Med Chem Res (2007) 16:370–379
DOI 10.1007/s00044-007-9049-0

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and evaluation of the 5-fluorouracil-pectin conjugate targeted at the colon

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Received: 2 April 2007 / Accepted: 5 November 2007 / Published online: 5 December 2007

Abstract This paper describes the first covalent synthesis of a 5-fluorouracil-pectin (FU-PT) conjugate. This conjugate with the potential for colon-targeted delivery has been evaluated. A sensitive high-performance liquid chromatography (HPLC) method was established for the determination of concentration of 5-FU-1-acetic acid in the gastrointestinal contents and plasma of rats. This method was also used to evaluate the colon-targeting properties of 5-FU-PT. 5-FU-PT was given to rats by oral administration at a dosage of 22.5 mg kg^{-1}. The different parts of gastrointestinal tract and plasma were taken after 1, 3, 5, 7, 9, 12, 24, 36, and 48 h of oral administration of 5-FU-PT to rats, and the concentration of 5-FU-1-acetic acid in gastrointestinal contents and plasma was measured by HPLC. 5-FU-1-acetic acid released from 5-FU-PT was mainly distributed in the cecum and colon. Therefore, the present results suggest that the 5-FU-PT conjugate has a good colon-targeting property.

Keywords 5-Fluorouracil · Pectin · Colon-specific delivery system

Abbreviations

FU	fluorouracil
PT	pectin
HPLC	high-performance liquid chromatography
DE	degree of esterification
DCC	dicyclohexylcarbodiimide
DMAP	dimethylaminopyridine

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DMSO	dimethylsulfoxide
DS	degree of substitution
TGA	thermogravimetric analysis
DSC	differential scanning calorimeter
PSI	proximal small intestine
MSI	middle small intestine
DSI	distal small intestine

Introduction

Colorectal cancer is the second or third most common malignancy, after breast cancer in women and after lung and prostate cancers in men, in developed countries (Kemeny, 1996). While chemotherapy in patients with advanced colorectal cancer has proven to be of only marginal benefit, 5-fluorouracil (5-FU) is the most effective treatment for this disease. 5-FU is useful alone or in combination with other drugs, but is associated with only a 15–20% likelihood of reducing measurable tumor mass by 50% or more (Piedbois *et al.*, 1992). Part of the reason for this is that the anticancer drug cannot reach an effective concentration in the colorectal area.

Several attempts have been made to increase effective concentration and reduce toxicity via oral colon-specific delivery systems (Huang *et al.*, 2001; Shantha *et al.*, 1995). Precise colonic drug delivery requires that the triggering mechanism in the delivery system only responds to the physiological conditions particular to the colon. To improve site specificity and versatile drug release kinetics to accommodate different therapeutic needs, certain types of polysaccharides can be used as carriers or to create the dosage forms. Pectins are nonstarch linear polysaccharides extracted from plant cell walls. They are intact in the upper gastrointestinal tract and are degraded by colonic microflora (Sinha and Kumria, 2001). Apart from the fact that pectin-derived drug carriers provide promising potential for colon-specific drug delivery (Sinha and Kumria, 2001), pectins also express extensive activity. Modified citrus pectin, given orally, inhibits carbohydrate-mediated tumor growth, angiogenesis, and metastasis (in vivo), presumably via its effects on galectin-3 function. These experiments stress the importance of pectin as agents for the prevention and/or treatment of cancer (Nangia-Makker *et al.*, 2002).

In the past years, many derivatives of 5-FU have been synthesized, among which glycosides are considered important (Ohya *et al.*, 1991). 5-FU-1-acetic acid, a derivative of 5-FU, which was used as an active fraction to be linked to saccharides, showed good inhibition of cancer cell growth rate (Yu, 2005; Lu *et al.*, 2004).

In the present study, an acetyl spacer was used to couple 5-FU covalently onto pectin. We first synthesized 5-FU-1-acetic acid, and then produced an ester from 5-FU-1-acetic acid and pectin. An HPLC method was described and validated to determine the 5-FU loading onto the pectin carrier. This pectin carrier, through its own intrinsic anticancer activity, is expected to act not only as a drug delivery agent, but also as an anticancer agent. The characteristic of the conjugates for colon targeting was investigated in vitro, using conditions chosen to simulate the pH and

time likely to be encountered during transit to the colon. The simulated test suggested that the conjugate might have a colon-targeting property. Finally, we investigated the release of the 5-FU-pectin conjugate in rats in vivo.

Experimental section

Materials

5-FU was supplied from Nan-tong Jing-hua Pharm. Corp., Ltd. (Nantong, China). Pectin (with DE 65%) was obtained from He-nan Fu-da Pectin Industry Corp., Ltd. (Xinxiang, China). Dicyclohexylcarbodiimide (DCC) was purchased from Nanjing Tianzun Chem. Corp., Ltd. Dimethylaminopyridine (DMAP) was supplied from Sinopharm Chemical Reagent Co., Ltd. Dimethylsulfoxide (DMSO) was dried over a 4A molecular sieve and distilled before use. Methanol and triethylamine were of HPLC grade. All other chemicals and solvents were of analytical reagent grade.

Animals

Male Sprague–Dawley (SD) rats were purchased from the Experimental Animal Center of the Fourth Military Medical University and the animals used in the experiment were approved by the institutional ethical committee of the Fourth Military Medical University. The rats were given standard food and water per day under controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%).

Instruments

Melting points were determined using XRC-1 micromelting point apparatus without correction. Infrared (IR) spectra were obtained with an AVATAR360 Fourier transform (FT)-IR spectrometer (Thermo, USA). Thermal analysis was performed by using a simultaneous thermal analyzer (SDT-Q600, TA Instruments Inc., USA) at a heating rate of 10°C/min with an open pan system from 40°C to 450°C referenced to alumina, both in a stream of N_2 gas. The HPLC system consisted of pumps, detector, and autoinjector from Waters.

Synthesis of 5-FU-pectin conjugates

5-FU-1-acetic acid was prepared by 5-FU reacting with chloroacetic acid giving an 82% yield (Masao, 1975), m.p. 278–279°C (Ref. 277–279°C). DCC (2.19 g, 10.6 mmol), DMAP (0.26 g, 2.12 mmol), and 5-FU-1-acetic acid (1 g, 5.3 mmol) were added to an anhydrous DMSO solution (20 mL) of pectin (0.5 g). After the mixture was stirred at 50°C for 96 hours, anhydrous ethanol was added, then

filtered. The precipitate was dissolved in anhydrous DMSO again, and anhydrous ethanol was added, then it was filtered. This process was repeated two times. The precipitate was dried at 60°C for 24 hours to yield 0.95 g 5-FU-PT. Fig 1

IR spectrum (KBr, cm^{-1}): 3439 (OH), 1706(C = O), 1147 ~ 1025(glucoside-O-).

Analytical methods

5-FU and 5-FU-1-acetic acid were measured by reverse-phase HPLC with an RP C-18 column (15 cm × 4.6 mm; particle size 5 μm). The mobile phase composition for the separation of 5-FU and 5-FU-1-acetic acid and DS was 95% water (0.15% triethylamine and 0.15% acetic acid) and 5% methanol. The flow rate was 1 mL/min with detection at 275 nm. In this HPLC method, the retention times of 5-FU and 5-FU-1-acetic acid were about 5.4 minutes and 6.5 minutes, respectively.

Degree of substitution

The degree of substitution (DS) was defined as the ratio of the number of hydroxyl groups of 5-FU-1-acetic acid bound among all hydroxyl groups of pectin. It was determined by measuring the amount of 5-FU-1-acetic acid by HPLC at 275 nm, which was released when 10 mg 5-FU-pectin was placed in 15 mL NaOH solution (2 mol/L) for one hour at 40°C. We also determined in parallel the amount of 5-FU-1-acetic acid that was released when 10 mg 5-FU-PT was placed in 15 mL water solution for one hour at 40°C. All of the hydrolysis solutions were adjusted to pH 7 with HCl solution (2 mol/L).

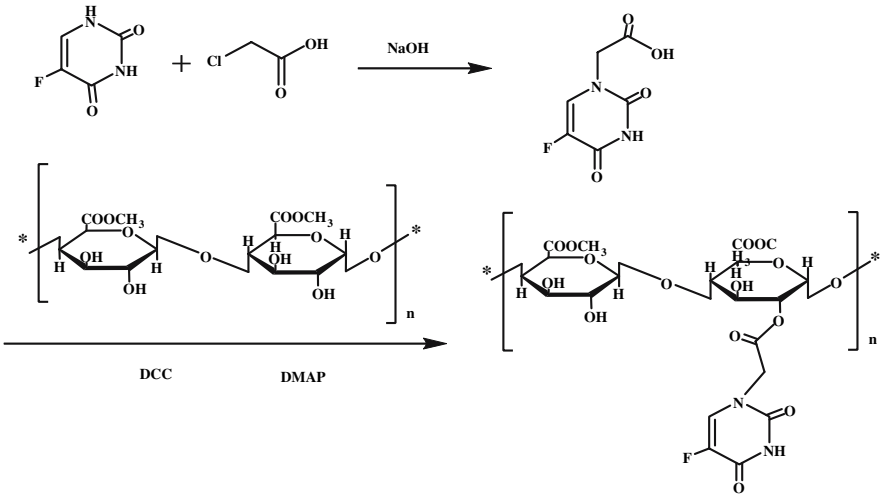

Fig. 1 Synthesis of 5-FU-pectin

Chemical stability

5-FU-PT (2 mg) was placed in solutions of pH 1.5, 4.9, 5.5, 6.8 ,and 7.4 buffer and pH 5.5 pectinase buffer, agitated, and reacted for 12 hours in a water bath at 37°C. Reaction mixture 0.1 mL and mobile phase 0.9 mL were vortexed for two minutes, centrifuged at 13,000 g for five minutes, and the amount of 5-FU-1-acetic acid in the supernatant was analyzed by HPLC.

In vivo drug release

The rats in this study were assigned randomly to ten groups, with eight rats in each. Each group corresponded to a time point. 5-FU-PT was suspended in water at a concentration of 9 mg/mL for intragastric administration. Whole blood was sampled from the abdominal aorta of ether-anesthetized rats at 1, 2, 3, 5, 7, 9, 12, 24, 36, and 48 hours after oral administration, respectively. Meanwhile, the contents were taken from the stomach, proximal small intestine (PSI), middle small intestine (MSI), distal small intestine (DSI), cecum, and colon at each time point.

The whole blood was centrifuged at 1,170 g for five minutes. One milliliter of supernatant plasma was taken, added to 4 mL ethyl acetate, agitated for two minutes, and centrifuged at 1,170 g for five minutes. The supernatant was dried under a stream of nitrogen and redissolved in 150 μL of mobile phase, 20 μL of which was subjected to HPLC analysis of 5-FU and 5-FU-1-acetic acid. The contents were homogenized in saline with a ratio of 1:10 (g/mL) using a mechanical homogenizer. The homogenate was filtered by microporous membrane (0.25 μm), and the filtrate was subjected to HPLC analysis.

Statistical analysis

Considering the inequality of variances between groups, we adopted analysis of variance (ANOVA) to analyze the average differences of the 5-FU-1-acetic acid concentrations in the different groups.

Results and discussion

Preparation of 5-FU-PT conjugate

Characterization of 5-FU-PT conjugate

5-FU-PT conjugate was prepared following the method mentioned in the experimental section. HPLC analysis showed that there was no free 5-FU-1-acetic acid in 5-FU-PT. The IR analysis indicated that the carboxyl group of 5-FU-1-acetic acid is covalently bound to one of hydroxyl groups of PT through an ester bond. Pectin has an exothermic peak at 252.29°C (Fig. 2a), 5-FU-1-acetic acid has two

endothermic peaks at 277.01°C and 341.75°C (Fig. 2b), the compounds of pectin and 5-FU-1-acetic acid have an exothermic peak at 277.05°C and an endothermic peak at 335.99°C (Fig. 2d), while 5-FU-PT only has a stronger exothermic peak at 255.41°C (Fig. 2c). All this confirms that a new substance was obtained.

Optimization of the reaction conditions

In the synthesis of 5-FU-PT conjugate, 4-dimethylaminopyridine was used as an acyl transfer catalyst, and DCC was used as a condensing agent. We obtained high yields with the excess of 5-FU-1-acetic acid and DCC. Adjusting the ratio of pectin, 5-FU-1-acetic acid and DCC varied the DS and the results are listed in Table 1. To obtain a higher DS, we also reviewed the effects of time and temperature. DS became higher with increases in time and temperature. After such comprehensive consideration, we chose a better reaction condition and obtained a conjugate with 34% DS. The 5-FU-PT conjugate was readily purified in high yields (greater than 90%). To avoid 5-FU-1-acetic acid enwrapped in PT, we determined the concentration of 5-FU-1-acetic acid in a water solution of 5-FU-PT.

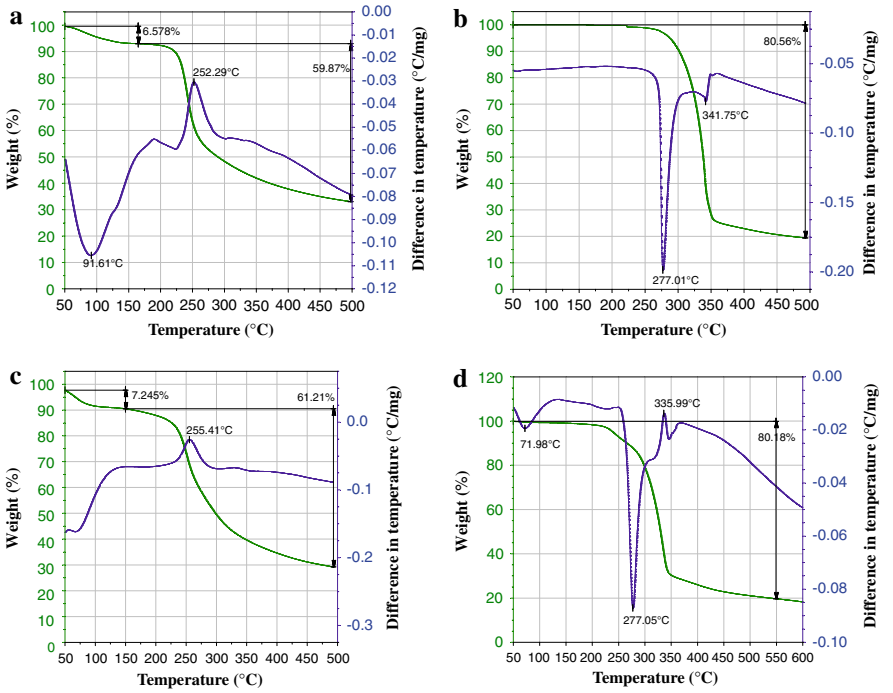

Fig. 2 TGA curve and DSC thermograms of samples of: **(a)** pectin, **(b)** 5-FU-1-acetic acid, **(c)** 5-FU-PT, and **(d)** the compound of pectin and 5-FU-1-acetic acid (1:1)

Table 1 Results of optimization experiment of the reaction condition

Experiment	Temperature (°C)	Time (hours)	Quality ratio of pectin and 5-FU-1-acetic acid	Molar ratio of DCC and 5-FU-1-acetic acid	DS (%)
1	30	48	1 : 2	2	22.6
2	30	72	1 : 3	3	27.4
3	30	96	1 : 4	4	38.5
4	40	48	1 : 3	4	31.7
5	40	72	1 : 4	2	34.7
6	40	96	1 : 2	3	41.2
7	50	48	1 : 4	3	27.6
8	50	72	1 : 2	4	28.8
9	50	96	1 : 3	2	44.3

Analytical methods

In the present study, the detection method for determining the exact amount of enzyme-dependant colon-targeting 5-FU-PT conjugate in rats and its colon-targeting property were explored. The findings suggest that the detection method adopted in this study is precise and reliable so that it can meet the needs of detection in vivo.

Chemical stability

It was noted that no free 5-FU and 5-FU-1-acetid acid were detected in the different buffers, which suggested that the conjugate was chemically stable at pH 1.5, 6.8, and 7.5 (Table 2). In the buffer with lower pH, little 5-FU-1-acetic acid was released within the incubation time. With increasing pH and incubation time, the release rate of 5-FU-1-acetic acid increased. During incubation, 98.9% 5-FU-1-acetic acid was released from the conjugate in nine hours in simulated pectinolytic enzymes fluid. 5-FU was not detected in another buffer or in simulated pectinolytic enzymes fluid.

In vivo drug release

5-FU-1-acetic acid was not detected in the contents of the stomach, proximal small intestine or middle small intestine, while in the contents of cecum and colon 5-FU-1-acetic acid was determined to be present at a high concentration (Table 3).

In the blood samples, 5-FU-1-acetic acid was not found. This may be accounted for as follows: (1) because it is macromolecular substance, 5-FU-PT cannot be absorbed in small intestine. Only a little 5-FU-1-acetic acid can be released from 5-FU-PT in DSI, so the amount of 5-FU-1-acetic acid absorbed is below the detection limit of the HPLC method; (2) absorption in colon is slower than in small intestine,

Table 2 Percentage cumulative amounts of 5-FU-1-acetic acid released from different simulated gastrointestinal fluids and pectinolytic enzymes fluid

Time (hours)	Simulated gastrointestinal fluids (pH)					Pectinolytic enzyme fluid, pH = 5.5
	1.5	4.9	5.5	6.8	7.4	
0.5	0. 048 ± 0.010	—	—	—	—	—
1	0. 089 ± 0.012	0.070 ± 0.012	0.182 ± 0.007	0.724 ± 0.127	1.528 ± 0.230	0.36 ± 0.08
2	0.101 ± 0.001	0.089 ± 0.027	0.267 ± 0.040	0.918 ± 0.037	1.993 ± 0.107	40.19 ± 1.12
3	0.119 ± 0.027	0.127 ± 0.0136	0.316 ± 0.019	1.548 ± 0.329	3.384 ± 0.658	48.32 ± 1.54
5	0.165 ± 0.010	0.199 ± 0.074	0.427 ± 0.102	2.381 ± 0.467	5.641 ± 0.974	60.34 ± 1.84
7	0.175 ± 0.047	0.239 ± 0.038	0.697 ± 0.067	2.734 ± 0.142	5.994 ± 0.066	94.82 ± 2.01
9	0.237 ± 0.025	0.287 ± 0.044	0.746 ± 0.087	3.942 ± 0.434	6.403 ± 0.181	98.89 ± 2.45
12	—	0.399 ± 0.061	0.828 ± 0.054	4.874 ± 0.113	8.199 ± 0.224	—

Mean ± standard deviation (SD), $n = 5$, — not detected

Table 3 Concentration of 5-FU-1-acetic acid in serum and contents of different parts of the rat GI tract after oral administration of 5-FU-PT

Time (hours)	Concentration ($\mu g\ mL^{-1}$)						
	Plasma	Stomach	PSI	MSI	DSI	Cecum	Colon
1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0
5	0	0	0	0	3.66 ± 0.84▲	15.41 ± 4.90*	16.79 ± 4.78*
7	0	0	0	0	8.06 ± 5.72▲	47.41 ± 10.59*	30.58 ± 8.45*
9	0	—	0	0	12.27 ± 11.90▲	25.16 ± 20.19*	57.57 ± 21.78*
12	0	—	0	0	0	39.13 ± 23.21*	43.59 ± 14.06*
24	0	—	0	0	0	1.58 ± 0.81*	2.94 ± 2.71*
36	0	—	—	—	—	0	0
48	0	—	—	—	—	0	0

Mean $\pm$ standard deviation (SD), $n = 8$

▲ only detected in the DSI contents of several rats

* $P < 0.01$ versus the concentration of 5-FU-1-acetic acid in DSI

because the effective absorbing surface area in the colon is smaller. Although there was lots of 5-FU-1-acetic acid in the contents of cecum and colon, only a small amount can enter the blood. So we adopted ethyl acetate to extract the maximum amount of 5-FU-1-acetic acid from the blood sample.

5-FU was not detected in all the biological samples, which shows that 5-FU-PT mainly releases 5-FU-1-acetic acid in colon.

Conclusions

In this study, pectin was used as a carrier. Through the optimization of the reaction conditions, we obtained 5-FU-PT with a higher DS. Our data demonstrate that pectin conjugation of 5-FU-1-acetic acid can reduce systemic absorption and effectively deliver 5-FU-1-acetic acid to the colon. On the basis of experiments in vivo, 5-FU-PT could survive passage through stomach and small intestine, to reach colon and be degraded by enzymes of the colonic microflora. The low concentration of 5-FU-1-acetic acid in blood shows that 5-FU-PT can accumulate in colon to reach an effective concentration.

Acknowledgements The authors gratefully acknowledge Professors Shen-Yong Zhang and Xiao-Li Sun for their laboratory assistance. This work was financially supported by PrimaNova BioSciences, Inc.

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