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Improved Stability and Tumor Targeting of 5-Fluorouracil by Conjugation with Hyaluronan

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ABSTRACT: To circumvent the rapid clearance *in vivo* and consequent low tumor targeting of 5-fluorouracil (5-Fu), hyaluronan-5-fluorouracil conjugate (HA-5-Fu) was firstly synthesized and characterized. The stability of HA-5-Fu *in vitro* was evaluated by incubation with phosphate buffered saline, hyaluronidase solution, and mice plasma, respectively. The tumor targeting was tested by *in vitro* cytotoxicity evaluation and *in vivo* pharmacokinetics study in plasma and tumor. HA-5-Fu with drug loading of 87.674 mg/g was successfully obtained and confirmed. HA-5-Fu showed high stability in acidic environment and moderate stability under enzymatic cleavage. The enhanced cytotoxicity of HA-5-Fu over 5-Fu depended on drug concentration, incubation time, and cell lines type. The $t_{1/2}$ of HA-5-Fu in plasma after injection of prodrug was extended up to 10 times compared with that of 5-Fu. Notably, AUC_{0-t} in tumors of HA-5-Fu was 3.6 times higher than 5-Fu, demonstrating its excellent tumor targeting and quite promising prospect in anti-cancer therapy. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 927–932, 2013

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INTRODUCTION

5-Fluorouracil (5-Fu) was commonly used to treat colorectal, breast, and ovarian carcinoma clinically as an anti-metabolite drug. 5-Fu could inhibit DNA biosynthesis which was accelerated in tumor tissue, via its derivative 5-fluorodeoxyuridine monophosphate (FdUMP). Although it was recommended to be injected intravenously due to low oral bioavailability, the maintenance time of effective drug concentration was still short because of rapid metabolism.[1,2] Moreover, attendant toxic reactions, such as gastrointestinal damage, bone marrow suppression, and sudden cardiac death, greatly restricted its further application.

Prodrug was proposed to provide an effective and practical strategy to improve effectiveness,[3,4] as evidenced by its various derivatives, such as tegafur,[5] carmofur,[6] and capecitabine.[7] As a macromolecule, polymeric prodrug investigated in this work exhibited several other merits.[8] The permeability was greatly changed after conjugation with hydrophilic polymer, hence causing active drug less susceptible to permeate through normal epithelial membrane but more accumulation in tumor tissues due to enhanced permeation and retention effect (EPR) in tumor.[9,10] Moreover, it was convenient to improve conjugate performance by modification with functional molecules on active groups such as carboxyl, hydroxyl, amino,[11] which were abundant on polymer. Furthermore, active drug gradually fell off from polymer backbone because of decelerated hydrolysis and enzymolysis due to stereospecific blockade, resulting in a sustained-release feature.[12,13]

Hyaluronan (HA) is a linear, negatively charged polysaccharide that is consisted of repetitive disaccharides and serves multifunction as pericellular and extracellular matrix.[14] It was reported that HA could target tumor cells by specifically binding to hyaladherin overexpressed on cell surface.[15–17] Herein, HA was used as both carrier material and targeting molecule aiming to increase tumor targeting. As compared with other polymeric prodrug or particle delivery system modified with limited targeting molecules, prodrugs based on HA polymer possessed better targeting benefiting from substantial repetitive units.[18] Meanwhile, time-consuming and labor intensive synthesis processes were avoided.

In this article, we coupled active drug 5-Fu with HA polymer and characterized its stability and tumor targeting, which had not been performed so far. Improved stability was demonstrated by *in vitro* hydrolysis and enzymolysis experiments. Cytotoxicity of HA-5-Fu to tumor cells was also increased examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The pharmacokinetics results obtained on mice

bearing hepatoma cancer further validated prolonged retention in plasma and targeting efficacy to tumor.

EXPERIMENTAL

Materials

5-Fu was purchased from Qilu pharmaceutical Co. Ltd. (Shandong, China). HA (MW ≈ 101 kDa) was purchased from Dali Co. Ltd. (Liuzhou, China). MTT was purchased from Sigma (St. Louis, Missouri). All other chemicals were obtained from commercial sources of analytical grade or better. RPMI-1640 (Roswell Park Memorial Institute) medium and DMEM (Dulbecco's Modified Eagle's Medium) were purchased from Gibco Co. (Carlsbad, California). A2780, HepG2, and H_{22} were kindly provided by the State Key Laboratory of Biotherapy.

Gel permeation chromatography (GPC) was performed by high performance liquid chromatography (HPLC) (Agilent, Santa Clara, California) equipped with Shodex OHPak SB2803HQ column. IR spectra were recorded on Nicolet 20SXB FT-IR spectrophotometer (Thermo Fisher, Waltham, Massachusetts). ^{1}H-NMR spectra were taken on UNITY INOVA 400 NMR spectrometer (Bruker, Billerica, Massachusetts).

All procedures *in vivo* were approved by Sichuan University Animal Ethical Experimentation Committee, in accordance with the requirements of the National Act on the use of experimental animals. Kunming Mice (20 ± 2 g) were provided by the Laboratory Animal Center of Sichuan University.

Synthesis of HA-5-Fu

Activation of 5-Fu by Esterification. 5-Fu (1.10 g, 8.5 mmol) was dissolved in 37% formaldehyde solution (1.53 g, 18.9 mmol) and the mixture was kept at 60°C for 2 h, furnishing product **1** (yield 82%) after concentration under vacuum. To the solution of **1** in anhydrous acetonitrile (10 mL) were successively added succinic anhydride (1.09 g, 10.9 mmol), 4-*N*,*N*-dimethylaminopyridine (0.06 g, 0.48 mmol) and triethylamine (0.71 mL, 5 mmol). The mixture was stirred in oil bath at 50°C for 24 h and concentrated under vacuum. The residue was purified via silica gel column chromatography (eluent: ethyl acetate/petroleum ether = 50/1), affording product **2** (yield 51%). Afterwards, to the solution of **2** in anhydrous acetonitrile (20 mL) were successively added *N*-hydroxysuccinimide (1.32 g, 11.5 mmol), dicyclohexylcarbodiimide (2.37 g, 11.5 mmol), and 4-*N*,*N*-dimethylaminopyridine (0.07 g, 0.575 mmol). The mixture was stirred in ice bath for 1 h followed by at room temperature for additional 24 h, and then filtered. The filtrate was concentrated under vacuum, purified via silica gel column chromatography (eluent: acetone/dichloromethane = 2/1), furnishing product **3** (yield 46.5%). ^{1}H-NMR (400 MHz, D_2O, δ, ppm): 7.648–7.661 (d, 1H, —CH=CF), 5.923 (s, 2H, —OCH_2), 2.784 (s, 2H, —$NCOCH_2$), 2.634–2.667 (t, 2H, —$COCH_2$), 2.542–2.573 (t, 2H, —$COCH_2CH_2$).

Derivatization of HA with Adipic Acid Dihydrazide. Adipic acid dihydrazide (1.73 g, 10 mmol) was added to a solution of HA (0.18 g) in water (45 mL). 1-Ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (0.39 g, 2 mmol) was then added after adjusting the pH of the reaction mixture to 4.75 with HCl solution. The reaction proceeded for 4 h under stirring at the constant pH of 4.75 and quenched by adjusting the pH to 7.0. After filtration, the mixture was successively dialyzed against 100 m*M* NaCl solution (1 × 12 h) and water (3 × 12 h), and final lyophilized to give Product **4** (yield 89%). ^{1}H-NMR (400 MHz, D_2O, δ, ppm): 2.459 (s, 4H, —$COCH_2$), 1.997–2.083 (t, 3H, —$NCOCH_3$), 1.717 (s, 4H, —$COCH_2CH_2$).

Synthesis of HA-5-Fu Conjugate. Product **3** (1.96 g) was added into the solution of product **4** (0.09 g) in dimethyl sulfoxide (20 mL). The reaction mixture was stirred at room temperature for 48 h and successively dialyzed against 50% ethanol/water (2 × 12 h) and water (3 × 12 h). Product **5** was obtained after filtration and lyophilization (yield 91.4%). IR (KBr, cm^{-1}): 3419.52 ($\nu_{—OH}$), 2931.95 ($\nu_{—CH}$), 1742.73 ($\nu_{—C=O}$), 1674.35 ($\nu_{—C=O}$), 1560.89 ($\delta_{—NHC=O}$), 1467.16 ($\delta_{—CH2}$), 1369.67 ($\delta_{—CH}$), 1267.69 ($\nu_{—C—CF}$), 1207.20 (νas$_{—C—O—C}$), 1144.08 (νs$_{—C—O—C}$), 1048.94 ($\nu_{—C—OH}$), 764.35 ($\gamma_{=C—H}$). ^{1}H-NMR (400 MHz, D_2O, δ, ppm): 8.023–8.056 (d, 1H, —CH=CF), 5.999 (s, 2H, —OCH_2), 2.487 (s, 4H, —$COCH_2$), 2.040 (t, 3H, —$NCOCH_3$).

HPLC Analysis of 5-Fu

5-Fu level was determined by HPLC system equipped with Kromasil C18-ODS column throughout the study. The detector was set at 266 nm corresponding to the maximum absorbance for 5-Fu. For drug loading and stability experiment, the mobile phase composed of methanol/water (95/5) was driven at a flow rate of 0.8 mL/min at 30 °C. Slightly differently, *in vivo* study, mobile phase was composed of 1/99 methanol/4 m*M* KH_2PO_4 (containing 0.1% triethylamine) and pH was adjusted to 5.4 with phosphoric acid. The flow rate and column temperature were both down-regulated to 0.6 mL/min and 25°C, respectively.

Drug Loading of HA-5-Fu

A homogeneous mixture of HA-5-Fu solution (1 mL, 0.05%) and NaOH (0.1 mL, 2*M*) was shaken at 40°C for 30 min and then neutralized. The solution was diluted to 0.025%, and then filtered and centrifuged at 8000 rpm for 20 min. 5-Fu content in the supernatant (20 μL) was analyzed by HPLC as described previously.

In Vitro Stability of HA-5-Fu

Hydrolysis Stability in PBS. HA-5-Fu (0.005 g) was dissolved in 50 m*M* phosphate buffer solution (PBS, 10 mL) at different pH of 2.4, 4.0, 6.0, 7.0, and 8.0, respectively. The solutions were incubated at 37°C with mild stirring. Samples (1 mL) were withdrawn at established time intervals and centrifuged at 8000 rpm for 10 min. The supernatant (20 μL) was used to determine the amount of released 5-Fu by HPLC analysis.

Enzymolysis Stability in HAase Solution and Plasma. The reaction solution was prepared by mixing 1 mL HA-5-Fu (0.5%) with isovolumetric hyaluronidase solution (HAase, 30.15 U/mL) and then diluted to 10 mL. All other procedures were the same as hydrolysis stability experiment expect for an elevated centrifugation speed of 12,000 rpm. For enzymolysis in plasma, 25 mg of HA-5-Fu was dissolved in 10 mL of 30% (vol/vol) mice plasma. About 800 μL of methanol was added to the samples (200 μL) withdrawn in predetermined intervals during the incubation as mentioned above. These solutions were also centrifuged at 12,000 rpm and analyzed by HPLC. All solvents used in this section were PBS of pH 7.4.

In Vitro Antitumor Activity

All cells were cultured in RPMI 1640 or DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C under a humidified atmosphere with 5% CO_2.

In vitro cytotoxicity of 5-Fu or HA-5-Fu was determined by MTT assay at incremental incubation times, that was 12, 24, 48, and 72 h. Briefly, the cells were seeded in 96-well plates at 10,000 cells/well. After 24 h, the cells were treated with 5-Fu or HA-5-Fu (refer to free 5-Fu component) at indicated concentrations (0.1188, 23.755, 95.02 μg/mL) for different time and then 20 μL MTT (0.5%). After incubation for 4 h, the MTT solution was removed and the crystals of viable cells were dissolved in 150 μL DMSO. The absorbance at 570 nm was measured on an ELISA plate reader (Bio-Rad, Microplate Reader 550). The inhibition rate (IR) was calculated as follows:

$$\mathrm{IR}(\%) = [1 - (C_e - C_0)/(C_c - C_0)] \times 100$$

where C_e, C_c, and C_o are the absorption value of the treatment group, control group and blank, respectively.

In Vivo Study

Biological Sample Extraction. Plasma or tumor tissue homogenates (0.5 mL) was mixed with 50 μL 5-bromouracil solution (102.0 μg/mL) as the internal standard and 50 μL NaOH (2*M*). The mixture was hydrolyzed at 40°C for 30 min and then neutralized with HCl (2*M*). Subsequently, the mixture was extracted with 6 mL ethyl acetate and then centrifuged for 10 min at 4000 rpm. After deproteinization, the supernatant was evaporated to dryness at 50°C. The residues were redissolved in 200 μL mobile phase, followed by centrifugation at 12,000 rpm for 10 min. Then 20 μL of the clear supernatant was injected into the HPLC system.

Pharmacokinetics and Tumor-Targeting. Hepatoma model was established by subcutaneous injection of 0.2 mL H_{22} cell suspension (1×10^6) into the right flank of each mouse and subsequent feeding until tumor volume achieved 1 cm^3. Tumor-bearing mice were injected with PBS solutions (pH = 7.4) containing 5-Fu or HA-5-Fu at a dose equivalent to 24 mg/kg body weight via the tail vein. The mice were sacrificed at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 12 h after injection. Blood samples were collected from ocular artery by eyeball removal and placed in tubes coated with heparin followed by

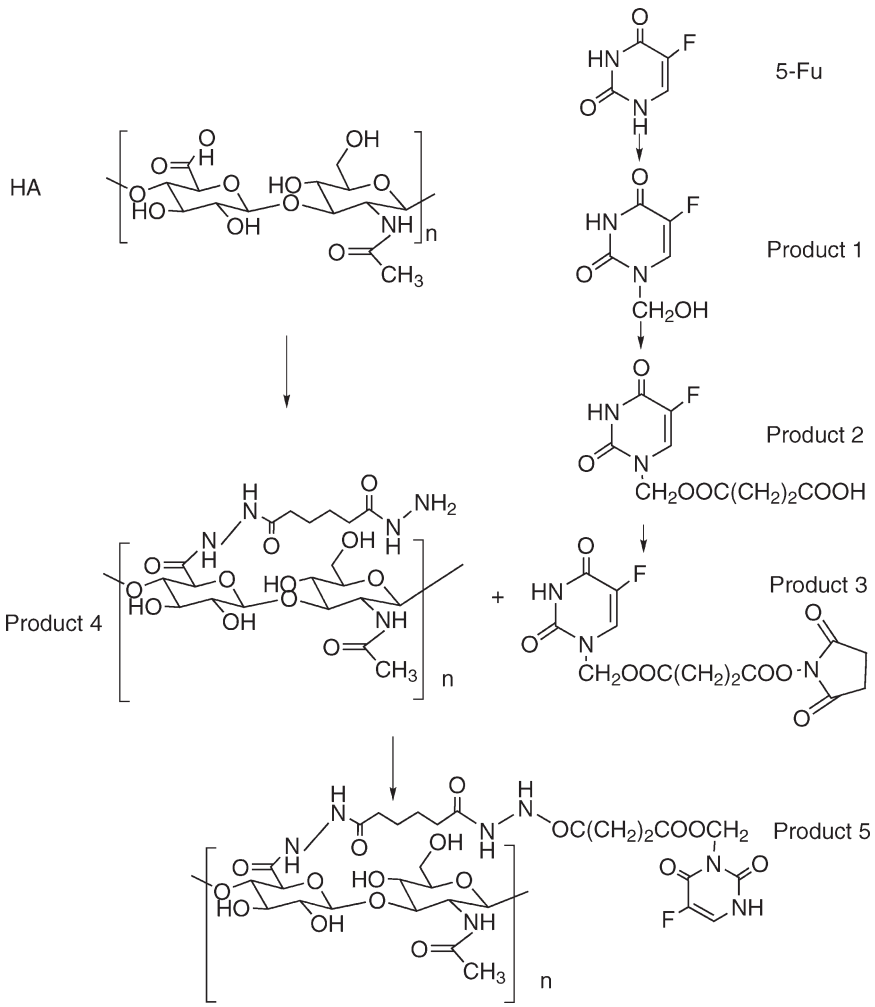

Figure 1. Schematic presentation of synthesis of HA-5-Fu conjugate.

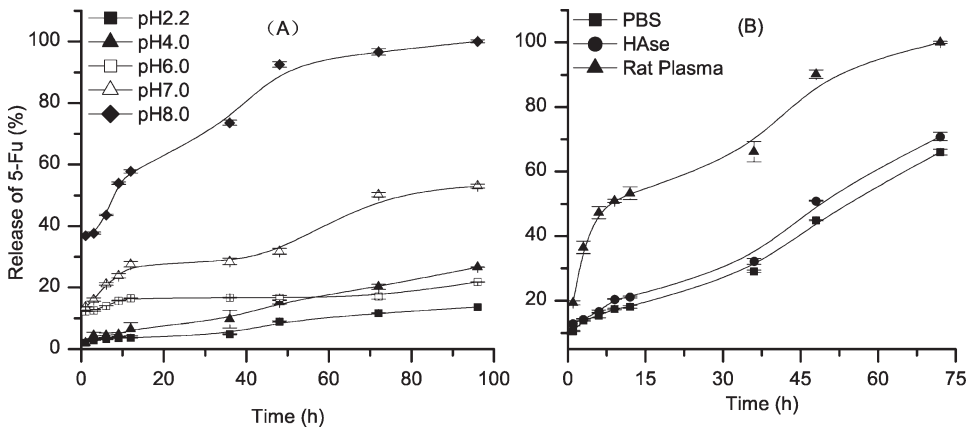

Figure 2. Release profiles of 5-Fu from HA-5-Fu in a serial of PBS of various pH values (A), PBS of pH 7.4, HAase solution, and plasma (B). Results were presented as means ± SD ($n = 3$).

immediate centrifugation at 8000 rpm for 10 min. Tumor tissue samples were harvested, washed, weighed, homogenized, and then diluted with saline to 1 : 2 (g/mL). All samples were stored at −20°C. HPLC was used to determine 5-Fu levels after extraction processes.

Data Analysis

Statistical evaluation was performed using student *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Synthesis and Confirmation of HA-5-Fu

HA-5-Fu was synthesized (Figure 1) by using 4-dimethylaminopyridine as the catalyst and dicyclohexylcarbodiimide as the condensing agent, and purified by dialysis to separate from free 5-Fu. GPC analysis showed that the molecular weight increased from 101 kDa to 136 kDa (molecular dispersity 1.65) indicating the successful grafted linkage of 5-Fu. Comparing to HA and 5-Fu, there were new absorption peaks at 1742.73 cm^{-1} ($\nu_{-C=O}$), 1560.89 cm^{-1} ($\delta_{-NHC=O}$) in the IR spectrum of HA-5-Fu. ^{1}H-NMR spectra of HA-5-Fu demonstrated that the peaks at 8.0 ppm belongs to —CH=CF. In conclusion, these results confirmed that 5-Fu was chemically conjugated to HA.

Drug loading of 5-Fu was determined by HPLC after complete alkaline hydrolysis. The retention time of 5-Fu in hydrolyzed prodrug was 3.75 min, which was similar to free 5-Fu, and as expected, no absorption appeared in unhydrolyzed prodrug. It was calculated that the drug loading of 5-Fu was 87.674 mg/g.

Stability of HA-5-Fu Conjugate

Figure 2 shows the cumulative release profiles of 5-Fu after incubation of HA-5-Fu with a serial of PBS. The prodrug appeared to be highly stable in pH 2.2, 4.0, and 6.0 buffer solutions, evidenced by maximum accumulative 5-Fu release less than 25% in 96 h. The degradation speeded up with the rise of pH, and was almost complete in 48 h at pH 8.0. This could be explained as enhanced hydrolytic activity of ester bond and facilitated rupture of hydrogen bond owing to higher OH^- concentration.[19,20]

The enzymolysis stability of HA-5-Fu was determined by monitoring its kinetics of degradation in HAase solution and plasma, as shown in Figure 2. Comparing to PBS, the release rate of

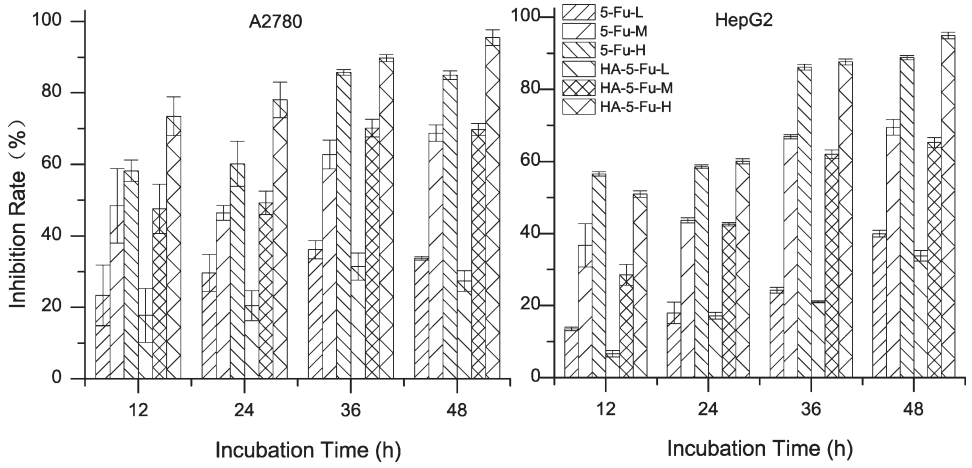

Figure 3. Effect of drug concentration and incubation time on cytotoxicity of 5-Fu and HA-5-Fu against A2780 (left histogram) and HepG2 (right histogram). Results were presented as means ± SD ($n = 3$).

Table I. The IC_{50} Values of 5-Fu and HA-5-Fu to Different Tumor Cells After Incubation for 48 h

	IC_{50} (μg/mL)		
Cell	5-Fu	HA-5-Fu	Ratio
A2780	35.270	27.353	1.290
HepG2	40.202	36.921	1.089

5-Fu from conjugate was slightly improved in HAase solution due to the shielding effect of side chains on HA substrate. Accordingly, integrated HA backbone enwrapped 5-Fu, resulting in similar release characteristics in HAase solution to that in PBS. Parent drug release rate in rat plasma was about 55% during the initial burst release on first 12 h, and rose to approximate 100% at 72 h due to synergistic effects of esterase and amidase in plasma. The release profile of HA-5-Fu in plasma also presented sustained-release property and fitted to first-order kinetics with $t_{1/2}$ of 10.09 h, predicting the stability of HA-5-Fu in blood circulation to some extent.

Cytotoxicity of HA-5-Fu to Cancer Cells

HA-5-Fu was evaluated for its anti-tumor activity against A2780 and HepG2 by MTT assay, and 5-Fu was employed as a reference drug (Figure 3). Conclusively, the cytotoxicity of HA-5-Fu and 5-Fu depended on drug concentration, incubation time, and cell type. Seemingly a paradox, 5-Fu was more effective than that of HA-5-Fu at low concentration and in a short incubation time. This was because HA-5-Fu acted as an anti-tumor agent only after undergoing a time-consuming degradation phase, hence the onset time was later.[21] With the increases of concentration and incubation time, the validity of HA-5-Fu approached to that of free 5-Fu gradually, and eventually exceeded.

Once treated with high concentration of HA-5-Fu or 5-Fu, we could conclude from Figure 3 that the prodrug was more cytotoxic than the free drug, which might be because of the enhanced active endocytosis mediated by the interaction between HA and hyaladherin. Additionally, improved stability in carcinoma cells and extracellular matrix where acidification aggravated was also achieved by extending duration time of 5-Fu. Besides, different cell types displayed various susceptibilities to HA-5-Fu (Table I), as listed below: A2780> HepG2, and differential expression of hyaladherin[18] and activating enzyme of 5-Fu[22] might account for the jagged results.

Table II. Pharmacokinetic Parameters of 5-Fu and HA-5-Fu in Plasma and Tumor

	Plasma		Tumor	
Parameter	5-Fu	HA-5-Fu	5-Fu	HA-5-Fu
AUC_{0-t} (mg/L min)	530.022	12,625.287	854.851	3088.937
$T_{1/2}$ (min)	44.125	435.137	28.914	211.334
MRT (min)	45.332	151.480	49.794	96.631
CL (L/min/kg)	0.035	0.001	0.025	0.005

In Vivo Study

The pharmacokinetic parameters in plasma of HA-5-Fu and free 5-Fu were shown in Table II. The area under the curve (AUC_{0-12}) of 5-Fu released from HA-5-Fu was 12,625.287 mg/L min, approximately 24-fold more than that of free 5-Fu. Also, the $t_{1/2}$ and mean retention time (MRT) of 5-Fu released from prodrug was 435.137 min and 151.480 min, significantly longer than that of the free drug.[2] The pharmacokinetic results were coincident to intensified stability of 5-Fu after combination with HA polymer described previously. The prolonged circulation time of 5-Fu and intrinsic macromolecular properties were beneficial to the accumulation of HA-5-Fu in tumor tissue via EPR effect.

The acidic intracellular and extracellular environment lowered the degradation of HA-5-Fu to a certain point, ensuring longer contact time. As expected, the AUC_{0-t}, $t_{1/2}$, MRT of the prodrug in tumor tissue were increased by 2.6, 6.3, and 0.9 times of those of 5-Fu, and clearance (CL) reduced by five times reasonably (also shown in Table II), demonstrating tumor targeting.

CONCLUSIONS

Adipic acid dihydrazide and succinic anhydride were used as the linkages to connect 5-Fu onto multifunctional HA polymer with high loading successfully. The polymeric prodrug presented high stability in acidic environment, and moderate stability in HAase solution and plasma. In addition to enhanced acid stability, the intrinsic active tumor targeting of HA mediated by its receptors overexpressed on tumor cell surface strengthened the anti-proliferative effect of HA-5-Fu, evidenced by reduced IC_{50} on different cancer cell lines. *In vivo* behavior of HA-5-Fu further verified extended circulation time and tumor targeting from several key parameters, such as AUC_{0-t}, $t_{1/2}$, MRT and CL. All results presented above concerning synthesis and *in vitro* and *in vivo* behaviors of HA-5-Fu were in consistent with expected consequences. Although further studies concerning tumor growth curve and mice survival curve were needed, this preliminary study provided a promising anti-cancer prodrug for targeted therapy.

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