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Selective bone targeting 5-fluorouracil prodrugs: Synthesis and preliminary biological evaluation

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ABSTRACT

Bone tumor is a notoriously difficult disease to manage, requiring frequent and heavy doses of systemically administered chemotherapy. Targeting anticancer drug to the bone after systemic administration may provide both greater efficacy of treatment and less frequent administration. In this paper, a series of bone targeting Asp oligopeptides 5-fluorouracil conjugates have been synthesized in a convergent approach and well characterized by NMR and MS techniques. Their hydroxyapatite (HAP) affinity, drug release and cytotoxicity characteristics were evaluated in in vitro conditions. All the prodrugs were water soluble and exhibited high affinity to HAP .The efficient release of the active drug moiety occurring by the cleavage of different linkage in physiological conditions significantly reduced the number of viable human cancer cells. From in vivo distribution, we get these compounds with high bone-selectivity and long halflife. These results provided an effective entry to the development of new bone targeting chemotherapeutic drugs.

1. Introduction

Bone tumors are classified as 'primary tumors' which originate in the bone, including osteoma, osteoid osteoma, osteochondroma, osteoblastoma, enchondroma, giant cell tumor of bone, etc. and 'secondary tumors' which originate elsewhere. Nowadays, bone tumors especially bone metastases become one of the most common cancers in the world.[1,2] However, it is generally difficult to treat through drug chemotherapy because of the special component of bone tissue.[3] Bones which are mostly composed of the inorganic compound hydroxyapatite (HAP), lack circulating systems and have a very low blood flow. Current methodology using selective bone targeting drug delivery through binding HAP is able to specifically activate prodrugs at precise bone locations and looks very promising.[4–6] Therefore, some scientists developed a series of drug targeting systems using bisphosphonates and other bone targeting moieties and achieved some results.[7–9] Recently, linear acidic oligopeptides which posses Asp repetitive sequences have been identified as high-affinity binding site for HAP.[10] (The chemical structures of bone targeting oligopeptides were shown in Fig. 1) In addition, unlike the P–C–P bond of bisphosphonates, the oligopeptides are biologically labile and enzymatically degraded. The advantageous property can make drug release more efficient and no unexpected long-term effects.[11] Bone targeting prodrugs based on Asp oligopeptides have been studied in the treatment of bone diseases by Miyamoto and co-workers,[12,13] Wang et al.[14,15] and our group[16,17] such as osteoporosis and osteomyelitis. As parts of our continuing efforts, in order to construct more effective bone targeting prodrugs based on Asp oligopeptides and find out a potential bone site-specific chemotherapeutic drug treatment, we chose a well-known broad spectrum anti-cancer drug 5-fluorouracil (5-FU) as a model drug and hope we can prepare a new kind of selective bone targeting 5-fluorouracil prodrugs bearing Asp oligopeptides and find out its use in bone tumor treatment. Like other bone-targeting drug delivery systems, this prodrug strategy is hoping to reduce the chemotherapeutic drugs administration dose and reduce the side effect in normal tissues.

A classic way of prodrug design is that the active molecules should be linked by labile and hydrolyzable bonds.[18] The active drugs were easily cleavable: the removal of the prodrug periphery groups, liberation of the terminal and subsequent unzipping of the scaffold lead to release the parent drugs. To create a 5-FU-aspatic oligopeptides conjugate, it was of primary importance to determine the manner in which 5-FU attached to the exposed amino

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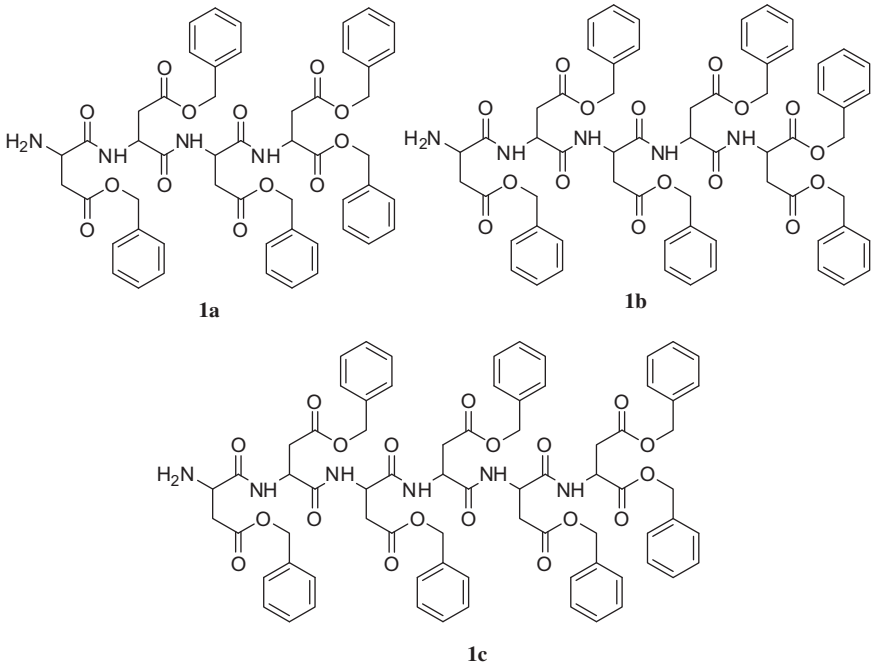


Figure 1. Structures of the fully protected Asp4–6 (**1a–c**).

of Asp. In the most case of 5-FU prodrugs designing, attaching directly to the N-1 of 5-FU by the use of a carbonate or carbamate linker was chosed.[19,20] However, some recent studies have shown that the N–C bond of N-1 alkyl-substituted 5-FU derivates was too stable to release and such prodrugs exhibited low therapeutic effect when tested against human tumor cell lines.[21,22] To solve this problem, experts proposed the concept of adding a labile longer carbon chain or more cleavable chemical bond as a self-immolative moiety which was further attached through a stable carbamate linkage to the amine group.[23] Based on the above viewpoints, we introduced a longer carbon chain to the N-1 of 5-FU: a succinate chain which was used as a simple self-immolative linker was connected between the drug and oligopeptides. We could compare the different characteristics of drug release and cytotoxicity of prodrugs with the two different linkers.

2. Results and discussion

2.1. Synthesis and characteristic of the prodrugs

The fully protected bone-targeting peptides **1a–c** were synthesized by a conventional liquid-phase peptide synthetic method from Boc–Asp–OBzl utilizing IBCF (isobutyl chloroformate) and NMM (*N*-methyl morpholine) (mixed anhydrides method) by a series of segment condensation which was described in our previous work.[16,17]

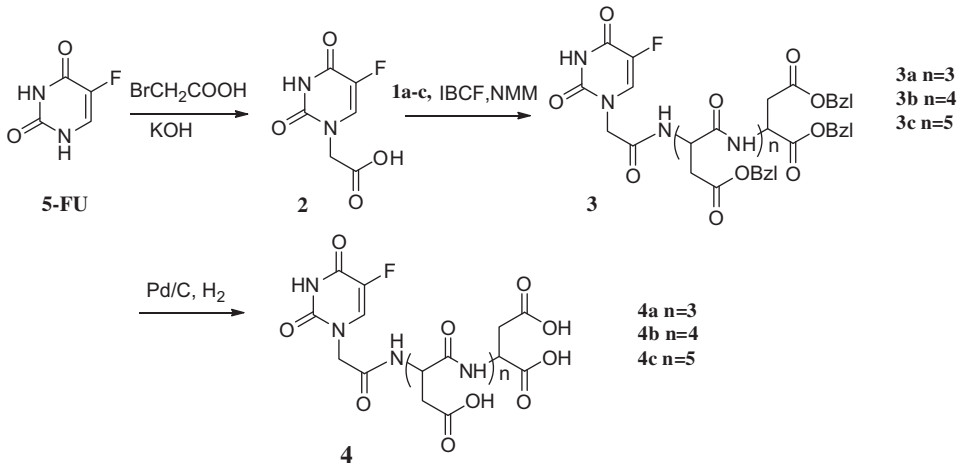
The synthetic procedure of the N-1 acetic acid modified 5-fluorouracil prodrugs was outlined in Scheme 1. First, the bromine acetic acid was conjugated to 5-FU through a simple substitution reaction to obtain the compound **2**. The protected 5-FU-oligopeptides conjugates **3a–c** was obtained by divergent synthesis of **1a–c** with **2** in the presence of IBCF and NMM in anhydrous tetrahydrofuran. The activation of the focal point was done by the removal of benzyl group by catalytic hydrogenolysis giving the target compound **4a–c**.

The synthetic procedure of the N-1 succinate acid modified 5-fluorouracil prodrugs involved several iterative protection–deprotection steps of orthogonally protected building blocks and was outlined in Scheme 2. 5-Fluorouracil reacted with formaldehyde to give N, N-1, 3-dimethylol-5-fluorouracil, the N-3 substituted 5-FU derivatives were relatively unstable and C–N bond was easily cleavable, and then condensed with benzyl succinate in the presence of DCC (dicyclohexylcarbodiimide) and DMAP (dimethylamino-pyridine) to obtain 5-fluorouracil derivatives compound **5** with a labile ester bond linkage. After purified by silica gel chromatography column and removed N-3 substituted coproducts, we use the method of Pd/C catalyzed hydrogenation to obtain compound **7**. Then these derivatives condensed with the protected aspartic acid oligopeptide **1a–c** under the condition of IBCF and NMM, and finally the target compounds **6a–c** were obtained after removal of the protecting groups of the peptide.

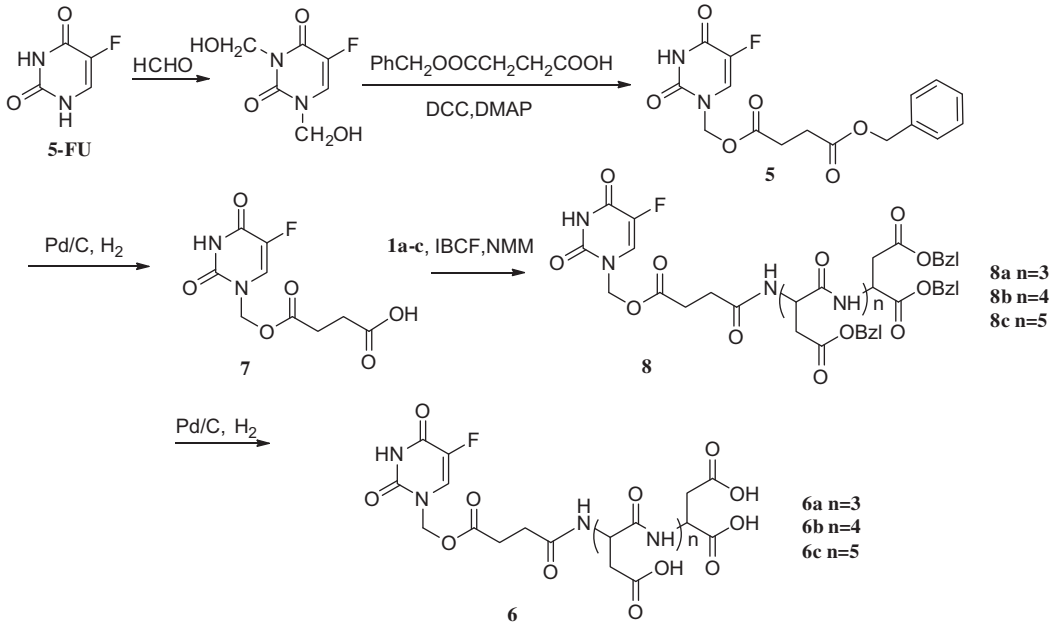
The high degree of symmetry in these molecules enabled facile confirmation of both structure and purity by NMR techniques. For example, in the ^{1}H NMR spectrum of compound **4a–c**, the 5-FU linker protons observed the resonance signals at 7.69 (d) and 4.12 (s) were clearly distinguishable from the resonances arising from the Asp oligopeptides at 2.72 (m) and 4.90 (m) ppm. In the ^{1}H NMR spectrum of compound **6a–c**, succinate protons were observed the resonance signals at 2.56 (br s). Integration of the respective areas of all the protons confirmed the complete coupling and the purity of the target compounds. In the ^{13}C NMR spectrum of all target molecules, 5-FU signals at near 124.5, 139.0, 145.7, 147.5 ppm. Furthermore, the structures of these compounds were further verified by electrospray ionization–mass spectrometry (ESI-MS). All the spectra displayed a very prominent peak corresponding to the compounds complexed with protons or sodium cation. Moreover, elemental analysis was also in good agreement with those of the signed structures.

2.2. Biological evaluation

To demonstrate in vivo activity, the 5-FU prodrugs need not only to bind to bone but also to release the active 5-fluorouracil.[24] These two requirements were evaluated in vitro to predict the therapeutic potential of these compounds. To study the binding of these conjugates to bone, an in vitro HAP binding assay was



Scheme 1. The synthesis of compound **4a**–**c**.



Scheme 2. The synthesis of compound **6a**–**c**.

set up using in vitro HAP binding methods described by Wang et al.[25] The conjugates were dissolved in water with various precise concentrations and the adsorption amounts were determined by a UV spectrophotometer at 254 nm to obtain the A–C linear regression equation. Tetracycline was taken as a positive control in this experiment. The bound percentage was calculated and the result was presented in Table 1. From The data presented, all conjugates of 5-FU exhibited HAP binding capability and the binding could take effect rapid within 0.5 h. Non-modified 5-FU hardly showed any binding demonstrating that oligopeptides played an important role in the HAP binding process. The prodrugs with Asp6 (**4c** and **6c**) were found to be more effective in binding HAP comparing the well-known bone-targeting substance: tetracycline. The binding trend was Asp6 >> Asp4 > or = Asp5, the preferential binding to bone of **4c** and **6c** exhibit the oligopeptides with six amino acid residues were more effective to be a bone-targeting moiety. The structures with multi-carboxy groups provide ionic interaction between negative charges and calcium ions in the bone mineral component. Therefore, we chose **4c** and **6c** in further biological evaluation.

Table 1
Relative binding of analogs **4a**–**c**, **6a**–**c** to hydroxyapatite compared to 5-fluorouracil and tetracycline binding

Compound	Binding index[a] (%)	
	0.5 h	Over 24 h
5-FU	0	0
Tetracycline	24.1 ± 2.6	56.2 ± 1.8
4a	14.3 ± 2.4	40.2 ± 3.2
4b	11.6 ± 0.7	19.8 ± 1.7
4c	31.4 ± 2.6	62.7 ± 4.3
6a	16.6 ± 3.2	42.5 ± 1.5
6b	13.4 ± 0.9	25.5 ± 2.6
6c	37.8 ± 1.7	69.7 ± 4.3

[a] The data represent the mean ± SD (n = 3).

Another important factor to be considered for the conjugate is the drug releasing profile.[26] Since the conjugate contains different covalent bond between the drug and the bone-targeting peptides, it should be enzymatically and/or hydrolytically degradable to

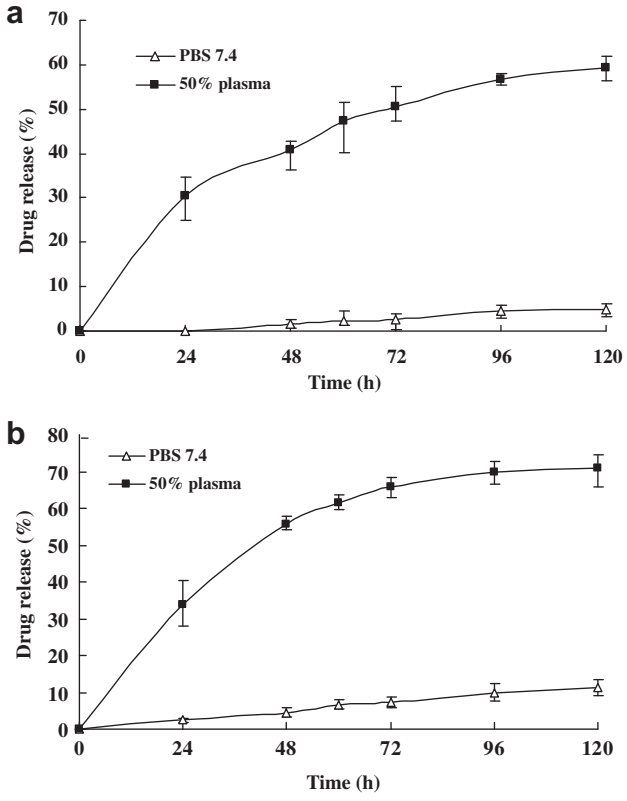


Figure 2. Release of 5-FU from compound **4c** (Fig. 2a) and compound **6c** (Fig. 2b) in physiological conditions. The percentage of drug release was deduced from the difference between the initial amounts of **4c** or **6c** and that of regenerated drug 5-FU. Error bars represented the mean and standard deviation of three independent experiments.

release the drug molecules to bone tissue and the different in vitro release capabilities of the two types of 5-FU prodrugs can be compared. The precipitate (**4c** and **6c**) was incubated in PBS pH 7.4 or 50% (v/v in PBS) human plasma (with a small amount of DMSO as hydrotropy agent) at 37 °C and the hydrolytic release of 5-FU in the solution was monitored by RP-HPLC.

The results from these in vitro assays (Fig. 2) suggest several trends. First, two different types of prodrugs were able to release the parent drugs in vitro. Specifically, the drug released rapidly from the compound **4c** in 50% human plasma, reaching 47.4% after 60 h and almost 60% within five days, whereas the drug release at PBS was much slower, implying 3.2% and 5% in the same period, respectively. Second, in 50% human plasma, the drug released from the compound **6c** reaching 60.3% after 60 h and almost 80% within five days. The release property of compound **6c** was much better than compound **4c**.

The trends observed with in vitro drug release data would suggest that the two linkages were labile under blood circulation conditions and relatively stable in PBS (pH 7.4) to allow the transport of the prodrug to bone issues and effectively release the free active drug from the carrier. Compound **6c** with succinate ester had a more rapid activation pathway, possibly because it had a longer and more labile carbon chain or the eliminate process was accelerating by a self-immolative disassembly pathway or the ester bond was instable than the amide bond.[27,28] Anyway, 5-FU prodrugs with succinate linkage were better substrates of enzymes in human plasma. The results shown in this study provide a proof of concept that succinate ester linkages modified bone-targeting 5-FU prodrugs had more effective release profiles and this concept could guide us to further prodrugs design.

Finally, the in vitro cytotoxicities of the compounds **4c** and **6c** in the 0% and 50% human plasma lipid were estimated using two 5-FU-sensitive cancer cell lines: human epithelial carcinoma cell line (HeLa) and human osteosarcoma cell line (MG63). In order to prove

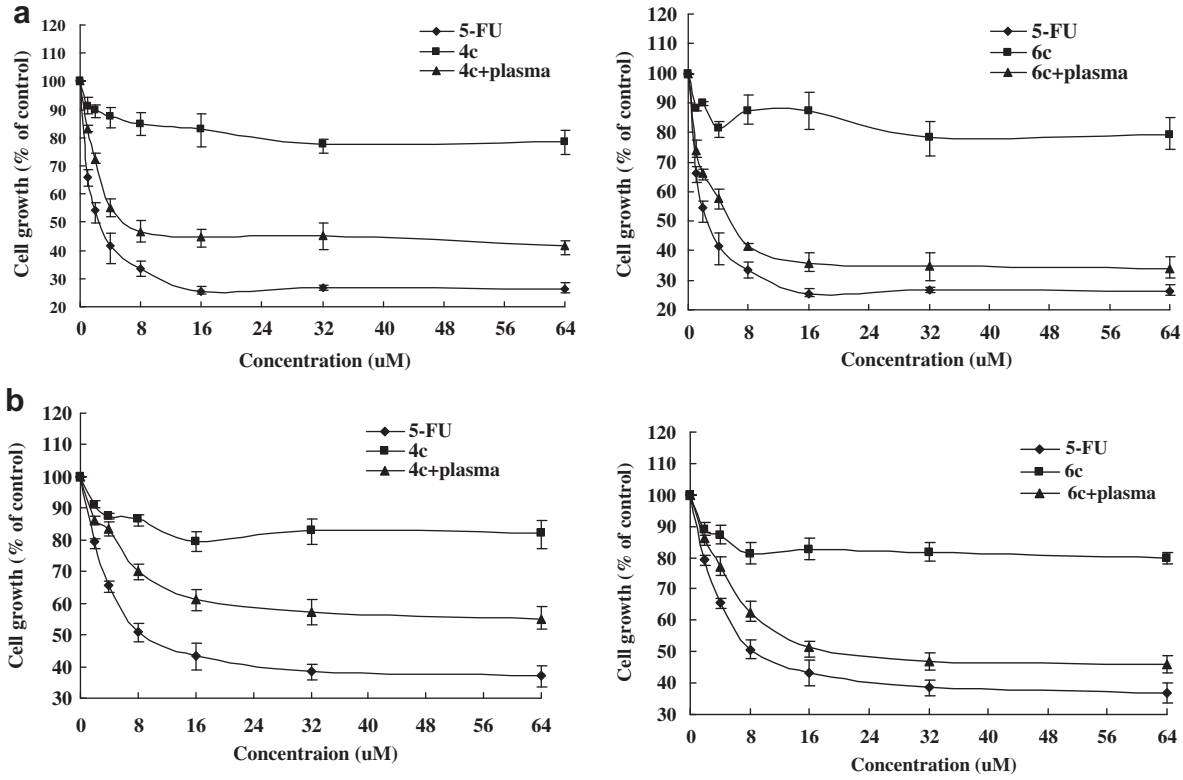


Figure 3. Cytotoxicities of the prodrugs, with and without human plasma, as functions of the human epithelial carcinoma cell line (HeLa) (a), and the human osteosarcoma cell line (MG63) (b). Error bars represented the mean and standard deviation of three independent experiments.

Table 2
Pharmacokinetic parameters of 5-FU-oligopeptides conjugate **4c**, **6c** and 5-FU in mice

Compound	AUC (mg∗h/L)	$T_{1/2}$ (h)	V1/*F* (L/kg)	C_{max} (mg/ml)	MRT
4c	76.55 ± 8.61	4.36 ± 0.62	24.12 ± 2.41	42.45 ± 5.41	7.41 ± 1.06
6c	90.08 ± 9.64	6.75 ± 1.22	32.18 ± 1.45	51.08 ± 4.78	8.25 ± 1.26
5-FU	31.17 ± 4.79	0.64 ± 0.13	64.74 ± 2.14	78.54 ± 8.11	1.61 ± 0.31

AUC: area under (the concentration time) curve; $T_{1/2}$: biological halflife; V1/*F*: apparent volume of distribution; C_{max}: maximum concentration; MRT: mean retention time. Data are represented as mean ± SD.

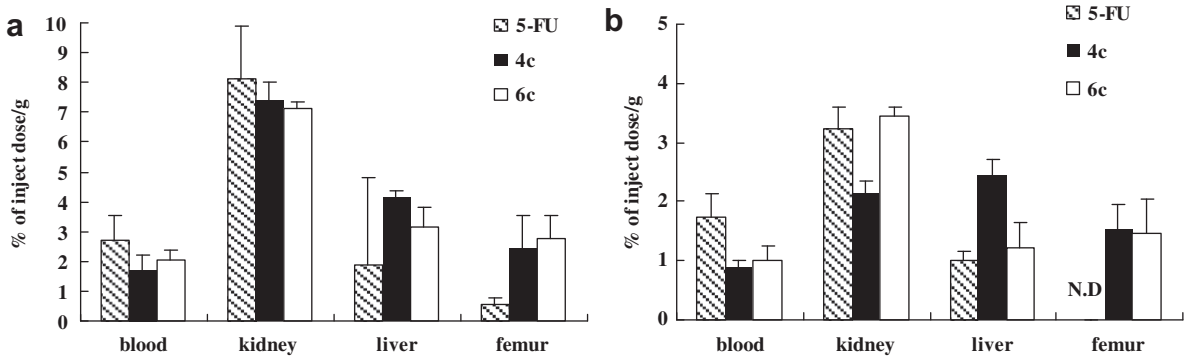


Figure 4. The biodistribution of the 5-FU oligopeptides conjugates with different linkers **4c**, **6c** and naked 5-FU. The biodistribution was analyzed at (a) 1 h and (b) 12 h after oral administration. Error bars represented the mean and standard deviation of three independent experiments. N.D. cannot be detected.

that the two conjugates are active through the cleavage by enzymes in human plasma, we performed a standard MTT cell-growth inhibition assay: cells were challenged for three days with free 5-FU, conjugate **4c**, conjugate **6c**, or the control conjugate over a range of concentrations. Cell viability was measured using colorimetric assay based on the MTT. The data were presented in Figure 3. Proliferation of HeLa cells were inhibited by free 5-FU, conjugate **4c** and conjugate **6c** (at 5-FU-equivalent concentrations) with IC50s of 4 μM, >64 μM and >64 μM, respectively. Proliferation of MG63 cells were inhibited with the same trend: 8 μM, >64 μM and >64 μM, respectively. 5-FU prodrugs **4c** and **6c** exhibited significantly reduced toxicity than free 5-FU in the absence of plasma. There was no noticeable change in the cytotoxicity of the conjugate **4c** and **6c** in the 0% plasma lipid throughout all concentration range. However, the cytotoxicities of the conjugates in 50% plasma lipid were significantly enhanced as the concentration of 5-FU increased. This result exhibited these bone targeting prodrugs themselves did not have cytotoxicity, but could be activated by enzymes in human plasma, the amide or esters were able to appreciably release their anticancer moieties and the process at least required the participation of enzymes.

An in vivo pharmacokinetic and biodistribution assay was taken to further exhibit the targeting ability of the prodrugs. The pharmacokinetic parameters for conjugates **4c** and **6c** were summarized in Table 2. The AUC and $T_{1/2}$ value of conjugate **4c** in blood were 76.55 mg∗h/L and 4.36 h, respectively, values much larger than those of 5-FU (31.17 mg∗h/L and 0.64 h, resp.). The longer circulation time of conjugate **4c** in the blood stream may be attributable to its larger molecular size, resulting in a lower glomerular filtration rate in the kidney. From this point of view, 5-FU-oligopeptides conjugates, with a longer retention time and a higher therapeutic window, was better than 5-FU for bone targeting chemotherapy.

Figure 4 shows the accumulation of the conjugates in the skeleton (represented by femur). All conjugates reached the skeleton within 1 h after administration. After 12 h, the amount of deposited conjugates began to decrease. It is obvious that the concentration of 5-FU in bone delivered by Asp oligopeptides is much higher than that by uncoupled control during 24 h. The results clearly show that, in contrast to nontargeted (no oligopeptides) controls, oligopeptides containing conjugates have a tendency of targeting and accumulation to the bone. From the in vivo data, the two linker's biodistribution is no significant difference, but compared to naked 5-FU, this bone-targeting prodrug strategy can reduce drug administration dose, reach a higher bone tissue drug accumulation and reduce side effects in other organs by chemotherapy drugs.

3. Conclusion

Site-specific bone drug delivery via a prodrug approach has generated considerable interest for enhancing the potency and diminishing the side effects of a drug. Effective release of chemotherapeutic drugs from a prodrug system is important if a high concentration of active drug is needed at the bone tissue. In conclusion, a number of methods were presented to tether a bone-targeting Asp oligopeptides group to the different carboxylic acid functionality of 5-fluorouracil to construct prodrugs as novel potential bone-targeting therapeutics. The resulting 5-FU oligopeptide conjugates were evaluated for the affinity to bone and the ability to release parent drug once bound to bone. In vitro investigations highlighted the strong affinity of Asp oligopeptide derivatives for bone, as opposed to the negligible bone affinities of the parent drugs. The bounded compound **4c** and **6c** could slowly release parent drug in physiological condition and significantly reduce the number of tumor cells. The in vivo biodistribution and pharmacokinetic data obtained support the effective targeting of 5-FU oligopeptides conjugates to bone with long half-life and the clearance rate of the conjugates from the organism possibly was dependent on molecular size and the different linkers. The preliminary results seem to be very promising and we are continuing to develop more suitable animal models for in vivo comprehensive evaluation of osteosarcoma treatment and possible harmful adverse effect.

4. Experimental

4.1. Chemistry

General: All reactions requiring anhydrous conditions were performed under an Ar or N_2 atmosphere. Chemicals and solvents were either A.R. grade or purified by standard techniques. Thin layer chromatography (TLC): silica gel plates GF_{254}; compounds were visualized by irradiation with UV light and/or by treatment with a solution of phosphomolybdic acid (20% wt in ethanol) followed by heating. Column chromatography was performed by using silica gel with eluent given in parentheses. ^{1}H NMR and ^{13}C NMR analysis was performed using $CDCl_3$ or D_2O as a solvent at room temperature. The chemical shifts are expressed in relative to TMS (= 0 ppm) and the coupling constants *J* in Hz. Hydroxyapatite (HAP) were purchased from Shanghai Institute of Biochemistry with surface area 9.12 m^2/g and average particle size 15 μm.

4.1.1. General procedure for the synthesis of compound 2

KOH (2.56 g, 45 mmol) and 5-fluorouracil (1.34 g, 10 mmol) dissolved in a clean flask, then 5 ml aqueous solution of bromoacetic acid (1.7 g, 18 mmol) was added under the temperature of 40 °C while stirred smoothly. The reaction mixture was stirred at 60 °C for 5 h and then cooled by ice-bath, adjusted to pH 5.5 with hydrochloric acid, after filtration through a membrane, the crude product was recrystallized by water, and compound **2** was obtained as white solid 1.53 g. Yield 85%, mp 255–257 °C. ^{1}H NMR (400 MHz, D_2O): 4.41 (s, 2H, N–CH_2), 7.54 (d, 1H, *J* = 5.2 Hz, 5-FU-H), ESI MS (*m*/*z*): calcd for 188.11. obsd 189.01 ([M+H]$^+$)

4.1.2. General procedure for the synthesis of compound 4

Compound **2** (0.1 g, 0.5 mmol) dissolved in anhydrous THF was cooled by ice-salt bath, then NMM (0.05 ml, 0.5 mmol) and IBCF (0.08 ml, 0.5 mmol) was added, after stirred for 30 min then, the protected oligopeptides NH_2–$Asp_{(4-6)}$ **1** (**1a**, 0.46 g, 0.5 mmol; **1b**, 0.57 g, 0.5 mmol; **1c**, 0.67 g, 0.5 mmol) in THF was added. The mixture was stirred for 3 h. After evaporated under reduced pressure, the residue was taken up in ethyl acetate and washed with 1 M HCl, 1 M $NaHCO_3$, and brine each for twice. The organic layer was dried and evaporated to give the crude product. The crude product was purified by silica gel column chromatography using DCM–methanol as an eluent to yield a ceraceous solid (Compound **3**).

A mixture of the obtained compound **3** and 10% Pd/C (10 mg) in CH_3OH (10 ml) was stirred at room temperature under a H_2 atmosphere. After 24 h, the mixture was passed through a membrane filter to remove the catalyst and then evaporated under reduced pressure to give the target molecules compound **4**. The total yield after two steps was about 56–68%.

4.1.2.1. Compound 4a. White wax (0.23 g, 68%) from **2** (0.1 g, 0.5 mmol).^{1}H NMR (400 MHz, D_2O): 2.72–2.92 (br s, 8H, Asp–βCH_2), 4.04 (s, 2H, N–CH_2), 4.56–4.90 (m, 4H, Asp–αCH), 7.69 (d, 1H, *J* = 5.2 Hz, 5-FU–CH). ^{13}C NMR (100 MHz, D_2O): 36.2, 36.6, 37.5, 51.0, 52.4, 53.0, 53.5, 54.2, 124.5, 139.0, 145.7, 147.5, 169.1, 172.3, 172.5, 172.7, 173.1, 173.4. ESI-MS (*m*/*z*): calcd for 648.46, obsd 671.65 ([M+Na]$^+$). Anal. Calcd for $C_{22}H_{25}FN_6O_{16}$: C, 40.75; H, 3.89; N, 12.96. Found: C, 40.11; H, 4.02; N, 12.17.

4.1.2.2. Compound 4b. White wax (0.25 g, 62%) from **2** (0.1 g, 0.5 mmol). ^{1}H NMR (400 MHz, D_2O): 2.69–2.90 (br s, 10H, Asp–βCH_2), 4.12 (s, 2H, N–CH_2), 4.44–4.91 (m, 5H, Asp–αCH), 7.71 (d, 1H, *J* = 5.2 Hz, 5-FU–CH). ^{13}C NMR (100 MHz, D_2O): 35.2, 35.7, 36..3, 37.4, 52.4, 52.3, 52.9, 53.4, 54.5, 55.1, 126.1.6, 139.9, 147.7, 149.5, 169.1, 171.2, 172.0, 172.5, 172.9, 173.3, 173.9, 174.2. ESI-MS (*m*/*z*): calcd for 763.55, obsd 763.41 ([M]$^+$). Anal. Calcd for $C_{26}H_{30}FN_7O_{19}$: C, 40.90; H, 3.96; N, 12.84. Found: C, 41.09; H, 3.88; N, 12.67.

4.1.2.3. Compound 4c. White wax (0.26 g, 56%) from **2** (0.1 g, 0.5 mmol).^{1}H NMR (400 MHz, D_2O): 2.64–2.96 (br s, 12H, Asp–βCH_2), 4.15 (s, 2H, N–CH_2), 4.43–4.96 (m, 6H, Asp–αCH), 7.77 (d, 1H, *J* = 5.2 Hz, 5-FU–CH). ^{13}C NMR (100 MHz, D_2O): 35.1, 35.4, 35.9, 36.7, 37.1, 37.3, 51.3, 51.7 52.4, 53.0, 53.5, 54.2, 55.4 125.5, 140.0, 149.7, 153.5, 169.1, 172.3, 172.5, 172.7, 173.1, 173.4. ESI-MS (*m*/*z*): calcd for 878.64, obsd 903.49 ([M+Na]$^+$). Anal. Calcd for $C_{30}H_{35}FN_8O_{22}$: C, 41.01; H, 4.02; N, 12.75. Found: C, 41.39; H, 3.78; N, 12.28.

4.1.3. General procedure for the synthesis of compound 5

To the solution of 37% formaldehyde/water (5 ml), 5-fluorouracil (0.52 g, 4 mmol) was added. The mixture was stirred at 60 °C to make the solid part dissolved completely, after 50 min, a colorless transparent thick oil 1,3-dimethylol-5-fluorouracil was obtained. Then anhydrous acetonitrile (12 ml), benzyl succinate (1.16 g, 5.6 mmol), DCC(1.15 g, 5.6 mmol) and DMAP(0.032 g, 0.26 mmol) was added under ice-bath, after stirred at 0 °C for 1 h, the mixture was then stirred at room temperature overnight. The dicyclohexylurea (DCU) was removed by filtration, then evaporated under reduced pressure. After concentration, the residue was taken up in 50 ml ethyl acetate and washed with 1 M HCl, 1 M $NaHCO_3$, and brine. The organic layer was dried and evaporated to give the crude product. The crude product was purified by silica gel column chromatography using petroleum ether–acetone as an eluent to yield compound **5** 1.00 g as a white solid-like substance. The total yield after two steps was about 71%. ^{1}H NMR (400 MHz, $CDCl_3$): 2.70 (s, 4H, SA-CH_2 × 2), 5.14 (s, 2H, N–CH_2), 7.30–7.50 (m, 5H, ph-H), 7.54 (d, 1H, *J* = 5.2 Hz, 5-FU-CH). ESI-MS (*m*/*z*): calcd for 350.30, obsd 373.34 ([M+Na]$^+$).

4.1.4. General procedure for the synthesis of compound 7

The obtained compound **5** (0.6 g, 1.71 mmol) was then dissolved in methanol (15 ml), 10%Pd/C (60 mg) was added. The mixture was stirred at room temperature under a H_2 atmosphere overnight. The Pd/C was removed by filtration, and then the mixture was evaporated under reduced pressure to give a colorless wax. The crude product was purified by silica gel column chromatography using petroleum ether–acetone as an eluent to yield a white ceraceous solid compound **7** (0.39 g). Yield 91%. ^{1}H NMR (400 MHz, $CDCl_3$): 2.64 (s, 4H, SA-CH_2 × 2), 5.31 (s, 2H, N–CH_2), 7.62 (d, 1H, *J* = 5.2 Hz, 5-FU–CH). ESI-MS (*m*/*z*): calcd for 260.18, obsd 283.20 ([M+Na]$^+$).

4.1.5. General procedure for the synthesis of compound 6

Same procedure as described above for preparation of the compound **4**. The total yield after two steps was about 47–61%.

4.1.5.1. Compound 6a. White wax (0.15 g, 56%) from compound **7** (0.1 g, 0.4 mmol).^{1}H NMR (400 MHz, D_2O): 2.59 (br s, 4H, SA-CH_2 × 2), 2.76–3.04 (br s, 8H, Asp–βCH_2), 4.66–4.97 (m, 4H, Asp–αCH), 5.50 (s, 2H, N–CH_2), 7.85 (d, 1H, *J* = 5.2 Hz, 5-FU–CH). ^{13}C NMR (100 MHz, D_2O): 27.1, 29.8, 35.2, 36.5, 52.0, 52.4, 53.5, 54.2, 76.4, 126.5, 139.4, 149.7, 154.5, 169.1, 171.3, 171.4, 172.3, 173.1, 174.4. ESI-MS (*m*/*z*): calcd for 720.53, obsd 719.38 ([M−H]$^-$). Anal. Calcd for $C_{25}H_{29}FN_6O_{18}$: C, 41.67; H, 4.06; N, 11.66. Found: C, 41.11; H, 4.48; N, 11.07.

4.1.5.2. Compound 6b. White wax (0.15 g, 47%) from compound **7** (0.1 g, 0.4 mmol).^{1}H NMR (400 MHz, D_2O): 2.47 (br s, 4H, SA–CH_2 × 2), 2.74–3.06 (br s, 10H, Asp–βCH_2), 4.76–5.01 (m, 5H, Asp–αCH), 5.48 (s, 2H, N–CH_2), 7.76 (d, 1H, *J* = 5.2 Hz,

5-FU–CH). ^{13}C NMR (100 MHz, D_2O): 28.1, 29.3, 34.7, 35.1, 35.7, 36.6, 53.3, 53.4, 53.5, 54.7, 77.1, 127.4, 140.2, 150.1, 157.1, 169.9, 170.1, 170.2, 170.3, 171.1, 171.4, 172.1, 173.1, 173.4. ESI-MS (*m*/*z*): calcd for 835.61, obsd 834.42 ($[M-H]^-$). Anal. Calcd for $C_{29}H_{34}FN_7O_{21}$: C, 41.68; H, 4.10; N, 11.73. Found: C, 41.92; H, 3.99; N, 11.41.

4.1.5.3. Compound 6c. White wax (0.22 g, 61%) from compound **7** (0.1 g, 0.4 mmol).^{1}H NMR (400 MHz, D_2O): 2.56 (br s, 4H, SA–CH_2 × 2), 2.72–3.01 (br s, 12H, Asp–βCH_2), 4.64–4.99 (m, 6H, Asp–αCH), 5.74 (s, 2H, N–CH_2), 7.86 (d, 1H, *J* = 5.2 Hz, 5-FU–CH). ^{13}C NMR (100 MHz, D_2O): 29.0, 31.3, 35.1, 35.2, 35.3, 35.5, 36.1, 53.0, 53.4, 53.5, 53.6, 53.7, 54.1, 78.2, 128.5, 140.4, 150.7, 158.5, 169.9, 170.0, 170.5, 170.6, 171.3, 171.5, 172.8, 173.7, 174.5, 175.4. ESI-MS (*m*/*z*): calcd for 950.70, obsd 951.43 ($[M+H]^+$). Anal. Calcd for $C_{33}H_{39}FN_8O_{24}$: C, 41.69; H, 4.13; N, 11.79. Found: C, 40.98; H, 4.25; N, 11.28.

4.2. Biological evaluation

4.2.1. Hydroxyapatite (HAP) binding study

The conjugates were dissolved in water with various precise concentrations and the adsorption amounts were determined by a UV spectrophotometer at 254 nm to obtain the A–C linear regression equation. In tubes 25 mg/ml HAP was added to 5 ml solutions of conjugates with the precise concentrations of 500 μg/ml (C0), followed by supersonic shake for 5 min, then placed in a water bath at 37 °C for 1 h and over 24 h. After the prescribed time, tubes were centrifuged for 1 min at 5000 rpm. The adsorption amounts were determined by UV at 254 nm and the Δ*C* was obtained by the special equation. The bound percentage was calculated by $(C0 - \Delta C)/C0 * 100\%$.

4.2.2. HPLC analysis

The HPLC system consisted of an SPD-10A variable UV–vis detetor and a set of Model LC-10AT liquid chromatograph includin a manometric module as well as a dynamic mixer from Agilent 1100 HPLC system. The mobile phase consists of pure water which was filtered through a 0.45 mm membrane filter beforuse. A Waters XTerra RP18 column (250 mm × 4.6 mm, 5 μm) was eluted with the mobile phase at flow rate of 1.0 mL/min. The eluate wasmonitored bymeasurin the absorption at 256 nm with a sensitivity of AUFS 0.01 at 25 °C. The retention time (RT) of 5-FU is 7.265 min, and the retention time of **4c** and **6c** was 5.465 min and 5.898 min.

4.2.3. Drug release study

The conjugates(compound **4c** and **6c**) were incubated in PBS pH 7.4 or 50% (v/v in PBS) human plasma (with a small amount of DMSO as hydrotropy agent) at 37 °C and the hydrolytic release of 5-FU in the solution was monitored by RP-HPLC. The concentration of 5-FU was analyzed using the HPLC conditions mentioned above.

4.2.4. In vitro cytotoxicity assay

The cytotoxic effects of 5-FU, compound **4c**, and compound **6c** in the absence or presence of human plasma was determined using the standard MTT assay. MG-63 or HeLa cells were harvested from culture flasks, resuspended in cell culture medium, and plated at a density of 5∗103cells/well in 200 μL onto 96-well culture plate. Cells were challenged with prodrug **4c** or prodrug **6c** (1–64 μM) in the presence or absence of human plasma enzyme and incubated for 72 h (5% CO_2). Activation solution (20 μL) was added to MTT reagent. The reaction solutions were added to each well. The plate was incubated for 2 h, shaken gently to evenly distribute the dye in the wells. Absorbance was measured at a wavelength of 490 nm.

4.2.5. Biodistribution in mice tissue in vivo

According to the requirements of the National Act on the usage of experimental animals (PR China), the Sichuan University Animal Ethical Experimentation Committee, approved all procedures of our in vivo studies. At the indicated time, the animals were sacrificed and blood samples were collected from the ocular artery directly after removing eyeball. Then the animals were dissected and each tested organ was removed, including kidney, liver, and femur. Organs were rinsed with cold normal saline, blotted dry with a paper towel, extracted with methanol, diluted, centrifuged, and dispensed in plastic sample vials. Both the samples were centrifuged at 3500 rpm for 15 min. After that, 20 μL of the supernatants were removed and the concentration of 5-FU was analyzed using the HPLC conditions mentioned above.

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