5-FU, P-FU-peptide or P-FU (containing equal amount of 5-FU) at various concentrations (ranging from 9.38 to 300.00 μg/mL of 5-FU) for 12 h and 36 h at 37 °C, respectively.

Then, 20 μL MTT (5 mg/mL) was added to each well. 4 h later, the solvent was removed, and 150 μL of DMSO was added to each well to dissolve the formazane of MTT. The absorption at 570 nm was measured using an ELISA plate reader (Bio-Rad, Microplate Reader 550). The growth inhibition rate (GI) was calculated as follows:

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

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 blank group (no cells). IC_{50} values (μg/mL) were calculated by SPSS software.

2.7. Competitive inhibition experiments

Competitive inhibition experiments were performed on SCC9 human head and neck squamous carcinoma cells. The cells were seeded onto 6-well plate at a density of 1×10^5 cell/well in 2 mL medium for 48 h in the CO_2 incubator. The medium was replaced with 0.20 mM free peptide first, then added with FITC-P-FU or FITC-P-FU-peptide and diluted with culture medium to give a concentration of 25 mM FITC equivalently. After being incubated at 37 °C for 15 min, 30 min, 60 min, 120 min, 180 min, the medium was pipetted off from the wells, and each well was rinsed with ice-cold PBS. Then, the cells were digested and washed twice. The blank sample was treated with culture medium and dealt with the same manner. The fluorescence intensity was measured by flow cytometry (Aria2, Becton, Dickinson and Company, USA).

2.8. Apoptosis studies

2.8.1. Morphological analysis of apoptosis by fluorescence microscopy

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using acridine organg/ethidium bromide (AO/EB) staining. Cells were seeded onto 6-well plate at a density of 1×10^5 cell/well in 2 mL medium for 48 h in the CO_2 incubator. After incubation with 5-FU, P-FU or P-FU-peptide at two dose of 18.75 mg/mL and 37.5 mg/mL (5-FU equiv.) for 12 h and 36 h, SCC9 cells were washed with PBS and incubated with 100 μL of a mixture of AO/EB (1:1, 4 μg/mL) solution for 15 min at 37 °C. Followed by PBS washing, cells were observed and photographed by Axiovert 40 CFL (Germany).

2.8.2. Apoptosis analysis with Annexin V-FITC/PI staining

Apoptosis assay was performed by flow cytometry according to the manufacturer's protocol (Roche Applied Science, Germany). Briefly, SCC9 cells were seeded onto a 6-well culture plate and treated with 5-FU, P-FU or P-FU-peptide for 12 h and 36 h. The harvested cells were washed twice with PBS and suspended binding buffer (10 mmol/L HEPES, pH7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$). Aliquots of 100 μL suspension (1×10^6 cells) were incubated with 2 μL Annexin V-FITC and 2 μL PI for 15 min at room temperature in the dark. Cell suspension was added with 400 μL of binding buffer, gently vortexed, and analyzed within 1 h by flow cytometry.

2.9. Statistical analysis

All data are expressed as means ± SD from at least three independent experiments. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA). Statistical significance was considered at $p < 0.05$. The SPSS version 13.0 program was used.

3. Results and discussion

3.1. Synthesis and characterization of polymeric conjugates

P-FU-peptide and P-FU were successfully synthesized and characterized (Table 1). To assay the internalization of both conjugates, MA-FITC was inducted into HPMA copolymers, and FITC-labeled conjugates (FITC-P-FU-peptide and FITC-P-FU) were synthesized.

In the preliminary study, HPMA copolymer backbone (P′) was firstly synthesized and then conjugated with 1,3-dimethylol-5-Fu according to our previous studies [34]. Although this method seemed to result in a higher 5-FU content (3.41 wt%), the reactive ONp ester groups in GG-ONp which would be reacted with targeting peptide in the next step were not stable during the reaction. Furthermore, it was found that the attachment of targeting peptide would also lead to the degradation of the ester bond between 5-FU and the HPMA copolymer. Therefore, it is a challenge to successfully attach the ligand to HPMA copolymer and maintain higher drug content. Consequently, several synthetic methods have been identified to effectively conjugate 5-FU and the targeting peptides to the conjugates. Finally, it was shown that during the procedure of 1,3-dimethylol-5-Fu conjugating to the HPMA copolymer backbone (P′), anhydrous conditions should be kept to inhibit the degradation of the ester bond for drug linkage. Moreover, anhydrous organic base DMF as solvent was used during the conjugation of the targeted peptide, which in turn kept the stability of the ester bond between drug and HPMA copolymer and promoted the reaction of GG-ONp with peptide. Furthermore, the reaction time was also important. The results indicated that the optimal time for the reaction of 1,3-dimethylol-5-Fu with P′ and reaction of targeting peptide with P-Fu was 18 h and 20 h, respectively .

In this synthetic route, 5-FU contents of P-FU-peptide and P-FU were 4.63 wt% and 8.41 wt%, respectively, which showed higher values than those of previous investigations (1.38 wt% and 3.41 wt%) [33,34]. The Hsp47/CBP2 binding peptide was successfully attached to the HPMA backbone via the non-degradable GG linker (Fig. 1) and found to be 0.08 mg/g polymer. The molecular weight and poly-dispersity index of the conjugates are important factors in a polymer carrier's biocompatibility. And there was a clear relationship among the molecular weight, the accumulation in tumor and rate of elimination [20,46]. The estimated average molecular weight (Mw) of P-FU-peptide and P-FU was 39.9 kDa and 25.4 kDa with poly-dispersity index (PDI) of 1.9 and 1.56, respectively. These measured Mw and PDI values were below the renal threshold to ensure their elimination, which were comparable to previous reports [47,48].

3.2. In vitro drug release studies

The stability of P-FU-peptide and P-FU in plasma (Fig. 2) indicated that the P-FU conjugates were relatively stable with only 27% drug released in the initial 8 h and the release continued to 120 h (77%), while P-FU-peptide conjugates were relatively stable with less than 20% drug released in the initial 15 h, and the drug release was only 30% until 120 h. The results showed that the circulation time can be significantly prolonged by attaching the drug to a macromolecular polymeric chain. Furthermore, data were fitted into five models (first-order model, zero-order model, Peppas equation, Hixson–Crowell equation and Higuchi equation), and the release profile could be best modeled to Higuchi equation, and the sustained-release of conjugates was demonstrated. Thus, the conjugates, especially for P-FU-peptide, can improve the short half-life of 5-FU (10–20 min) in plasma circulation and decrease the degradation of free 5-FU. Moreover, the drug release of P-FU was significantly higher than P-FU-peptide ($p < 0.05$) at all time points and is consistent with previous reports [49].

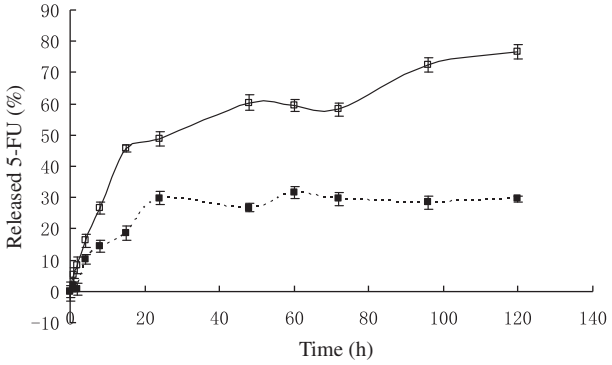

Fig. 2. Release profile of 5-FU from P-FU-peptide and P-FU in plasma (concentration of 5-FU was measured). Closed squares: P-FU-peptide; open squares: P-FU. All data are the mean ± SD ($n = 3$).

3.3. Cytotoxicity evaluation

The growth inhibitions of 5-FU, P-FU-peptide and P-FU on SCC9 cells were investigated at various concentrations for 12 h and 36 h by MTT assay (Fig. 3). The IC_{50} values among three samples were compared (Table 2).

The results showed that all the compounds were capable of inhibiting cell growth, and both conjugates, compared to free 5-FU, displayed superior cytotoxicities against SCC9 cells with equivalent concentrations to 5-FU from 18.75 μg/mL to 300 μg/mL. After 36 h of culturing, even though the IC_{50} of 5-FU decreased significantly, P-FU-peptide still showed higher cytotoxicity. With extension of culturing time, P-FU-peptide expressed higher toxicity against SCC9 cells at 36 h compared with the other compounds (Table 2). It was well known that the disadvantages of 5-FU in clinic included its high dosage and dose-limitation; thus, these results indicated the HPMA copolymer conjugates may improve the therapeutic efficacy of 5-FU.

As was known, for HPMA copolymer doxorubicin conjugates, the IC_{50} of the conjugates was higher than free drug since the drug

Table 1
Physicochemical characteristics of HPMA copolymer conjugates.

Sample[a]	Feed comonomer composition (mol%)			Polymer characteristics			
	HPMA	Targeting peptide	Spacer	5-FU[b] content (mg/g polymer)	Peptide[b] content (mmole/g polymer)	Mw[c] (g/mole)	Mw/Mn[c]
P-FU-peptide	80	10	−GFLG-(10) −GG-(10)	46.27	0.08	39.9	1.9
P-FU	80	–	−GFLG-(10),	84.14	–	25.4	1.56

[a] For structures of polymer–drug conjugates, see Fig. 1.
[b] Polymer–drug conjugate.
[c] Polymer precursor.

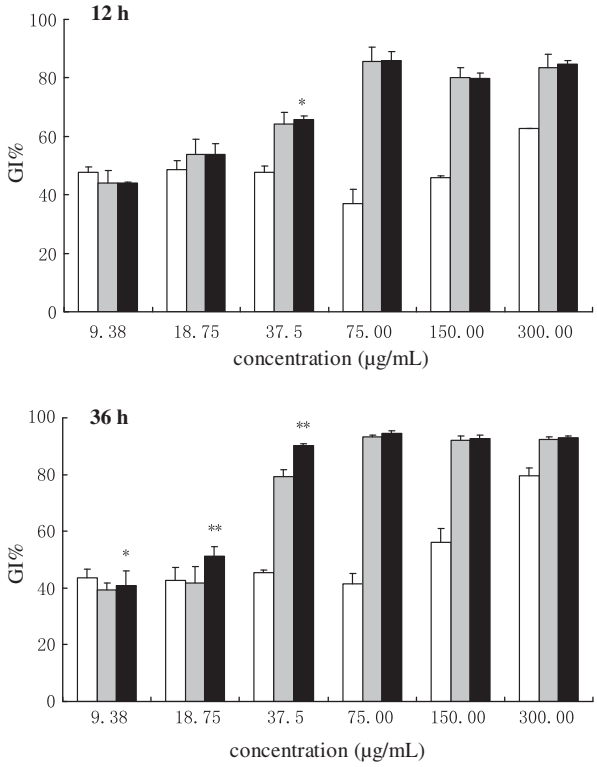

Fig. 3. Growth inhibition (GI) effect of 5-FU, P-FU-peptide and P-FU on SCC9. (5-FU,white. P-FU, gray. P-FU-peptide, black.) The results represent the average ± SD of at least three independent experiments. Significant differences noted as compared to P-FU for P-FU-peptide ($^{*}p < 0.05$; $^{**}p < 0.01$).

Table 2
The IC_{50} values of P-FU, P-FU-peptide and 5-FU against SCC9 cancer cell lines. All data are mean ± SD ($n = 3$).

SCC9	IC_{50} (μg/mL)			Ratio[a]	Ratio[b]	Ratio[c]
	5-FU	P-FU	P-FU-peptide			
12 h	113.49 ± 8.79	12.36 ± 0.92*	10.52 ± 1.47*	9.18	10.79	1.17
36 h	43.25 ± 6.57	12.50 ± 0.58*	9.70 ± 1.96*	3.46	4.46	1.29

[a] Ratio, IC_{50} for 5-FU vs. IC_{50} for P-FU.
[b] Ratio, IC_{50} for 5-FU vs. IC_{50} for P-FU-peptide.
[c] Ratio, IC_{50} for P-FU vs. IC_{50} for P-FU-peptide.
* $p < 0.05$, compared to 5-FU.

needs to release first from conjugates. But it should be noted that doxorubicin is sensitive to several reported tumor cells, and the drug is not easily to be degraded in these tumor cells [19]. In the present study, SCC9 was less sensitive to 5-FU, and the rate-limiting enzymes in 5-FU catabolism mainly existed in cytoplasm, which resulted in its instability. Thus, the IC_{50} values for 5-FU were up to 113.49 μg/mL (12 h) and 43.25 μg/mL (36 h). With the conjugation of the drug to HPMA copolymer, the mechanism of cellular uptake was changed compared with free drug, and 5-FU was mainly released in lysosome (the GFLG spacer between 5-FU and HPMA copolymer was mainly degraded by lysosomal proteases) [40]. Since the amount of rate-limiting enzymes in 5-FU catabolism was less in lysosome compartment, the stability of 5-FU was improved in some degree. In addition, because of the influence of HPMA conjugates on P-glycoprotein pump, the exclusion of the polymer–drug conjugate from the cytoplasm of the cell should render the efflux pump ineffective [19], and then the susceptibility of the SCC9 cells toward the therapeutic agent (5-FU) was improved. Consistent with previous reports, HPMA conjugates have the

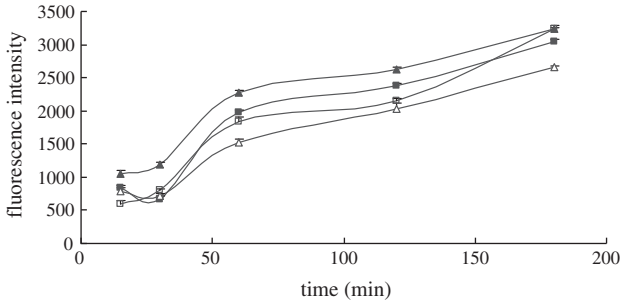

Fig. 4. The fluorescence intensity of SCC9 cells treated with FITC-P-FU-peptide and FITC-P-FU (with or without free peptide). Closed triangles: FITC-P-FU-peptide; closed squares: FITC-P-FU; open triangles: FITC-P-FU-peptide with free peptide; open squares: FITC-P-FU with free peptide. Data presented as the average ± SD of three independent experiments.

similar increased cytotoxicity toward tumor cells that are less sensitive to 5-FU such as HepG2 and Hela cells [35]. These may explain our observed phenomena that the HPMA conjugates were more effective for the treatment of tumor cells that are less sensitive to free drug.

In addition, the data in Fig. 3 demonstrated that at 5-FU equivalent concentration of 37.5 μg/mL, the GI% of P-FU-peptide was significant higher than P-FU ($p < 0.05$) at 12 h. Furthermore, with extension of culturing time, P-FU-peptide showed higher toxicity to SCC9 cells compared to P-FU at 5-FU equivalent concentration of 9.38 μg/mL ($p < 0.05$), 18.75 μg/mL ($p < 0.01$) and 37.5 μg/mL ($p < 0.01$). Since the previous reports already demonstrated the targeting peptide (WHYPWFQNWAMA) alone did not inhibit proliferation of SCC9 cell lines to any significant extent nor was it cytotoxic [50], these results may illustrate that an optimum concentration of the Hsp47/CBP2 targeting peptide and a longer culturing time are necessary to enhance the anti-tumor effect in the cell culture model.

3.4. Competitive inhibition experiments

In order to demonstrate active receptor-mediated targeting activity of the polymeric conjugates (HPMA-FU-peptide), the quantitative estimation of FITC-HPMA conjugates internalized by SCC9 cells was studied, and control groups that were treated by free peptides first then treated by the HPMA conjugates were designed.

Fig. 4 showed the fluorescence intensity of SCC9 cells treated with FITC-P-FU-peptide and FITC-P-FU (with or without free peptide). The results clearly suggested that FITC-P-FU-peptide was internalized much faster than FITC-P-FU ($p < 0.05$), especially after being incubated for 30 min. Furthermore, after adding the free peptides to weaken the peptide binding ability, both of the HPMA conjugates (equivalent to 25 mM of FITC) were internalized by SCC9 in a time-dependent manner (Fig. 4). It was found that with the same FITC concentrations, the fluorescence intensity of SCC9 cells treated with FITC-P-FU-peptide showed significantly lower uptake ($p < 0.02$) when the cells were first treated by free peptides. The fluorescence intensity of FITC-P-FU was less affected by the presence of the free peptides. These results indicated the internalization of P-FU-peptide conjugates seemed to be enhanced via a receptor-mediated uptake, thus resulting in higher activity against SCC9 cells.

3.5. Apoptosis studies

3.5.1. Morphology assay by AO/EB double staining

On the basis of overall cell morphology and cell membrane integrity, necrotic and apoptotic cells can be distinguished from

each other using fluorescence microscope. Double staining allowed discrimination of live (L) cells from early apoptotic (EA), late apoptotic (LA), early necrosis (EN) and necrotic cells (N). As is known, AO permeates all cells and stains the nuclei green as a result of intercalation in double-stranded DNA, or orange when it binds to single-stranded RNA and accumulates in lysosomes [51]. EB is absorbed only by cells with a damaged cytoplasmic membrane, and the nucleus of LA and N cells are stained red. EB dominates over AO. L cells have a normal green nucleus. EA cells display a bright green nucleus with condensed or fragmented chromatin. In LA cells, condensed and fragmented chromatin is stained orange. Finally, N cells have an organ-stained, structurally normal nucleus.

Hence, after AO/EB double staining, morphological changes indicative of apoptosis were observed in SCC9 cultures, where L, EA, LA, EN and N cells were identified (Fig. 5). 5-FU-treated cultures showed the largest number of L at 12 h (Fig. 5A) and small number of LA, EA and EN at 36 h (Fig. 5D). P-FU-peptide-treated cultures exhibited the smallest number of L cells at all time points, and EA cells had already appeared at 12 h (Fig. 5C). Until 36 h, EN and LA cells were numerous, and there were less L cells (Fig. 5F). Similarly, after being exposed to P-FU, the number of L cells decreased from 12 to 36 h (Fig. 5B and E). The amount of LA and EN cells was also higher than those of 5-FU-treated cultures, but less than P-FU-peptide-treated cultures. These results showed an increased therapeutic potential of P-FU-peptide compared to P-FU and 5-FU as more apoptosis was detected in P-FU-peptide-treated cells. Furthermore, it was also observed that with the prolonged time of treatment on SCC9 cells, polymeric conjugates induced more apoptosis and necrosis, indicating that the activity of P-FU and P-FU-peptide against SCC9 cells seemed to be enhanced following by prolonged incubation time via apoptosis/necrosis induction.

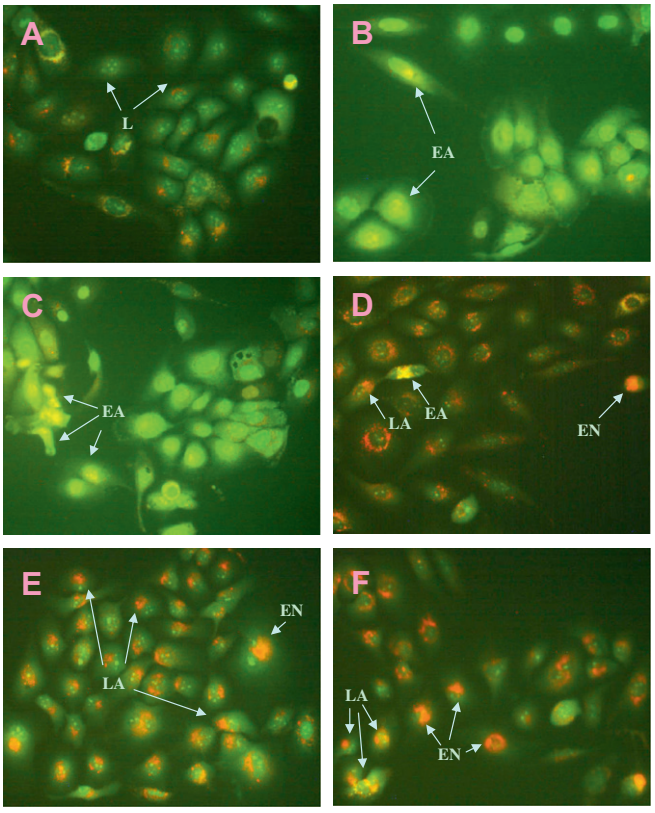

Fig. 5. Morphological and nuclear changes of SCC9 cells treated with 5-FU, P1 and P2 for 12 h and 36 h as determined by Acridine orange/Ethidium bromide staining (20× original magnification). (A) 5-FU (18.75 μg/ml, 12 h); (B) P-FU (18.75 μg/ml 5-FU equiv., 12 h); (C) P-FU-peptide (18.75 μg/ml 5-FU equiv., 12 h); (D) 5-FU (18.75 μg/ml, 36 h); (E) P-FU (18.75 μg/ml 5-FU equiv., 36 h); (F) P-FU-peptide (18.75 μg/ml 5-FU equiv., 36 h). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5.2. Analysis of apoptosis by flow cytometry

The above AO/EB staining experiments exhibited apoptotic induction by the treatment of two HPMA conjugates. Therefore, to further quantitatively investigate the apoptotic and early necrotic cells of these treated cultures, fluorescent probe Annexin V-FITC and PI were used, and then apoptotic and early necrotic cells were determined and quantified by flow cytometry analysis which could clearly differentiate normal cells with low Annexin V-FITC and low PI staining, apoptotic cells with high Annexin V-FITC and low PI staining, and necrotic cells with high Annexin V-FITC and high PI staining.

The apoptotic cells were observed in SCC9 cells treated with all the compounds, but the percentage of apoptotic cells varied from each other (Fig. 6). Cells treated with P-FU-peptide showed 30.58% and 7.91% of early apoptotic (Annexin V-FITC+/PI−) and late apoptotic/early necrotic (Annexin V-FITC+/PI+) populations, respectively, compared to 5-FU (15.70% and 10.84%) and P-FU (25.35% and 8.76%) at 18.75 μg/mL dose for 12 h, and there were no notable differences between cells treated with both conjugates after being incubated for the first 12 h. Then, increased percentage of apoptotic cells treated with both populations was observed at 36 h. The percentage of early apoptotic (Annexin V-FITC+/PI−) and late apoptotic/early necrotic (Annexin V-FITC+/PI+) populations in cells treated with 5-FU, P-FU and P-FU-peptide at 18.75 μg/mL dose was 18.86% and 8.10%, 33.68% and 6.50%, 38.06% and 6.98%, respectively.

Apparently, more early apoptotic cells (Annexin V-FITC+/PI−) than late apoptotic/early necrotic cells (Annexin V-FITC+/PI+) were generated in these three compounds treated cells, suggesting the progression from early apoptotic to late apoptotic/early necrotic population. These results demonstrated the apoptotic cell pathways, but not directly necrotic one, seemed to be activated by these compounds. Moreover, data indicated that during 12 h, the number of apoptotic and early necrotic cells were similar in

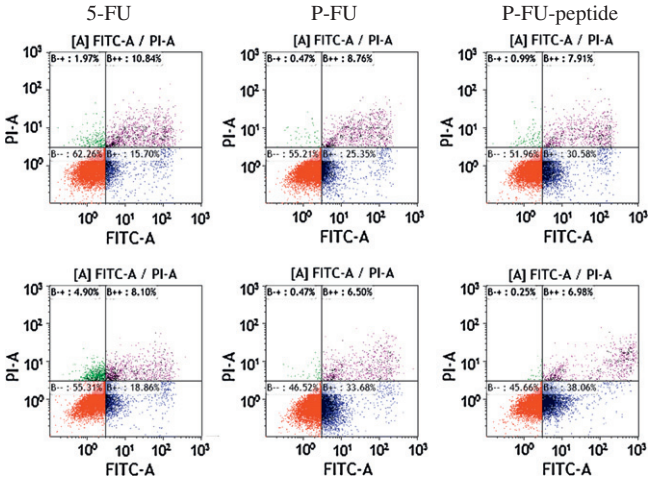

Fig. 6. Time-dependent effect on apoptosis of SCC9 cells by treatment with 5-FU, P-FU-peptide and P-FU at 18.75 μg/mL dose for 12 h and 36 h was determined by flow cytometry analysis. Results of dose-dependent effect are expressed as dot plot of Annexin V-FITC vs. PI, and representative values from three experiments are shown. Dot plot from flow cytometry analysis reveals the four different populations of cells. Top right: necrotic/late apoptotic cells (Annexin V-FITC+/PI+); bottom left: live cells (Annexin V-FITC−/PI−); and bottom right: early apoptotic cells (Annexin V-FITC+/PI−). Cells, incubated for 12 h, were listed at first line while for 36 h at second line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

P-FU-peptide and P-FU-treated cultures while much higher in 5-FU treated culture which was also very close to the GI% measured in cytotoxicity assay. Furthermore, it also showed that treatment with P-FU-peptide after 36 h resulted in more early apoptotic cells and late apoptotic/early necrotic cells than P-FU at same concentration (18.75 μg/mL), which could be attributed to the diffusion and accumulation of 5-FU directly at the site of action causing more necrosis. This phenomenon seemed to explain the results in the GI% measurement in cytotoxicity assay that the P-FU-peptide copolymer with targeting peptide attachment showed greater efficacy, compared to non-targeted copolymer P-FU and 5-FU at lower 5-FU equivalent concentrations.

4. Conclusion

In this study, water-soluble HPMA copolymer–(Hsp47/CBP2 binding peptide)-5-FU conjugates were synthesized and characterized. In comparison with the previously described HPMA copolymer conjugates, the attachment of Hsp47/CBP2 binding peptide had few effect on the content of 5-FU, and thus, a higher drug contents were achieved. Both HPMA copolymer–5-FU conjugates significantly increased the cytotoxicity and improved therapeutic efficacy of free drug. It should be noted that P-FU-peptide exhibited greater ability to inhibit the growth of SCC9 cells *in vitro* than P-FU conjugates, especially at lower concentrations, due to a combination of receptor-mediated process and passive endocytosis. Furthermore, treatment with P-FU-peptide resulted in more apoptotic and early necrotic cells than P-FU or 5-FU, and apoptosis induced by P-FU-peptide was higher than necrosis for prolonging incubation time. In summary, the HPMA copolymer–Hsp47/CBP2 binding peptide conjugates showed a promising future for the treatment of HNSCC with improved efficacy.

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