Full Paper

Synthesis and Bioevaluation of 5-Fluorouracil Derivatives

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Abstract: A series of six novel 5-fluorouracil derivatives **1-6** were synthesized and their structures confirmed by ^{1}H- and ^{13}C-NMR, MS and elemental analysis. The preliminary *in vitro* antitumor activities against B16, K562 and CHO cells and the *in vivo* inhibitions of liver cancer H_{22} demonstrated that some of these compounds effectively inhibit the growth of tumor cells, but the *in vivo* trials in mice revealed that the compounds also exhibited serious liver and lung tissue toxicity. The hydrolysis experiments indicated that this type of compound did not readily liberate 5-fluorouracil, as expected.

Keywords: 5-Fluorouracil, prodrug, synthesis, bioevaluation

Introduction

5-Fluorouracil (5-Fu, **7**), first synthesized in 1957 [1], is one of the antitumor agents frequently used for treating solid tumors such as breast, colorectal and gastric cancers [2]. 5-Fu is poorly tumor selective, and therefore its therapeutic use results in high incidences of bone marrow, gastrointestinal

tract and central nervous system toxicity. To tackle these problems, numerous modifications of the 5-Fu structure have been performed. Thus, a series of 5-Fu prodrugs in which 5-Fu is attached to amino acids, peptides, phospholipids, and polymers have been reported [3-7]. These *N*-1 and/or *N*-3 substituted derivatives have exhibited improved pharmacological and pharmacokinetic properties, including increased bioactivity, selectivity, metabolic stability, absorption and lower toxicity.

Recently, the concept of mutual prodrugs in which two different antineoplastic agents are coupled directly or by means of a spacer has become well accepted [8-17]. This technique can be used to overcome many problems including poor solubility or absorption, patient acceptability, drug instability and toxicity, and especially drug resistance [11]. After the entry of a mutual prodrug into a cancer cell, the two active components can reach a target simultaneously and are liberated concomitantly, whereas they might be transported to the same site with different efficacy when administered individually. This kind of prodrugs can display a broader antitumor spectrum, less drug resistance and less toxicity [18].

Many DNA intercalators have been evaluated as antineoplastic agents in chemotherapy, where they constitute one of the most important drug classes. These agents are characterized by the presence of flat chromophores bearing π electron-deficient polyconjugated plane bound to polar groups. Their antitumor action results from DNA distortion and altered nuclear protein interaction as a consequence of reversible complex formation. Because the cellular target of 5-Fu is DNA, the combination of 5-Fu and some antineoplastic DNA binders through an ester bond [8, 14] helps the resulting dual prodrug reach the target and optimize the efficiency of both 5-Fu and the DNA binders. According to this strategy, we prepared six novel 5-fluorouracil derivatives and evaluated their antitumor activity both *in vitro* and *in vivo*.

Results and Discussion

Chemistry

The synthetic route towards the target compounds **1-6** is shown in Scheme 1. 5-Fu was reacted with formaldehyde to form a mixture of N^1-hydroxymethylene-5-fluorouracil, N^3-hydroxymethylene-5-fluorouracil and N^1,N^3-dihydroxymethylene-5-fluorouracil. Without separation, the mixture was directly coupled with the appropriate carboxylic acids to give the target compounds. *N,N'*-dicyclo-hexylcarbodiimide (DCC) was the first reagent chosen to facilitate the esterification, but the resulting 1,3-dicyclohexylurea (DCU) byproduct was hard to remove by flash chromatograph and recrystallization. On the other hand, when *N,N'*-diisopropylcarbodiimide (DIC) was used, the target compounds could be purified through flash chromatograph and recrystallization from acetone.

Two kinds of drugs with flat structures were attached to 5-Fu. First, nonsteroidal antiinflammatory drugs such as naproxen and biphenylacetic acid, which have been reported to inhibit the growth of cancer cells. In addition, naproxen and biphenylacetic acid can bind with DNA [19]. Second, imides, which also exhibit efficient DNA-binding properties and antitumor activities on a variety of murine and human tumor cells [20]. The imides were first converted to the corresponding carboxylic acids through the reactions of 1,8-naphthalic or phthalic anhydride with relevant amino acids [21], then the imide moiety was linked to 5-Fu *via* an (acyloxy)methylene group, known to be easily removable [3,8]. All structures were verified by ^{1}H-NMR, ^{13}C-NMR, MS and elemental analysis.

Scheme 1. Synthesis of compounds **1-6**.
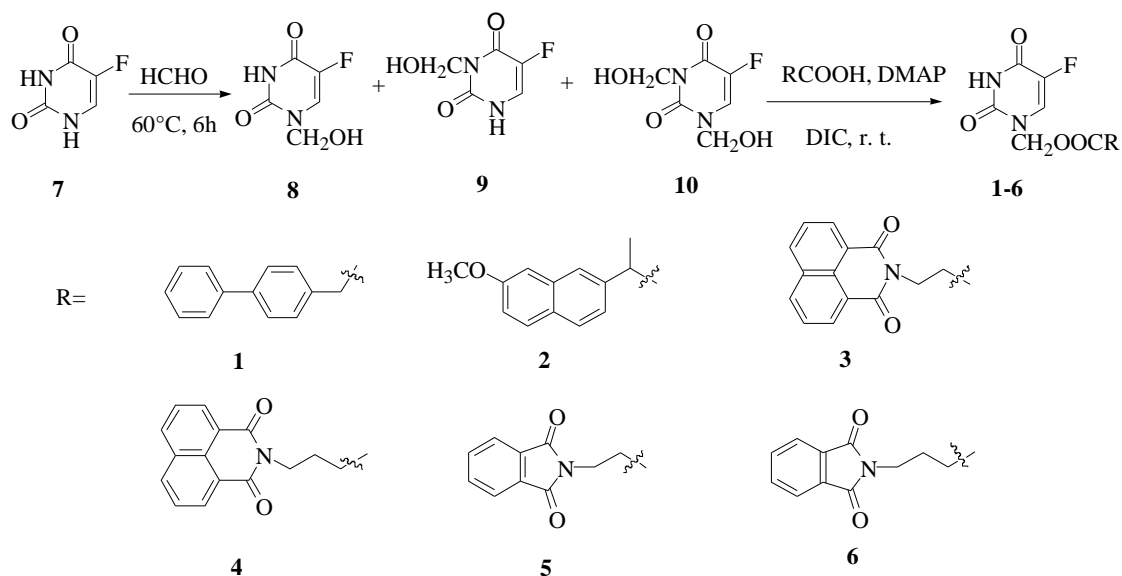
In vitro antitumor activity

All target compounds **1-6** were evaluated for their antitumor activity *in vitro* against K562, B16, and CHO cell lines by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay method [6,22]. The reference drug used was 5-fluorouracil. The activity of the samples and the reference drug was assayed under identical conditions at concentrations of 0.1, 1, 10, 30 and 50 µM. The lower its IC_{50} value, the more efficiently a compound kills cancer cells. As shown in Table 1, all compounds' *in vitro* activity was significantly decreased because the *N*-1 position, the active area of 5-Fu, was occupied. Compounds **2** and **6** still possessed some moderate to potent inhibition rates on the three tested cell lines and compounds **3, 4** and **5** exhibited some selectivity against B16 and/or CHO cells.

Table 1. The *in vitro* inhibition activity of compound **1-6** against three cell lines.

Sample	IC_{50} (µM)		
	K562	**B16**	**CHO**
5-Fu	0.27	0.23	0.35
1	16.83	12.15	12.86
2	43.25	36.93	>50
3	>50	3.72	32.30
4	30.46	4.44	16.83
5	>50	3.97	7.41
6	14.48	7.35	6.86

In vivo antitumor activity

The *in vivo* antitumor activities of three compounds on mice bearing liver cancer H_{22} are listed in Table 2. The T/C ratio is used as an index of the antitumor activity: *T/C* (%) = (C−T) / C x 100 % (T:

Average tumor weight of treated mice; C: Average tumor weight of control mice). As shown in Table 2, compounds **2**, **5** effectively inhibited tumor growth (ratio of tumor inhibition >40%) although they were less active than 5-Fu. Compound **2** was special because it was ineffective against all three cell types *in vitro* but was effective *in vivo*. We also found that surprisingly all three tested compounds caused more liver and lung tissue toxicity in mice than 5-Fu. This could happen if the hydrolysis of compound **2**, which contained only two clinically used drugs (5-Fu and naproxen), occurred readily under physiological conditions, liberating toxic formaldehyde. To verify this the hydrolysis of compound **2** was monitored by UV spectroscopy at physiological pH, but no 5-Fu was detected, thus indicating that the (acyloxy)methylene group in target compounds is stable [8], and target compounds might exert their biological functions as a new kind of "drugs".

Table 2. The *in vivo* inhibition activity of compounds **2**, **5** and **6** against H_{22} liver cancer.

Sample (T/C, %)	Dose (µmol/kg)	Weight of tumor (g)	Ratio of tumor inhibition
Control	0	1.63±0.31	0
5-Fu	150	0.59±0.19	64.07
2	150	0.95±0.30	41.64
5	150	0.89±0.53	45.17
6	150	1.26±0.54	22.85

Conclusions

In conclusion, a novel series of 5-fluorouracil derivatives was synthesized and some compounds were found to be active against tumor cell lines *in vitro* and *in vivo*. Three tested compounds caused more *in vivo* toxicities on the liver and lung tissues of mice than 5-Fu and were less effective than 5-Fu. This might be explained by the difficult hydrolysis of target compounds to give free 5-fluorouracil under physiological pH conditions.

Experimental

General

Melting points were determined on an X-6 hot stage microscope and were uncorrected. Mass spectra were obtained on an ESQUIRE-LC instrument. ^{1}H-NMR spectra and ^{13}C-NMR were run in DMSO-d6, with TMS as the internal standard, on a Bruker AV-400 instrument operating at 400 MHz. Elemental analysis was performed with a Gmbe VarioEL Elemental analysis instrument.

Synthesis of the target compounds **1-6**

Synthesis of compounds **1-6** was accomplished as shown in Scheme 1. 5-Fu (**7**, 1.3 g, 10 mmol), formaldehyde (37 wt.%, aq., 1.26 g, 15.5 mmol) and water (10 g) were added to a round-bottom flask, which was then immersed in water bath at 60°C and agitated for 6 h. The product solution was concentrated under reduced pressure to give an oily mixture containing **8-10**. The oil was dissolved in

dry acetonitrile (50 mL), and the corresponding carboxylic acids (10 mmol), DMAP (*N,N*-dimethylpyridin-4-amine, 80 mg, 14 mmol) and DIC (2.2 mL, 14 mmol) were added and the mixture was stirred at room temperature for 48-72 h. The white precipitate was filtered off and the solvent was removed by evaporation. The residue was dissolved in ethyl acetate (30 mL) and washed by diluted hydrochloric acid (pH = 3-4), saturated aqueous $NaHCO_3$ (pH = 7-8) and water. The organic layer was collected, dried over sodium sulphate, filtered and finally concentrated under reduced pressure. The target compounds were obtained through flash column chromatography: petroleum ether-acetone = 7:3 and recrystalization from acetone [8,14].

(5-Fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl-2-(biphenyl-4-yl)acetate (**1**). Yield: 31.1%; m. p.: 180.5-182°C; ^{1}H-NMR δ: 12.00 (s, 1H, NH of 5-Fu), 7.3-8.1 (m, 10H, 9×ArH, CH=CF of 5-Fu), 5.58 (s, 2H, CH2O), 3.7 (s, 2H, CH2C=O); ^{13}C-NMR δ: 170.89, 157.37 (d, J_{CCF} = 26 Hz), 149.22, 139.78, 139.41 (d, J_{CF} = 229 Hz), 138.84, 132.92, 130.00, 129.42 (d, J_{CCF} = 34 Hz), 128.89, 127.38, 126.61, 126.57, 70.86, 40.22; ESI-MS: *m/z* 377 (M^++Na); Anal. calcd. for $C_{19}H_{15}FN_2O_4$: C, 64.40; H, 4.27; N, 7.91; found: C, 64.33; H, 4.29; N, 7.81.

(5-Fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl-2-(7-methoxynaphthalen-2-yl)propanoate (**2**). Yield: 60.0%; m. p.: 171.5-172.5°C; ^{1}H-NMR δ:11.96 (s, 1H, NH of 5-Fu), 7.10-8.1 (m, 7H, 6xArH, CH=CF of 5-Fu), 5.60 (s, 2H, CH2O), 3.92 (s, 3H, CH3O), 3.84 (q, 1H, *J* = 6.6 Hz, CH3C<u>H</u>), 1.40 (d, *J* = 7.6 Hz, 3H, C<u>H</u>3CH); ^{13}C-NMR δ: 173.53, 157.28 (d, J_{CCF} = 26 Hz), 157.23, 149.09, 139.34 (d, J_{CF} = 229 Hz), 135.02, 133.33, 129.34 (d, J_{CCF} = 34 Hz), 129.08, 128.34, 127.00, 126.10, 125.64, 118.79, 105.70, 70.84, 55.15, 44.13, 18.13; ESI-MS: *m/z* 395 (M^++Na); Anal. calcd. for $C_{19}H_{17}FN_2O_5$: C, 61.29; H, 4.60; N, 7.52; found: C, 61.36; H, 4.33; N, 7.29.

(5-Fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl-3-(1,8-naphthalimido)propanoate (**3**). Yield: 20.0%; m. p.: 215.5-217°C; ^{1}H-NMR δ: 11.93 (s, 1H, NH of 5-Fu), 8.39-8.51 (4H, m, ArH), 8.02 (d, 1H, *J* = 6.0 Hz, CH=CF), 7.83-7.87 (2H, m, ArH), 5.56 (s, 2H, OCH2), 4.29 (t, 2H, NCH2), 2.74 (t, 2H, CH2CO); ^{13}C-NMR δ: 170.72, 163.3, 157.43 (d, J_{CCF} = 26 Hz), 149.20, 139.34 (d, J_{CF} = 229 Hz), 134.40, 131.24, 130.68, 129.43 (d, J_{CCF}=34 Hz), 127.31, 127.14, 121.84, 70.42, 35.56, 32.19; ESI-MS : *m/z* 434 (M^++Na); Anal. calcd. for $C_{20}H_{14}FN_3O_6 \cdot 0.30H_2O$: C, 57.64; H, 3.53; N, 10.08; found: C, 57.71; H, 3.34; N, 9.75.

(5-Fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl-4-(1,8-naphthalimido)butyrate (**4**). Yield: 20.0%; m. p.: 233.5-235°C; ^{1}H-NMR δ: 11.97 (s, 1H, NH of 5-Fu), 8.12-8.43 (m, 4H, ArH), 8.07 (d, 1H, *J* = 6.0 Hz, CH=CF), 7.83-7.85 (m, 2H, ArH), 5.54 (s, 2H, OCH2), 4.06 (t, 2H, NCH2), 2.37 (t, 2H, CH2CO), 1.84-1.91 (m, 2H, C<u>H</u>2CH2CO); ^{13}C-NMR δ: 172.23, 163.59, 157.46 (d, J_{CCF} = 25 Hz), 149.27, 139.31 (d, J_{CF} = 229 Hz), 134.23, 133.96, 131.23, 130.61, 129.58 (d, J_{CCF} = 34 Hz), 127.26, 122.07, 70.23, 39.11, 30.58, 22.50; ESI-MS: *m/z* 448 (M^++ Na); Anal. calcd. for $C_{21}H_{16}FN_3O_6 \cdot 0.39H_2O$: C, 58.33; H, 3.91; N, 9.72; found: C, 58.73; H ,3.93; N, 9.26.

(5-Fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl-3-(phthalimido)propanoate (**5**). Yield: 46.7%; m. p.: 233-235°C; ^{1}H-NMR δ: 11.92 (s, 1H, NH of 5-Fu), 8.10 (d, 1H, *J* = 6.0 Hz, CH=CF),

7.82 (m, 4H, 4×ArH), 5.53 (s, 2H, OCH$_2$), 3.81 (t, 2H, NCH$_2$), 2.72 (t, 2H, CH$_2$CO); ^{13}C-NMR δ: 170.84, 168.04, 157.92 (d, J_{CCF} = 26Hz), 149.69, 139.83 (d, J_{CF} = 229 Hz), 134.90, 131.99, 129.91 (d, J_{CCF} = 34 Hz), 123.48, 70.93, 33.82, 32.74; ESI-MS: *m/z* 384 (M$^+$+Na); Anal. calcd. for C$_{16}$H$_{12}$FN$_3$O$_6$: C, 53.19; H, 3.35; N, 11.63; found: C, 53.16; H, 3.22; N, 11.37.

(5-Fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl-4-(phthalimido)butyrate (**6**). Yield: 71.5%; m. p.: 181.5-184°C; ^{1}H-NMR δ: 11.99 (s, 1H, NH of 5-Fu), 8.09 (d, 1H, *J* = 6.0 Hz, CH=CF), 7.76-7.84 (m, 4H, 4×ArH), 5.55 (s, 2H, CH$_2$O), 3.60 (t, 2H, NCH$_2$), 2.43 (t, 2H, CH$_2$CO),1.82-1.86 (m, 2H, CH$_2$); ^{13}C-NMR δ: 172.58, 168.29, 157.90 (d, J_{CCF} = 26 Hz), 149.71, 139.83 (d, J_{CF} = 229 Hz), 134.67, 132.04, 129.96 (d, J_{CCF} = 34 Hz), 123.31, 70.82, 36.89, 30.81, 23.33; ESI- MS: *m/z* 398 (M$^+$+ Na); Anal. calcd. for C$_{17}$H$_{14}$FN$_3$O$_6$: C, 54.40; H, 3.76; N, 11.20; found: C, 54.54; H, 3.56; N, 11.91.

In vitro antitumor activity experiments

Cell lines (K562, B16, CHO) were obtained from the American Type Culture Collection (ATTC) and cultured in RPMI1640, supplemented with 10% heat-inactivated fetal calf serum and antibiotics (penicillin, 100 units/mL; streptomycin sulfate, 100 µg/mL) at 37°C, in an atmosphere of 95% air and 5% CO$_2$ under humidified condition. All chemicals were purchased from Sigma, unless otherwise indicated. RPMI1640 and fetal calf serum (FCS) were purchased from Gibco. Stock solution (10 µM) of samples was prepared in dimethylsulfoxide (DMSO) and diluted with various concentrations with serum-free culture medium. The *in vitro* antitumor activity of compounds **1-6** and 5-Fu were determined by MTT assay method [10]. Briefly, exponentially growing B16 or CHO cells were seeded in 96-well plates (5000 to each well) and allowed to attach overnight. After 24 h, the cells were treated with indicated concentrations of samples for 48 h, and then MTT (100 µL, 1 mg/mL) was added. After incubation for 4 h at 37°C, the MTT solution was removed and the crystals of viable cells were dissolved with DMSO (150 µL) in each well; 4000/well exponentially growing K562 were seeded in 96-well plates and treated with indicated concentrations of samples for 48 h, and then MTT (10 mg/mL, 10 µL) was added. After incubation for 4 h at 37°C the crystals of viable cells were dissolved overnight with SDS (sodium dodecylsulfonate, 10%, 100 µL) in each well. The absorbance spectra were measured on an ELISA Processor II Microplate Reader at a wavelength of 570 nm. The percentage of cytotoxicity was defined with treated and untreated cell lines. The 50% antitumor activity dose (IC$_{50}$) was defined as the concentration of samples that reduced the absorbance of the treated cells by 50% [22].

In vivo antitumor activity experiments

To evaluate the *in vivo* antitumor activity of 5-fluorouracil derivatives **2**, **5** and **6** mice bearing H$_{22}$ tumor cells were used. Kunming mice (*n* = 10) were first injected subcutaneously (s.c.) with liver cancer H$_{22}$ cell line (2×10^5 cells/mL). The mice bearing the liver cancer H$_{22}$ tumor cell line were then injected intraperitoneally (i.p.) with water solutions of compounds **2**, **5** or **6** for 8 days. For comparison, antitumor activities of 5-fluorouracil (5-Fu) also were tested by the same method. A control group was treated with H$_{22}$ tumor cells along with the same volume of NaCl (0.9 wt.%, aq.). The ratio (T/C) obtained by dividing the number of mice treated with 5-fluorouracil derivatives (T)

showing tumor inhibition to that of mice in the control groups (C) was used as the antitumor activity index [22].

Hydrolysis experiments

Ultraviolet and visible spectral measurements were performed with a Varian DMS 300 spectrophotometer. Buffers (trishydroxymethylaminomethane-HCl, Tris-HCl, pH = 7.4) were made in our laboratory. The UV method is defined as recording the absorbance changes over time for reactions in which the absorbance of substrate and product differ maximally at a particular wavelength. This method was used to study the activation of compound **2** [8]. Reactions were performed on aliquots of buffer solution (3 mL) in a round-bottom flask by addition of compound **2** in chloroform at 25°C for 2 hours. Release of 5-Fu of compound **2** was determined from the absorbance at 266 nm. Initial concentration of all substrates studied by the UV method was 5×10^{-5} M. No absorbance of 5-Fu was observed at 266 nm.

Acknowledgements

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Sample Availability: Contact the authors.