DRUG BIOCHEMISTRY

SYNTHESIS AND EVALUATION OF NOVEL BONE-TARGETING IBUPROFEN PRODRUG BASED ON DENDRITIC ASPARTIC ACID

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Abstract: Bone diseases, such as osteoarthritis, osteomyelitis, are notoriously difficult diseases to treat due to the comparatively low blood flows in bone tissue. Ibuprofen is a well-known potent non-steroid anti-inflammatory drug (NSAID), which plays an important role in the treatment of osteoarthritis. However, its poor bone-targeting ability hinders its further application. Herein, several novel dendritic aspartic acid-modified ibuprofen prodrugs were designed and further synthesized. The stability of these prodrugs was investigated in buffer solutions and plasma, respectively. In addition, the solubility, hydroxyapatite (HAP) binding, cytotoxicity, acute toxicity, and bone targeting ability *in vivo* were all evaluated. All the novel prodrugs showed the superior physicochemical property *in vitro*. The bone-targeting study *in vivo* provided the evidence that these prodrugs could increase ibuprofen levels in bone tissue, among which the Ibu-Asp₈ showed the best affinity due to the increased aspartic acid residues conjugated to the drug. The dendritic Asp carriers were efficient as bone-targeting moieties, and the conjugation with ibuprofen ensured the increased accumulation of drugs in the bone region. In general, our findings provided a novel and effective drug for the treatment of bone diseases.

Keywords: bone targeting, ibuprofen, osteoarthritis, prodrug, dendritic aspartic acid

With the development of the medical level, society has begun entering into aging and the ratio of old people has increased dramatically in most countries (1). Meanwhile, bone diseases are becoming increasingly common (2-3). If not treated effectively, most of the diseases, especially osteoarthritis, may cause persistent pain and significantly lower the quality of patients' life. Osteoarthritis, the chronic inflammation of bones, is mainly treated by surgery, drug therapy, and non-drug therapy at present. However, its precise mechanism is still unclear (4).

Recently, non-steroidal anti-inflammatory drugs (NSAIDs) have been widely used for osteoarthritis treatment (5-6). Nevertheless, it often needs high dose and long-term use due to the low blood flows in bone tissue and low targeting ability of drugs. Although increasing the dose of drugs in osteoarthritis treatment could cause stronger side effects and more severe toxicity to the gastrointestinal tract, liver, and kidneys. Therefore, strategies that

could effectively deliver drugs, such as ibuprofen, to the site of bone tissue are of great interest for osteoarthritis treatment (7-8).

Bone tissue is composed of inorganic salts (50-70%), organic bone (20-40%), water (5-10%), and esters (1-5%), and the inorganic compound hydroxyapatite (HAP) is known as the main component of bone, which has been used as a promising target of bone for selective drug delivery (9-11). In our previous report, it was found the acidic amino acids, especially glutamic oligopeptide, and aspartic (Asp) oligopeptide, had a superior affinity to the bone (12-13). The mechanism for the bone-targeting ability of these oligopeptides is that the multi-carboxy groups of peptides provide ionic interaction between negative charges and calcium ions in the HAP (14). Moreover, this affinity usually depends on the number of exposed amino acid residues. Compared with the linear peptide, the dendritic peptide has more carboxy groups outside, which could enhance the affinity to bone and the water solubility.

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Figure 1. Schematic illustration of prodrug Ibu-Asp₈ to target bone.

Based on the affinity mechanism and the dendritic effect, we hypothesize that the more amino acid residues conjugated to the drug, the stronger affinity to bone exhibited. Over the past years, we have been working on designing bone-targeting drugs, especially based on the acidic amino acid, which is biocompatible, non-toxic, and biodegradable (2-3, 12-13). We are still exploring how to further heighten the bone targeting ability of drugs. In this study, a series of novel dendritic aspartic acid-modified ibuprofen prodrugs (Ibu-Asp₁, Ibu-Asp₂, Ibu-Asp₄, and Ibu-Asp₈) were designed and synthesized. These prodrugs could utilize the dendritic Asp to target bone efficaciously (Figure 1). The stability of these prodrugs was investigated in buffer solutions and plasma, respectively. What's more, the solubility, hydroxyapatite (HAP) binding, cytotoxicity, acute toxicity, and bone targeting ability *in vivo* were also evaluated.

MATERIALS AND METHODS

General Procedures

All the starting materials, unless otherwise specified, were used from commercial resources. The thin-layer chromatography (TLC) was performed using precoated silica gel GF254 (0.2 mm), and column chromatography was performed using silica gel (100-200 mesh). Elemental analyses were performed by Atlantic Microlab (Atlanta, GA, USA). In this study, all the animal procedures were performed after being

approved by the Experiment Animal Administrative Committee (P.R. China).

Chemistry

Synthesis of compounds 3-8 (Figure 2)

The synthesis of compounds 3-8 was reported in our previous report (13).

Synthesis of compound 10 (dibenzyl (2-(4-isobutylphenyl)propanoyl)aspartate)

To a solution of ibuprofen 9 (1.00 g, 4.85 mmol) in CH₂Cl₂ (30 mL) were added isobutyl chloroformate (IBCF, 0.92 mL, 7.27 mmol) and 4-methylmorpholine (NMM, 0.81 mL, 7.27 mmol). After the mixture was stirred for 15 min at -15°C, Asp(OBn)-OBn (2.28 g, 7.27 mmol) in CH₂Cl₂ (20 mL) was added dropwise and the reaction mixture was stirred at r.t. for 6 h. The solution was filtrated, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography to get compound 10 (2.24 g, 92%). The resulting compound purity was 95% detected by HPLC. ESI-MS (*m/z*): calculated for C₃₁H₃₅NO₅Na [M+Na]⁺ 524.24, found 524.35. Elemental Analysis: C, 74.23; H, 7.03; N, 2.79, found C, 74.35; H, 7.11; N, 2.68.

Synthesis of prodrug Ibu-Asp1 ((2-(4-isobutylphenyl)propanoyl)aspartic acid)

The compound 10 (1.50 g, 2.99 mmol) was dissolved in CH₃OH (20 mL), and 10% Pd/C (0.15 g) was added into the solution. Subsequently, the mixture

Figure 2. Synthesis of prodrugs Ibu-Asp₈ and Ibu-Asp₁. Reagents and conditions: (a) C₂H₅OH, r.t. (b) i) HCOONH₄, Pd/C, CH₃OH, r.t.; ii) Boc-Gly-OH, IBCF, NMM, -15°C - r.t.; iii) NaOH, H₂O, r.t. (c) Asp(OBn)-OBn, EDCI, HOBT, CH₂Cl₂, r.t. (d) TFA, CH₂Cl₂, r.t. (e) 4, HBTU, DIPEA, CH₂Cl₂, r.t. (f) TFA, CH₂Cl₂, r.t. (g) Asp(OBn)-OBn, IBCF, NMM, CH₂Cl₂, -15°C - r.t. (h) H₂, Pd/C, CH₃OH, r.t. (i) 8, IBCF, NMM, CH₂Cl₂, -15°C - r.t. (j) H₂, Pd/C, CH₃OH, r.t.

was stirred at ambient temperature under H₂ atmosphere for 1 h and then was filtered. The filtrate was concentrated and purified by flash column chromatography to yield prodrug Ibu-Asp₁ (0.96 g, 90%). The resulting compound purity was 98% detected by HPLC. ESI-MS (*m/z*): calculated for C₁₇H₂₃NO₅Na [M+Na]⁺ 344.15, found 344.22. Elemental Analysis: C, 63.54; H, 7.21; N, 4.36, found C, 63.68; H, 7.14; N, 4.45.

Synthesis of compound 11

To a solution of prodrug Ibu-Asp₁ (0.30 g, 0.93 mmol) in CH₂Cl₂ (10 mL) were added IBCF (0.28 mL, 2.24 mmol) and NMM (0.25 mL, 2.24 mmol). After the mixture was stirred for 15 min at -15°C, the dendron 8 (4.18 g, 2.33 mmol) in CH₂Cl₂ (30 mL) was added dropwise and the

reaction mixture was stirred at r.t. for 20 h. The solution was filtrated, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography to give compound 11 (2.25 g, 62%). The resulting compound purity was 96% detected by HPLC. MALDITOF-MS (*m/z*): calculated for C₂₀₉H₂₃₁N₂₁O₅₃Na [M+Na]⁺ 3905.59, found 3905.66. Elemental Analysis: C, 64.61; H, 5.99; N, 7.57, found C, 64.50; H, 5.86; N, 7.69.

Synthesis of prodrug Ibu-Asp8

The compound 11 (0.50 g, 0.13 mmol) was dissolved in CH₃OH (10 mL), and 10% Pd/C (0.20 g) was added into the solution. Subsequently, the mixture was stirred at ambient temperature under H₂ atmosphere for 5 h, and then was filtered. The filtrate

Figure 3. Synthesis of prodrugs Ibu-Asp₂ and Ibu-Asp₄. Reagents and conditions: (a) **6**, IBCF, NMM, CH₂Cl₂, -15°C - r.t. (b) H₂, Pd/C, CH₃OH, r.t. (c) **8**, IBCF, NMM, CH₂Cl₂, -15°C - r.t. (d) H₂, Pd/C, CH₃OH, r.t.

was concentrated and purified by flash column chromatography to yield prodrug Ibu-Asp₈ (0.25 g, 80%). The resulting compound purity was 96% detected by HPLC. MALDITOF-MS (*m/z*): calculated for C₉₇H₁₃₅N₂₁O₅₃Na [M+Na]⁺ 2464.84, found 2464.75. Elemental Analysis: C, 47.69; H, 5.57; N, 12.04, found C, 47.55; H, 5.50; N, 12.17.

Synthesis of compound 12

To a solution of ibuprofen **9** (0.85 g, 4.12 mmol) in CH₂Cl₂ (10 mL) were added IBCF (0.68 mL, 5.37 mmol) and NMM (0.60 mL, 5.37 mmol). After the mixture was stirred for 15 min at -15°C, the dendron **6** (4.67 g, 5.77 mmol) in CH₂Cl₂ (20 mL) was added dropwise and the reaction mixture was stirred at r.t. for 5 h. The solution was filtrated, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography to get compound **12** (3.20 g, 78%). The resulting compound purity was 94% detected by HPLC. ESI-MS (*m/z*): calculated for C₅₇H₆₄N₄O₁₂Na [M+Na]⁺ 1019.44, found 1019.57. Elemental Analysis: C, 68.66; H, 6.47; N, 5.62, found C, 68.54; H, 6.38; N, 5.74.

Synthesis of prodrug Ibu-Asp₂

The compound **12** (0.20 g, 0.20 mmol) was dissolved in CH₃OH (10 mL), and 10% Pd/C (30 mg) was added to the solution. Subsequently, the mixture was stirred at ambient temperature under H₂ atmosphere for 2 h and then was filtered. The filtrate was concentrated and purified by flash column chromatography to yield prodrug Ibu-Asp₂ (0.11 g, 88%). The resulting compound purity was 96% detected by HPLC. ESI-MS (*m/z*): calculated for C₂₉H₄₀N₄O₁₂Na [M+Na]⁺ 659.254, found 659.33. Elemental Analysis: C, 54.71; H, 6.33; N, 8.80, found C, 54.65; H, 6.44; N, 8.69.

Synthesis of compound 13

To a solution of ibuprofen **9** (0.20 g, 0.97 mmol) in CH₂Cl₂ (10 mL) were added IBCF (0.16 mL, 1.26 mmol) and NMM (0.14 mL, 1.26 mmol). After the mixture was stirred for 15 min at -15°C, the dendron **8** (2.44 g, 1.36 mmol) in CH₂Cl₂ (20 mL) was added dropwise and the reaction mixture was stirred at r.t. for 6 h. The solution was filtrated, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography to get compound **13** (1.33 g, 69%). The resulting compound purity was 93% detected by HPLC. MALDITOF-MS (*m/z*): calculated for C₁₀₉H₁₂₂N₁₀O₂₆Na [M+Na]⁺ 2009.84, found 2009.77. Elemental Analysis: C, 65.85; H, 6.19; N, 7.04, found C, 65.73; H, 6.31; N, 6.92.

Synthesis of prodrug Ibu-Asp₄

The compound **13** (0.10 g, 0.05 mmol) was dissolved in CH₃OH (10 mL), and 10% Pd/C (20 mg) was added to the solution. Subsequently, the mixture was stirred at ambient temperature under H₂ atmosphere for 3 h and then was filtered. The filtrate was concentrated and purified by flash column chromatography to yield prodrug Ibu-Asp₄ (43 mg, 67%). The resulting compound purity was 95% detected by HPLC. MALDITOF-MS (*m/z*): calculated for C₅₃H₇₄N₁₀O₂₆Na [M+Na]⁺ 1289.47, found 1289.40. Elemental Analysis: C, 50.23; H, 5.89; N, 11.05, found C, 50.38; H, 5.76; N, 11.17.

Stability of prodrugs in phosphate buffer and plasma

The high-performance liquid chromatography (HPLC) employed was an Agilent 1100 HPLC liquid chromatographic system. The analysis was carried out on a Waters XTerra RP18 column

(250 mm × 4.6 mm, 5 μm). The mobile phase was composed of methanol/water (70: 30), and the pH was adjusted to 3.0 with 10% phosphoric acid. The UV wavenumber was 265 nm and the flow rate was set at 1.0 mL/min with a 20 μL injection volume.

The chemical stability of the prodrugs including Ibu-Asp₁, Ibu-Asp₂, Ibu-Asp₄, and Ibu-Asp₈, was determined *in vitro* through incubating in the indicated phosphate buffers (pH 2.5, 5.0, 7.4, and 8.0), respectively. In general, 1 mL of the prodrugs methanol solution (500 μM) was added into 4 mL different buffer separately with the final concentration at 100 μM. The entire system was kept at 37°C with continuous shaking at 50 rpm. The medium sample (200 μL) was taken at the predetermined time points (0, 1, 2, 4, 6, 12, 24, 36, and 48 h) and then it was replaced with an equal volume of fresh medium. These samples were analyzed by the aforementioned HPLC method.

What's more, we also incubated these prodrugs in plasma to investigate their metabolic stability. In brief, 1 mL of the prodrugs methanol solution (500 μM) was added into 4 mL plasma (Gibco, USA) with the final concentration at 100 μM. The entire system was kept at 37°C with continuous shaking at 50 rpm. The medium sample (200 μL) was taken at the predetermined time points (0, 1, 2, 4, 6, 12, 24, 36, and 48 h) and then it was replaced with an equal volume of fresh medium. These samples were monitored by the HPLC method described above.

Solubility assessment

The solubility of the prodrugs (Table 2) was investigated in PBS at pH 7.4. In general, excessive amounts of the prodrugs were added into 2 mL PBS, respectively. And the mixtures were kept at 37°C with continuous shaking at 200 rpm for 30 min. After centrifugation at 12000 rpm for 20 min, the drug in the supernatant was monitored by the HPLC method described above.

In vitro HAP binding assay

To determine the binding ability (Figure 4) of these prodrugs to bones, an *in vitro* HAP binding assay was set up using the method described in our previous report (2, 12). Briefly, the different prodrugs were dissolved in 1 mL PBS with the concentration at 5 μM and incubated with 20 mg HAP (Aladdin, China) in tubes. The mixture was shaken gently at 37°C. At the predetermined time (1 h or 24 h), the unbound prodrug was separated from the mixture by centrifugation at 5000 rpm for 3 min and quantified through

HPLC. The binding efficiency was calculated by HAP binding rate (%) = $(C_0 - C)/C_0 \times 100\%$, where C_0 is the concentration at the beginning and C is the concentration of unbound prodrug determined by HPLC.

Cytotoxicity evaluation

The cytotoxicity of the synthesized prodrugs (Ibu-Asp₁, Ibu-Asp₂, Ibu-Asp₄, and Ibu-Asp₈) was evaluated on human embryonic kidneys (HEK293, Chinese Academy of Sciences, China) cells using (3,4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium (MTT) assay. The cells were incubated in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin (Aladdin, China), and streptomycin (Aladdin, China). The cells were cultured at 37°C in a 5% CO₂ humidified environment incubator.

In this process, the cells were seeded into 96-well plates. In each well, a density of approximately 1000 cells was maintained at 37°C for about 24 h. Then these drugs were diluted to predetermined concentrations (1, 10, 20, 50, and 100 μM) with phosphate-buffered saline (PBS). The diluted solutions were then added to each well. After 24 h of incubation, the cells were treated with MTT (20 μL, 5.0 mg/mL) for 4 h. Then, they were thrice washed with PBS. These cells were then isolated using 200 μL DMSO. The OD value of the samples was measured at a wavelength of 490 nm on the microplate reader.

Figure 4. Percentage of prodrugs bound to HAP. ***P < 0.001. (n = 3, mean ± SD)

Acute toxicity *in vivo*

The acute toxicity of these prodrug formulations was investigated using Kunming mice weighing 20–24 g. The mice were randomly divided into five groups ($n = 10$). Before the study, the animals abstained from food for 12 h but they had free access to water. The different prodrugs were injected into animals at the dose of 100, 500, 1000, 5000 mg/kg (calculated as ibuprofen, in PBS) body weight via tail vein. After 24 h, the dead animals were counted in each group. The animals were under observation for another two weeks for any morbidities or mortalities.

Targeting bone *in vivo*

The evaluation of the bone-targeting ability of these Asp-modified prodrugs was investigated according to our previously reported methods (3). Generally, the Kunming mice weighing 20–24 g were randomly divided into five groups ($n = 3$). All animal experiments were performed by following institutional guidelines and approved by the Animal Ethical Experimentation Committee. The animals were kept in well-spaced ventilated cages, and they were fed a healthy and balanced diet. Before injecting the animals, we starved them for 12 h. However, free access to water was provided during the starvation period. A tissue distribution study was conducted in rats to evaluate and compare the bone targeting potential of the prodrugs. Ibuprofen and ibuprofen prodrugs (50 $\mu\text{mol/kg}$, calculated as ibuprofen) dissolved in PBS were injected into the tail vein of each mice.

At the indicated time-points (1, 2, 4, and 8 h), the mice were sacrificed, and the lower tibia bones were removed and rinsed with saline. Then, the tibia bone tissues were dried on a filter paper and subsequently homogenized with twice the amount of saline. After vortex with an equal volume (200 μL) of methanol for five minutes, 10 μL NaOH solution (5 mol/L) was added into the homogenate (200 μL). Then, the hydrolysis was carried out for 10 min at room temperature to release ibuprofen from the prodrugs. Thereafter, the hydrochloric acid solution (10 μL , 5 mol/L) was added to neutralize the NaOH. After vortex for another 5 min, the mixture was centrifuged at 13000 rpm for 10 min and the supernatant was dried by nitrogen gas flow. Subsequently, the residues were dissolved in 200 μL methanol and mixed by the vortex. The solution was centrifuged at 13000 rpm for 10 min, and 20 μL of the supernatant was injected into the HPLC system for analysis.

Statistical analysis

GraphPad Prism 5.0 software (GraphPad, USA) was used for statistical analysis. All data in this study were represented as mean $\pm$ standard deviation (SD). Statistical comparisons were performed by analysis of variance (ANOVA) for multiple groups followed by Student's *t*-test. The significance was defined as follows: *** $P < 0.001$.

RESULTS

Chemistry

The synthesis procedure of prodrugs Ibu-Asp₈ and Ibu-Asp₁ was shown in Figure 2. The bone-targeting dendritic aspartic acid (Asp₂ and Asp₄) derivatives were prepared according to our previously reported work (13). Generally, the ethyl acrylate **1** and benzylamine **2** underwent six steps to generate the dendritic intermediate **6** (13), which further then underwent two steps to yield the dendritic intermediate **8** (13). What's more, the commercially available ibuprofen **9** was coupled with Asp(OBn)-OBn to give the compound **10**, which was debenzylated with 10% Pd/C to generate the prodrug Ibu-Asp₁. Subsequently, coupling Ibu-Asp₁ with the dendron **8** under IBCF/NMM was performed to give the compound **11**, which was deprotected to obtain the prodrug Ibu-Asp₈.

The synthetic routes of prodrugs Ibu-Asp₂ and Ibu-Asp₄ were outlined in Figure 3. In brief, the ibuprofen was respectively conjugated with the intermediate **6** or **8** in the presence of IBCF and NMM to yield compound **12** or **13**, which were then debenzylated with 10% Pd/C to give the desired ligand prodrugs Ibu-Asp₂ and Ibu-Asp₄.

Stability and solubility of prodrugs

Stability study of these prodrugs was performed in various phosphate buffers to simulate the different physiological environments. As shown in Table 1, the pseudo-first-order rate constants (K_{disapp}) and half-lives ($t_{1/2}$) of these prodrugs in PBS were calculated by linear regression of the peak area against time. It could be seen that all the prodrugs were highly stable in pH 7.4 buffer solution, wherein its $t_{1/2}$ value was more than 40 h, moderately stable in pH 5.0, and unstable in pH 2.5 and 8.0 buffer solution. What's more, the metabolic stability of the prodrugs was also investigated in mice plasma at 37°C. Interestingly, all the prodrugs exhibited considerable stability in mice plasma, wherein its $t_{1/2}$ value was about 4.8–6.6 h. All the results indicated that the superior stability of these prodrugs ensured the long circulation in plasma and

Table 1. Stability of ibuprofen prodrugs at 37°C.

Media	Ibu-Asp ₁		Ibu-Asp ₂		Ibu-Asp ₄		Ibu-Asp ₈	
	K _{disapp} (h ⁻¹)	t _{1/2} (h)	K _{disapp} (h ⁻¹)	t _{1/2} (h)	K _{disapp} (h ⁻¹)	t _{1/2} (h)	K _{disapp} (h ⁻¹)	t _{1/2} (h)
pH 2.5	6.7 × 10 ⁻²	10.4	5.0 × 10 ⁻²	13.8	7.1 × 10 ⁻²	9.7	6.0 × 10 ⁻²	11.5
pH 5.0	2.7 × 10 ⁻²	25.8	2.9 × 10 ⁻²	23.6	2.5 × 10 ⁻²	27.4	2.3 × 10 ⁻²	30.1
pH 7.4	1.7 × 10 ⁻²	40.5	1.5 × 10 ⁻²	46.3	1.5 × 10 ⁻²	45.1	1.4 × 10 ⁻²	47.8
pH 8.0	4.3 × 10 ⁻²	16.2	4.5 × 10 ⁻²	15.4	3.7 × 10 ⁻²	18.6	4.0 × 10 ⁻²	17.2
Plasma	14.4 × 10 ⁻²	4.8	13.3 × 10 ⁻²	5.2	11.3 × 10 ⁻²	6.1	10.5 × 10 ⁻²	6.6

enough time to reach the bone tissue before they were metabolized.

The results of the solubility assessment were presented in Table 2, which clearly demonstrated the solubility of the prodrugs. It was revealed that the dendritic aspartic acid could significantly increase the solubility of ibuprofen. And this highly increased solubility could be attributed to the surface carboxy groups that were available to interact with water molecules. The decoration of dendritic aspartic acid increased the solubility of ibuprofen to 1.05–3.89 mM in water, which indicated that the hydrophilic dendrimers were effective vehicles to heighten the solubility of insoluble drugs. It also revealed that the Asp₈ had more carboxy groups at the surface area to interact with water molecules with higher ability than that of other groups. To sum up, all the results suggested that dendritic Asp is a superior strategy to enhance the solubility and delivery capacity of poorly water-soluble drugs.

In vitro HAP binding assay

As shown in Figure 4, it was revealed that all of the Asp-modified ibuprofen prodrugs exhibited excellent HAP affinity, whereas the free ibuprofen was at best negligibly bound, which suggested that these dendritic aspartic acid moieties played a crucial role in the HAP binding process. The binding rates of these prodrugs evidently exceeded 60%, especially for the group of Ibu-Asp₈ with binding rates of over 90%. In addition, the results in Figure 4 also showed that the binding rates dramatically culminated rapidly. And the number of Asp that modified on the drug was correlated with the binding efficiency. Besides, no significant difference was found between the HAP affinity within 1 h and 24 h. All the results suggested that the bone-targeting property of ibuprofen was

Table 2. Water solubility of prodrugs in PBS.

	Number of -COOH	Water solubility (mM)
Ibuprofen	1	0.22
Ibu-Asp ₁	2	1.05
Ibu-Asp ₂	4	1.80
Ibu-Asp ₄	8	2.57
Ibu-Asp ₈	16	3.89

enhanced, which was attributed to the modification of dendritic Asp.

Cytotoxicity evaluation

Cytotoxicity is one of the most critical factors to be considered in selecting a carrier for drug delivery in the biological system. In this study, the natural amino acids (Asp) were chosen to conjugate with ibuprofen to target bone tissue. As shown in Figure 5, all the prodrugs did not exhibit any toxicity on HEK293 cells at the dose level of 1–100 µM, which suggested that the dendritic

Figure 5. Cytotoxicity of Asp-modified prodrugs against HEK293 cells. (n = 3, mean ± SD)

Figure 6. Ibuprofen concentration after injection of free ibuprofen, Ibu-Asp₁, Ibu-Asp₂, Ibu-Asp₄, and Ibu-Asp₈ in the femur tissue. *** $P < 0.001$, ($n = 5$, mean $\pm$ SD). ND means not detected in the ibuprofen group at 1 h.

aspartic acid was non-toxic or low-toxic in tested concentrations.

Acute toxicity

It is important to investigate the acute toxicity *in vivo* to ensure the biocompatibility of these prodrugs. The drugs were injected into the mice respectively with different doses to establish their safety for application in drug delivery. After 14 days of observation, no serious adverse effects or signs of morbidity were found in any animal in all groups, which confirmed the safety of the Asp-modified prodrugs.

Targeting bone *in vivo*

To further make sure whether the dendritic Asp-modified prodrugs could target bone tissue, the bone-targeting ability *in vivo* was investigated at the fixed time intervals after injection. The ibuprofen was quantified calculated as the percentage of injected dose (%ID). As shown in Figure 6, modification of dendritic Asp could obviously enhance the accumulation of ibuprofen in the femur, which was due to the outstanding bone-targeting ability of aspartic acid. And it is interesting to note that an increasing trend was exhibited with the increase of the number of aspartic acid residues. The concentrations of ibuprofen in the Ibu-Asp₁, Ibu-Asp₂, Ibu-Asp₄, and Ibu-Asp₈ groups were 2.7, 3.3, 3.9, and 5.0 times higher than that of the ibuprofen group, respectively. This

effect was attributed to the peculiarity of Asp, which had a strong affinity to bones. Overall, the dendritic Asp carriers were efficient as bone-targeting moieties, and the conjugation with ibuprofen ensured the increased accumulation of the drug in the bone region, which could reduce the terrible side-effects of the drug on other organs. In general, the results provided a novel and effective entry to the development of bone-targeting drugs.

DISCUSSION

Bone diseases are the most common degenerative disease in bone tissue (15). The incidence of osteoarthritis has dramatically increased all across the world. This disease has affected about 10% of people over the age of 60. Osteoarthritis is mainly characterized as joint pain and stiffness,

which could seriously affect patients' health and quality of life (16). It is also one of the main causes that result in disability. Currently, the treatment for osteoarthritis is usually to relieve pain, prevent and delay disease progression, protect joint function and improve quality of life (17). There is no radical treatment for it and the pathogenesis of osteoarthritis is still unclear at present.

In recently, many studies have reported that NSAIDs, especially ibuprofen, have been widely used in treating osteoarthritis (18-19). Owing to the low blood flows in bone tissue and the low targeting ability of ibuprofen, it is prescribed in higher doses to achieve satisfactory therapeutic effects, which may cause strong side effects and more severe toxicity to the gastrointestinal tract, liver, and kidneys. Therefore, new promising strategies must be developed to effectively deliver drugs to the site of bone tissue for the treatment of osteoarthritis.

In order to develop an efficient bone-targeting drug delivery system to greatly enhance the capacity of ibuprofen to target bone tissue, a series of novel dendritic aspartic acid-modified ibuprofen prodrugs were designed and synthesized in this study. These prodrugs had superior properties, such as the moderate stability in PBS and plasma, which could ensure the drugs had plenty of time to reach the bone before being hydrolyzed. Furthermore, it was also found that the prodrugs had low cytotoxicity both *in vitro* and

in vivo, indicating that these prodrug drug delivery systems were safe and non-toxic to be further used *in vivo*. Moreover, the Asp-modified prodrugs had an obvious increase in targeting ability to bone *in vitro* and in mice, and this targeting capacity was positively correlated with the number of Asp residues.

Interestingly, this work proves that the dendritic aspartic acid was a superior carrier and enhances the delivery of ibuprofen to target the bone tissue. All the results *in vitro* and *in vivo* suggested that these prodrugs indeed had the potential to improve the clinical therapeutic effects of osteoarthritis. The results are very promising and more work is in progress, that is, better animal models are being developed for comprehensive evaluations *in vivo*. We will surely report the biological activities of this conjugate in due time.

CONCLUSION

Site-specific bone drug delivery via targeting function approach has generated considerable interest. This strategy not only enhances the potency of drugs but also diminishes their side effects and toxicity. Here, several novel dendritic prodrugs were designed and synthesized for bone-targeting effects. These drugs possess good biosecurity and high HAP binding efficiency *in vitro*. And the results *in vivo* showed that all the prodrugs could obviously enhance the accumulation of ibuprofen in the femur, among which, the Ibu-Asp₈ groups showed the concentration of ibuprofen was 5.0 times higher than that of the ibuprofen group. Therefore, the results provided a promising and effective entry to the development of bone-targeting drugs.

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Conflict of interest

The authors declare no competing financial interest.

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