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Original Article

In vitro and *in vivo* investigation of dexibuprofen derivatives for CNS delivery

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Aim: Dexibuprofen, the S(+)-isomer of ibuprofen, is an effective therapeutic agent for the treatment of neurodegenerative disorders. However, its clinical use is hampered by a limited brain distribution. The aim of this study was to design and synthesize brain-targeting dexibuprofen prodrugs and to evaluate their brain-targeting efficiency using biodistribution and pharmacokinetic analysis.
Methods: *In vitro* stability, biodistribution and pharmacokinetic studies were performed on male Sprague-Dawley rats. The concentrations of dexibuprofen in biosamples, including the plasma, brain, heart, liver, spleen, lung, and kidney, were measured using high pressure lipid chromatography (HPLC). The pharmacokinetic parameters of the drug in the plasma and tissues were calculated using obtained data and statistics.
Results: Five dexibuprofen prodrugs that were modified to contain ethanolamine-related structures were designed and synthesized. Their chemical structures were confirmed using ^{1}H NMR, ^{13}C NMR, IR, and HRMS. In the biodistribution study, 10 min after intravenous administration of dexibuprofen (11.70 mg/kg) and its prodrugs (the dose of each compound was equivalent to 11.70 mg/kg of dexibuprofen) in male Sprague-Dawley rats, the dexibuprofen concentrations in the brain and plasma were measured. The C_{brain}/C_{plasma} ratios of prodrugs 1, 2, 3, 4, and 5 were 17.0-, 15.7-, 7.88-, 9.31-, and 3.42-fold higher than that of dexibuprofen, respectively (P<0.01). Thus, each of the prodrugs exhibited a significantly enhanced brain distribution when compared with dexibuprofen. In the pharmacokinetic study, prodrug 1 exhibited a brain-targeting index of 11.19 {$DTI=(AUC_{brain}/AUC_{plasma})_1/(AUC_{brain}/AUC_{plasma})_{dexibuprofen}$}.
Conclusion: The ethanolamine-related structures may play an important role in transport across the brain blood barrier.

Keywords: dexibuprofen; ethanolamine; prodrugs; brain targeting

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Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), including ibuprofen, naproxen and flubiprofen, are widely used as neuroprotective agents in the treatment of neurodegenerative disorders[1-3]. However, the clinical application of NSAIDs is impeded by limited brain accessibility. Several *in vivo* animal studies have shown that the concentrations of ibuprofen or flurbiprofen in the brain achieved at normal plasma concentrations are only 0.9–1.5 μmol/L[4–6]. It has also been found that most NSAIDs, including naproxen, ibuprofen, flurbiprofen, ketoprofen, and indomethacin, exhibit very limited distribution in the CNS, with steady state brain/plasma concentration ratios of 0.01– 0.05[7]. Several factors account for the low CNS distribution of NSAIDs, including high plasma protein binding rates, limited passive transport and efflux transport[8, 9]. Therefore, it is critical to enhance the brain distribution of NSAIDs to achieve therapeutic concentrations at normal doses.

Over 90% of therapeutic agents are excluded from CNS by the blood brain barrier (BBB), which is composed of the tightly connected endothelial cells of brain capillaries and the surrounding astrocytes and pericytes[10, 11]. Typically, molecules that are capable of crossing the BBB by passive diffusion are small (M_r<500 Da), lipophilic and uncharged at physiological pH[12, 13]. Various facilitated or energy-dependent transporters and receptors are highly expressed at the BBB; examples of such transporters include members of the organic cation transporter (OCT) families, organic anion transporters (OATs) and multidrug resistance proteins (MRPs)[14, 15]. A number of studies aimed at enhancing the distribution of therapeutics in the brain based on carrier- and receptor-mediated transport systems have been performed[16–18]. Due to the negative charge carried by the BBB, which expresses acetylneuraminic acid on the capillary luminal membrane and heparin sulfate on

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the basal lamina, compounds carrying positive charges have also been designed to cross through adsorption-mediated transport[19, 20].

Although several NSAID prodrugs have been synthesized to enhance the anti-inflammation activity or reduce the gastrointestinal toxicity of their parent compounds, few studies have investigated the targeted delivery of NSAIDs to the CNS[21, 22]. In a previous study, a series of NSAID prodrugs were synthesized to enhance transport to the brain. The carboxylic groups of NSAIDs were coupled with a 1,4-dihydro-1-methylpyridine-3-carboxylate moiety[23]. The results of this study revealed that all of the prodrugs were more lipophilic than their corresponding parent compounds, suggesting that prodrugs may exhibit better brain penetration. In another study, six ibuprofen prodrugs containing a proline moiety or related structures were synthesized and evaluated[24]. The results revealed that the synthesized prodrugs possessed anti-inflammatory properties; furthermore, three of these compounds inhibited lipoxygenase.

In this study, the *S*(+)-isomer of ibuprofen, (2*S*)-2-(4-isobutylphenyl)propionic acid (dexibuprofen), was used as a model drug because it exhibits better anti-inflammatory effects and fewer side effects than ibuprofen. Ethanolamine and its related structures were chosen as modifications to dexibuprofen in the preparation of prodrugs. Although some ethanolamine derivatives of NSAIDs were synthesized to improve the anti-inflammatory activity of parent compounds and decrease gastrointestinal toxicity[25, 26], an investigation of their potential as brain targeting agents has not yet been performed. Ethanolamine is a commonly found small molecule. Ethanolamine and its related structures are not only present in endogenous substances including phospholipids (*eg*, neurotransmitters) but also in various CNS-accessible therapeutics, such as meclofenoxate, cetirizine and procaine. Containing an amino group, ethanolamine-related structures are positively charged under physiological conditions, which may allow them to interact with the negatively charged BBB. Moreover, prodrugs that are modified by ethanolamine-related structures may act as substrates of the organic cation or choline transporters that are located at the BBB. After being transported across the BBB, the prodrugs can be further hydrolyzed by esterases in the brain to release dexibuprofen.

In this study, a series of dexibuprofen prodrugs modified by ethanolamine-related structures were designed and synthesized. The active carboxyl group of dexibuprofen was coupled with ethanolamine via an ester bond to give prodrug **4**. The amino group of ethanolamine was substituted by one methyl group, two methyl groups, two ethyl groups or one acetyl group to yield prodrugs **3**, **1**, **2**, and **5**, respectively. The stability of these prodrugs in the plasma and brain homogenates of Sprague-Dawley rats was tested. Biodistribution and pharmacokinetic studies of dexibuprofen and the prodrugs were performed in male Sprague-Dawley rats to evaluate the brain-targeting efficiency of the prodrugs. The influence of their chemical structures on their transport efficiency is discussed.

Materials and methods

Materials

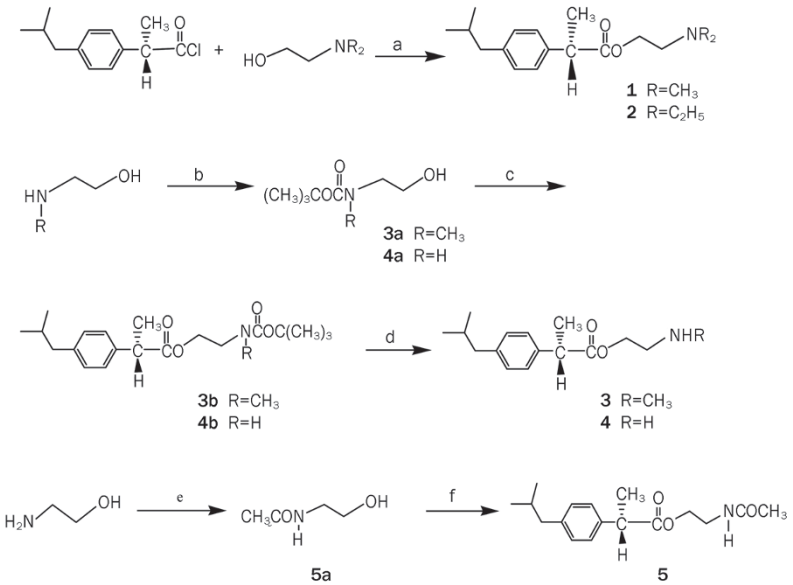
Dexibuprofen (99.8% purity) was purchased from the Fourth Pharmaceutical Company of Suzhou (Suzhou, China). Acetonitrile (HPLC grade) was purchased from Kemiou (Tianjin, China). Water was prepared using an Aquapro water purification system (Chongqing, China). All of the other chemicals and reagents used in this study were of analytical grade and obtained commercially. TLC (thin-layer chromatograpy) (silica gel GF254) was used to detect spots using UV radiation. The purification of intermediates and final synthesized compounds was achieved by flash chromatography on silica gel. ^{1}H-NMR and ^{13}C-NMR analyses were performed using an AMX-400 Bruker Spectrometer. Infrared (IR) spectra were obtained using a Perkin-Elmer 983 Infrared Spectrophotometer (USA). MS spectroscopy was recorded on a Waters Q-TLF Premier mass spectrometer (USA) utilizing electrospray ionization (ESI). Optical rotations were determined using a Perkin–Elmer 341 polarimeter.

Chemistry

The synthesis routes of the dexibuprofen derivatives (**1**, **2**, **3**, **4**, and **5**) are outlined in Scheme 1. Dexibuprofen chloride was prepared as described by Song *et al*[27]. In brief, dexibuprofen was treated with thionyl chloride ($SOCl_2$) at reflux for 4 h. The reaction mixture was concentrated under reduced pressure. The resulting dexibuprofen chloride was coupled with the hydroxyl group of ethanolamine-related structures via an ester bond. To generate *N*,*N*-disubstituted ethanolamine, esterification was performed in a one-step reaction by mixing commercially available *N*,*N*-dimethylaminoethanol or *N*,*N*-diethylaminoethanol with dexibuprofen chloride in anhydrous dichloromethane (CH_2Cl_2) in the presence of triethylamine (TEA) to give target prodrug **1** or **2** in high yield[26]. For aminoethanol or 2-(methylamino)ethanol, which has a free H atom at the amino group, the amino group was protected by ditertbutyldicarbonate in the first step. The obtained intermediate, either **3a** or **4a,** was then coupled with dexibuprofen chloride in the presence of TEA to give either prodrug **3b** or **4b**. Prodrug **3b** or **4b** was then deprotected by HCl in ethyl acetate to yield prodrug **3** or **4**, respectively. In the preparation of *N*-acetyl ethanolamine dexibuprofen ester, *N*-acetylethanolamine was synthesized in the first step. Ethanolamine was dissolved in dioxane with added acetyl chloride in the presence of sodium hydride (NaOH) to yield prodrug **5a**. Prodrug **5a** was then condensed with dexibuprofen chloride to yield final compound **5**.

Animals

Male Sprague-Dawley rats (200–230 g) were provided by the Laboratory Animal Center of Sichuan University (Chengdu, China). All rats were maintained under standard conditions with access to food and water *ad libitum*. All animal study procedures were approved by the Sichuan University Animal Ethical Experimentation Committee according to the require-



Scheme 1. Synthesis of dexibuprofen derivatives modified by ethanolamine related structures (**1**, **2**, **3**, **4**, **5**). Reagents and conditions: (a) CH_2Cl_2, TEA; (b) $(Boc)_2O$, NaOH; (c) dexibuprofen chloride, TEA; (d) EtOAc, HCl, RT; (e) CH_3COCl, NaH, dioxane; (f) dexibuprofen chloride, TEA.

ments of the National Act on the use of experimental animals (China).

In vitro stability

To determine whether ester prodrugs of dexibuprofen were hydrolyzed into free dexibuprofen in rat plasma and brain homogenates, we incubated prodrugs **1**, **2**, and **3** with the prepared cultures at 37 °C. At a specific time point, aliquots of rat plasma or brain homogenate were collected and processed. The released dexibuprofen concentrations were determined using high pressure liquid chromatography (HPLC) with a UV detector.

Rat plasma was prepared immediately before each experiment. Briefly, blood was obtained from the ocular artery and placed in a heparinized EP tube prior to centrifugation at 1110×*g* for 5 min. The resulting plasma was incubated with prodrugs without dilution. Rat brains were removed and homogenated with 0.9% sterile saline (g/mL) at a ratio of 1:2 immediately before use.

Prodrugs **1**, **2**, and **3** (2 mg each) were dissolved in a mixed solvent solution (2 mL) of ethanol, 0.9% saline and propylene glycol at a ratio of 1:2:2. The resulting solution (0.2 mL) was then added in the plasma or brain homogenates (9.8 mL), resulting in a final concentration of 20 μg/mL. The mixtures were incubated at 37 °C. Samples were prepared at predetermined time points (plasma: 0, 5, 15, 20, 25, 30, and 40 min; brain homogenates: 0, 0.5, 1, 2, 3, 4, 6, and 12 h). Aliquots of plasma (200 μL) or brain homogenate (500 μL) were withdrawn to determine the concentration of released dexibuprofen. Naproxen solution in methanol (1.266 μg/mL, 150 μL) was added immediately. A total of 1 mL or 2 mL methanol was then added to the plasma or brain homogenates, respectively, to prevent further hydrolysis of the prodrugs. The biosample processing method and chromatographic conditions are described in the following sections.

Hydrolysis half-life measurements were used to evaluate the stability of the prodrugs. These values were obtained using the formula $t_{1/2}$=0.693/K. The K values were calculated from the slope rate of the profile, which was obtained from a plot of time versus the natural logarithm of the remaining prodrug in the medium. The remaining prodrug concentration was determined by subtracting the released dexibuprofen concentration from the initial concentration of added prodrug in the medium.

Processing of biosamples

In an EP tube, an aliquot of 200 μL plasma or 500 μL tissue homogenates was mixed with naproxen solution in methanol (1.266 μg/mL, 150 μL), which was used as an internal standard. A total of 1 or 2 mL of methanol was then added to the plasma and tissue homogenates, respectively. The mixtures were then vortexed for 10 min, followed by centrifugation at 1665×*g* for 10 min. The supernatant was then transferred to an EP tube and dried at 35 °C under air flow. The residue was then ultrasonically redissolved in 150 μL of methanol for 5 min. After centrifugation at 10010×*g* for 10 min, 20 μL of the obtained supernatant was injected into an HPLC system to determine the dexibuprofen concentration.

Chromatographic conditions

An HPLC-based method was developed to determine the concentration of dexibuprofen in biosamples including plasma, brain, heart, liver, spleen, lung and kidney. The HPLC system (Waters, USA) was equipped with a Waters Delta 600 pump, a Waters 2487 Dual λ absorbance detector, a Waters 600 controller, a model 100 column heater (CBL photoelectron technology) and a 20-μL injector loop. The Kromasil C-18 column (150 mm×4.6 mm, ODS, 5 μm) was protected by a Phenomenex guard column (4 mm×3 mm, ODS). The column was eluted using a mobile-phase system containing acetonitrile/water (49/51), with 0.2% (*v*/*v*) triethylamine added. Phosphoric acid was used to adjust the pH to 2.5. The mobile

phase was then filtered and degassed by sonication before use. The flow rate used was 1.0 mL/min, and the diode-array detector was operated at 220 nm.

Naproxen was used as an internal standard. An HPLC assay for determining the concentration of dexibuprofen in different tissues was developed, including protocols for generating calibration curves and determining intra- and inter-day precision and extraction recoveries. Under the optimized HPLC conditions, the retention times of naproxen and dexibuprofen were 5.5 and 13.83 min, respectively. These values were well separated from endogenous substances and prodrugs **1**, **2**, **3**, **4**, and **5,** which had retention times of 2.730, 3.595, 2.265, 2.730, and 12.710 min, respectively (Figure 1).

Biodistribution and pharmacokinetic studies of dexibuprofen and its prodrugs in rats

To further evaluate the brain targeting efficiency of the prodrugs, a biodistribution and pharmacokinetic study was performed *in vivo*. Male Sprague-Dawley rats (200–230 g) were provided by the Laboratory Animal Center of Sichuan University. Prodrugs and dexibuprofen were injected through the caudal vein. For each sampling time point, five non-fasted male rats were treated with a single dose of either dexibuprofen (11.70 mg/kg) or prodrug **1** (15.73 mg/kg), **2** (17.34 mg/kg), **3** (14.94 mg/kg), **4** (14.15 mg/kg), or **5** (16.53 mg/kg). Each prodrug dose was equivalent to 11.70 mg/kg of dexibuprofen. Prior to administration, the compounds were dissolved in a mixed solvent containing a 1:2:2 ratio of ethanol, 0.9% saline and propylene glycol, to a final concentration of 60 mmol/L. In the biodistribution study, the rats were sacrificed 10 min after the injection of either dexibuprofen or prodrugs **1**, **2**, **3**, **4**, or **5**. For the pharmacokinetic investigations of dexibuprofen and prodrug **1**, the rats were sacrificed at 5, 10, 15, 30, 45, 60, 120, and 240 min post-dosing.

At a specific time point, the rats were bled from the ocular artery and sacrificed by cervical dislocation. Blood samples were collected and placed into heparinized EP tubes. Immediately following collection, the samples were centrifuged at 1110×*g* for 5 min. The plasma was then stored at -20 °C until assays were performed. Tissue samples, including samples from the brain, heart, liver, spleen, lung, and kidney, were removed and flushed with saline. The tissue samples were then homogenized using 0.9% saline (g/mL) at a ratio of 1:2. The homogenates were stored at -20 °C until assays were performed. Aliquots of 200 μL plasma or 500 μL tissue homogenates were processed, and the released dexibuprofen was measured as described above.

Data analysis

The pharmacokinetic parameters of dexibuprofen and the prodrugs were described as a two-compartment open model. The pharmacokinetic parameters in the plasma and tissues were calculated using Data and Statistics software pakage (DAS, Shanghai, China). The area under the curve (AUC), the maximal concentration (C_{max}) and the mean residence time (MRT_{0-t}) for the plasma, brain, heart, liver, spleen, lung and kidney were calculated separately. Statistical evaluation was performed using analysis of variance followed by a *t*-test. *P* values less than 0.01 and 0.05 were considered significant. The data shown represent mean values±SD.

The relative uptake efficiency (RE), concentration efficiency (CE) and drug targeting index (DTI) were calculated to evaluate the brain targeting efficiency of the prodrugs. The ratio of the dexibuprofen concentration in the brain compared to that in the plasma $\{(C_{brain}/C_{plasma})_{dexibuprofen}\}$ was also used to evaluate brain targeting efficiency. The values of RE, CE, and DTI are defined as follows:

$RE=(AUC)_{prodrug}/(AUC)_{dexibuprofen}$,

$CE=(C_{max})_{prodrug}/(C_{max})_{dexibuprofen}$ and

$DTI=(AUC_{brain}/AUC_{plasma})_{prodrug}/(AUC_{brain}/AUC_{plasma})_{dexibuprofen}$

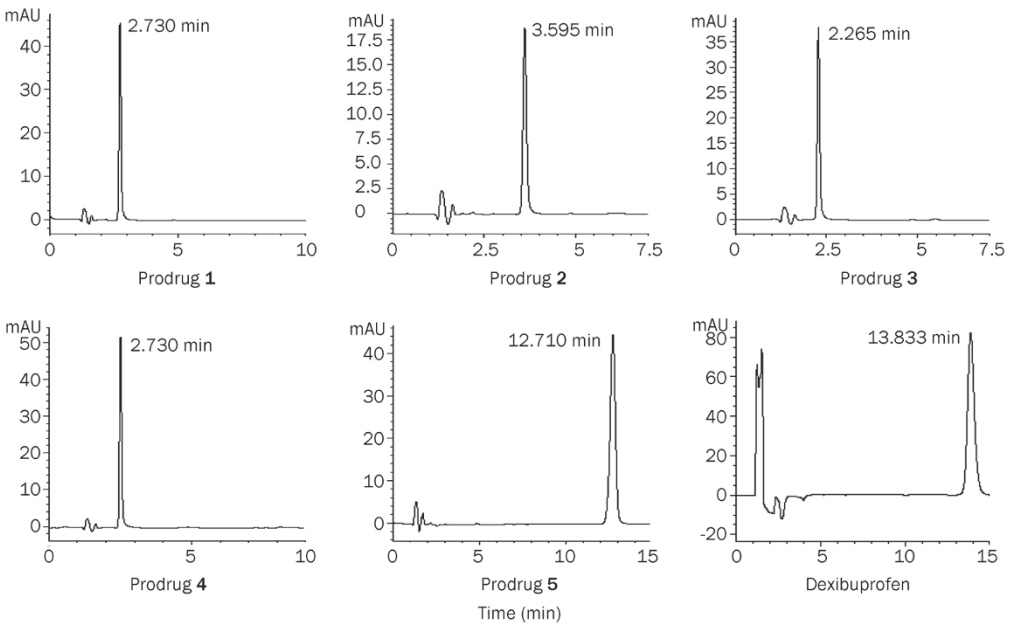


Figure 1. HPLC chromatograms of dexibuprofen and prodrugs **1**, **2**, **3**, **4**, and **5**. Compound was dissolved in methanol with concentration of 5 μg/mL.

Results

In vitro stability

The *in vitro* stability studies revealed that all of the tested dexibuprofen derivatives released dexibuprofen when incubated with rat plasma and brain homogenates (Table 1). These result suggest that compounds **1**, **2**, and **3** were rapidly hydrolyzed in rat plasma. The half-lives of compounds **1** and **2** were less than 5 min. The half-life of prodrug **3** was 11.63±1.04 min. These results imply that prodrugs **1**, **2**, and **3,** which all contain ester bonds, may be substrates of specific esterases in the plasma and are efficiently hydrolyzed. Investigation of the stability of the prodrugs in rat brain homogenates showed that compounds **1**, **2**, and **3** were cleaved by enzymes at different rates. The hydrolysis half-lives of compounds **1**, **2**, and **3** were 0.273±0.009, 1.150±0.061, and 3.712±0.174 h, respectively. The results suggested that prodrug **3** was more stable in brain homogenates than either compound **1** or **2**, which was consistent with the findings for the plasma.

Table 1. Hydrolysis half-life ($t_{1/2}$) of prodrugs **1**, **2**, **3** in rat plasma or brain homogenate at 37 ℃ (n=3).

Compound	$t_{1/2}$	
	Plasma	Brain homogenate
1	<5.0 min	0.273±0.009 h
2	<5.0 min	1.150±0.061 h
3	11.63±1.04 min	3.712±0.174 h

Biodistribution and pharmacokinetic study of dexibuprofen and prodrugs in rats

To evaluate the brain-targeting efficiency of prodrugs **1**, **2**, **3**, **4**, and **5** (Scheme 1), a biodistribution study was performed. We determined the concentrations of dexibuprofen in the plasma, brain, heart, liver, spleen, lung and kidney 10 min after intravenous administration of dexibuprofen (11.70 mg/kg) or prodrugs (at doses equivalent to 11.70 mg/kg of dexibuprofen). The concentration of dexibuprofen in the brain versus that in the plasma $(C_{brain}/C_{plasma})_{dexibuprofen}$ was used to evaluate the brain-targeting efficiency of the compounds. The concentrations of dexibuprofen in the brain and plasma 10 min after the injection of dexibuprofen or prodrug **1**, **2**, **3**, **4**, or **5** are shown in Figure 2 (n=5). Prodrugs **1**, **2**, and **3** exhibited enhanced drug concentrations in the brain in comparison with dexibuprofen (P<0.01). The $(C_{brain}/C_{plasma})_{dexibuprofen}$ ratios 10 min after administration of dexibuprofen or prodrug **1**, **2**, **3**, **4**, or **5** were 0.037±0.011, 0.626±0.091, 0.576±0.119, 0.290±0.114, 0.342±0.132, and 0.126±0.026, respectively (Figure 3; n=5). The $(C_{brain}/C_{plasma})_{dexibuprofen}$ ratios of prodrugs **1**, **2**, **3**, **4**, and **5** were 17.0-, 15.7-, 7.88-, 9.31-, and 3.42-fold higher, respectively, than that of dexibuprofen. Thus, the prodrugs exhibit good brain-targeting efficiency (P<0.01), with a relative efficiency of **1>2>4>3>5**. Among prodrugs (2*S*)-2-(4-isobutylphenyl) propionic acid 2-dimethylaminoethyl ester (prodrug **1**), exhibited the highest distribution efficiency in the brain.

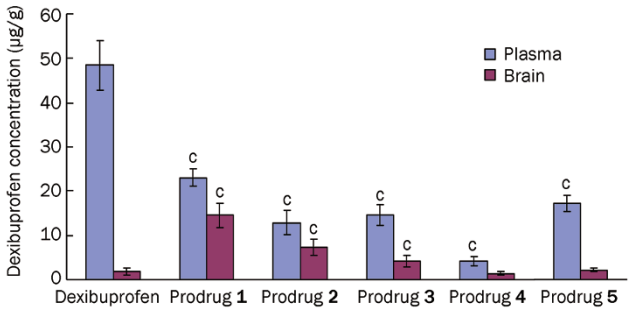


Figure 2. Dexibuprofen concentrations (μg/g) in the plasma and brain 10 min after intravenous administration of dexibuprofen (11.70 mg/kg) or prodrugs **1**, **2**, **3**, **4**, **5** (each compound was equivalent to 11.70 mg/kg of dexibuprofen) in rats. Each point represents the mean±SD of five experiments. ^cP<0.01 *vs* dexibuprofen.

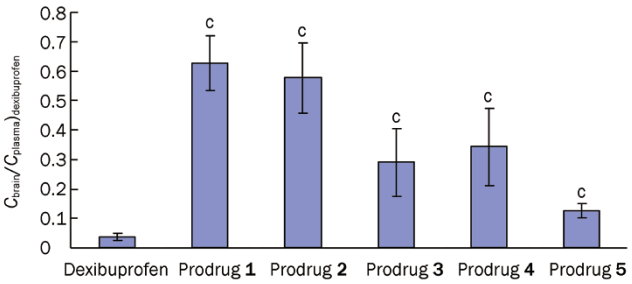


Figure 3. Comparison of $(C_{brain}/C_{plasma})_{dexibuprofen}$ ratios of dexibuprofen and prodrugs. C_{brain}, C_{plasma}: dexibuprofen concentration (μg/g) in the brain or plasma 10 min after intravenous administration of dexibuprofen (11.70 mg/kg) or prodrugs **1**, **2**, **3**, **4**, **5** (each compound was equivalent to 11.70 mg/kg of dexibuprofen) in rats. Each point represents the mean±SD of five experiments. ^cP<0.01 *vs* dexibuprofen.

We further investigated the biodistribution and pharmacokinetic parameters of dexibuprofen and prodrug **1** in Sprague-Dawley rats. The concentrations of dexibuprofen after the systemic administration of dexibuprofen (11.70 mg/kg) and prodrug **1** (15.73 mg/kg, a dose equivalent to 11.70 mg/kg of dexibuprofen) were determined and statistically analyzed. The dexibuprofen concentration profile versus time is shown in Figure 4. The C_{brain}/C_{plasma} ratio versus time is shown in Figure 5. The pharmacokinetic parameters are reported in Table 2. An evaluation of the targeting efficiency of prodrug **1** is shown in Table 3.

The results revealed that 5 min after the administration of dexibuprofen, the concentration in the plasma was initially very high, with a C_{plasma} of 64.44±14.00 μg/g (Figure 4). However, at the same time point, the concentration of dexibuprofen in the brain was only 2.81±1.16 μg/g. At different time points, the C_{brain}/C_{plasma} ratios were consistently low after the administration of dexibuprofen, never exceeding 0.05 (Figure 5). The AUC_{brain}/AUC_{plasma} ratio was only 0.025. These results indicated that the BBB was able to strongly prevent the entry of dexibuprofen into the brain. This finding is in agreement with

Table 2. Pharmacokinetic parameters (calculating from the measured dexibuprofen concentrations) in rat plasma and tissues after intravenous administration of dexibuprofen (11.70 mg/kg) or prodrug **1** (15.73 mg/kg, equivalent to 11.70 mg/kg of dexibuprofen). Each point represents the mean±SD of five experiments. $^{b}P<0.05$, $^{c}P<0.01$ *vs* dexibuprofen.

Tissue	Dexibuprofen			Prodrug **1**		
	$AUC_{0\to t}$ (μg/g·h)	C_{max} (μg/g)	MRT_{0-t} (h)	$AUC_{0\to t}$ (μg/g·h)	C_{max} (μg/g)	MRT_{0-t} (h)
Plasma	44.79±7.73	64.44±14.00	0.789±0.046	28.70±2.35^{b}	29.74±2.97^{c}	0.900±0.119
Brain	1.10±0.41	2.81±1.16	0.274±0.023	7.89±1.23^{c}	21.79±3.37^{c}	0.237±0.034
Heart	8.11±1.39	11.99±3.70	0.767±0.158	6.66±1.04	10.46±1.78	0.733±0.167
Liver	14.03±3.91	16.94±3.25	0.961±0.086	12.98±5.12	10.34±1.49^{b}	1.052±0.16
Spleen	4.01±0.62	5.60±1.27	1.128±0.357	4.12±1.12	6.24±3.15	0.591±0.096^{b}
Lung	10.60±2.10	13.19±2.56	1.220±0.072	22.07±4.84^{c}	29.03±4.41^{c}	0.853±0.037^{c}
Kidney	6.34±1.10	9.43±2.72	0.700±0.077	7.58±1.34	11.59±2.68	0.592±0.043

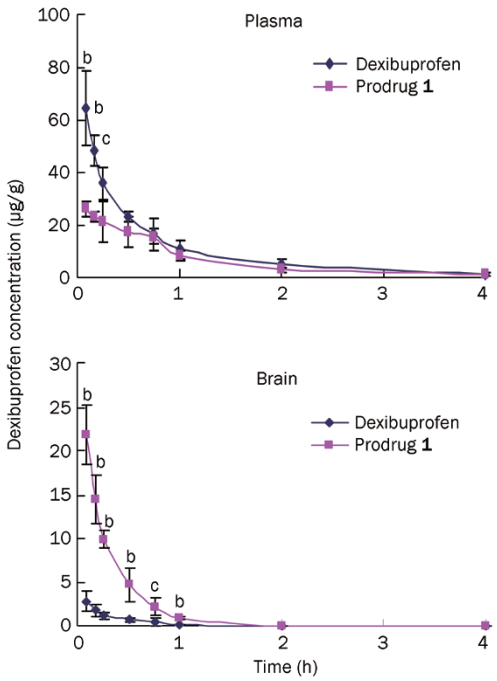


Figure 4. Dexibuprofen concentrations in the plasma and brain (μg/g) *vs* time (h) after intravenous administration of dexibuprofen (11.70 mg/kg) and prodrug **1** (15.73 mg/kg, equivalent to 11.70 mg/kg of dexibuprofen) in rats. Each point represents the mean±SD of five experiments. $^{b}P<0.05$, $^{c}P<0.01$ *vs* dexibuprofen.

Table 3. Targeting efficiency of Prodrug **1** compared with dexibuprofen after intravenous administration of dexibuprofen (11.70 mg/kg) and 1 (15.73 mg/kg, equivalent to 11.70 mg/kg of dexibuprofen) in male rats (mean±SD, n=5). $^{a}RE=(AUC)_1/(AUC)_{dexibuprofen}$. $^{b}CE=(C_{max})_1/(C_{max})_{dexibuprofen}$. $^{c}DTI=(AUC_{tissue}/AUC_{plasma})_1/(AUC_{tissue}/AUC_{plasma})_{dexibuprofen}$.

	Brain	Heart	Liver	Spleen	Lung	Kidney
RE^{a}	7.17	0.82	0.93	1.4	1.24	1.49
CE^{b}	7.75	0.87	0.61	1.11	2.20	1.23
DTI^{c}	11.19	1.28	1.44	2.18	1.94	2.32

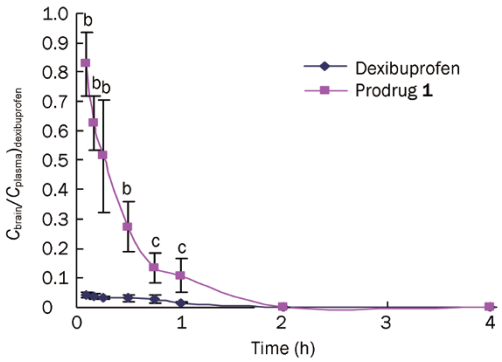


Figure 5. $(C_{brain}/C_{plasma})_{dexibuprofen}$ ratios of dexibuprofen or prodrug **1** versus time (h). C_{brain}, C_{plasma}: dexibuprofen concentration (μg/g) in the brain or plasma after intravenous administration of dexibuprofen (11.70 mg/kg) or prodrug **1** (15.73 mg/kg, equivalent to 11.70 mg/kg of dexibuprofen) in rats. Each point represents the mean±SD of five experiments. $^{b}P<0.05$, $^{c}P<0.01$ *vs* dexibuprofen.

a previous study that revealed that most NSAIDs exhibited very limited distribution in the CNS, with steady state brain/plasma concentration ratios of 0.01–0.05[7].

Compared with the administration of free dexibuprofen, the concentration of dexibuprofen in the plasma after administration of prodrug **1** was moderate. The concentration in the plasma was 29.74±2.97 μg/g 5 min after the administration of prodrug **1** (Figure 4), which was lower than that of dexibuprofen (P<0.01). With moderate concentrations in the plasma, prodrug **1** exhibited a high concentration of dexibuprofen (21.79±3.37 μg/g) in the brain 5 min after administration (Figure 4). The $(C_{brain}/C_{plasma})_{dexibuprofen}$ ratio of prodrug **1** was 0.829 at 5 min, much higher than that of dexibuprofen, which was only 0.044 (Figure 5) (P<0.01). These results revealed almost no barrier effect of the BBB in response to prodrug **1**. However, the high concentration of dexibuprofen observed decreased rapidly, with an MRT of 0.237±0.034 h, showing an active exclusion effect of the BBB toward dexibuprofen.

The AUC_{0-t} values after administration of dexibuprofen and prodrug **1** were 1.10±0.41 and 7.89±1.23 μg/g·h in the brain, respectively. The C_{max} values after the administration

of dexibuprofen and prodrug **1** were 2.81±1.16 and 21.79±3.37 μg/g in the brain, respectively (Table 2). The relative efficiency (RE=$AUC_1/AUC_{dexibuprofen}$) was 7.17, and the $(C_{max})_1/(C_{max})_{dexibuprofen}$ ratio was 7.75 in the brain (Table 3). The drug targeting index {DTI=$(AUC_{brain}/AUC_{plasma})_1/(AUC_{brain}/AUC_{plasma})_{dexibuprofen}$} was 11.19. Taken together, these data indicated a remarkable brain targeting efficiency of prodrug **1** with a marked enhancement of brain distribution, accompanied by a decreased plasma distribution. This finding supports the use of such compounds as therapeutic agents, permitting enhanced therapeutic concentrations in the brain with decreased peripheral side effects.

Discussion

In this study, ethanolamine and its related structures were coupled with dexibuprofen to generate potential brain-targeting agents. The *in vitro* stability and *in vivo* biodistribution of these compounds were investigated and biopharmacokinetic studies were performed to evaluate their brain-targeting efficiency. The results obtained were compared with that of free dexibuprofen. The influence of their chemical structures on the transport efficiency was also discussed.

In the stability study, the results showed a rapid hydrolysis of prodrugs **1**, **2**, and **3** in the plasma (Table 1). Prodrugs **1**, **2**, and **3,** which each contain ester bonds, were likely degraded by specific esterases in the plasma. Because *N,N*-dimethylaminoethanol, *N,N*-diethylaminoethanol and 2-(methylamino) ethanol have structures that are similar to that of choline, prodrugs **1**, **2**, and **3** could be substrates of plasma esterases, such as cholinesterase. These findings were in agreement with those of a previous study that showed compounds with structures similar to that of prodrug **1** undergoing very fast hydrolysis in 80% human plasma, with half-lives less than 5 min[28]. The hydrolysis profiles of the prodrugs in rat brain homogenates revealed that prodrug **3** was more stable than either prodrug **1** or **2**, suggesting that prodrugs **1** and **2** are better substrates of specific esterases in the brain than prodrug **3** and underwent hydrolysis with high efficiency to release dexibuprofen.

In our preliminary investigations, we investigated the *in vitro* stability of prodrugs **4** and **5**. The hydrolysis profiles of prodrugs **4** and **5** revealed that they were not stable and did not follow the linear kinetic equation. Therefore, we have only presented *in vitro* stability data relating to prodrugs **1**, **2**, and **3**.

In our biodistribution studies, each prodrug exhibited an enhancement relative to dexibuprofen in brain-targeting efficiency (Figure 3, $P<0.01$). As observed from the results of our HPLC studies, the lipophilicity of prodrugs **1**, **2**, **3**, **4**, and **5** are not increased when compared with dexibuprofen (Figure 1); thus, the observed enhancement of brain distribution is probably due to movement by specific transporters located at the BBB. Because ethanolamine-related structures are similar to choline and small molecular cations, the high transport efficiency of prodrugs is probably facilitated by the action of organic cations or choline transporters at the BBB. In addition, prodrugs are positively charged under physiological conditions, which may allow them to interact with the negatively charged BBB.

Among the prodrugs tested, prodrug **1**, which was prepared by coupling *N,N*-dimethylaminoethanol and dexibuprofen via an ester bond, exhibited the highest targeting efficiency. Because prodrug **1** was rapidly hydrolyzed in the rat plasma with a half-life less than 5 min, its high distribution in the brain indicated remarkable brain-transport efficiency.

During the synthesis of prodrugs **1**, **2**, **3**, **4**, and **5**, the active carboxyl group of dexibuprofen was coupled with ethanolamine-related structures via an ester bond. The present study reveals that although prodrugs have similar structures, their stability in media and brain-transport efficiency were quite different. It is interesting to note that many CNS-accessible therapeutics, such as meclofenoxate, cetirizine and procaine, contain *N,N*-dimethylaminoethanol structures. Because the structure of *N,N*-dimethylaminoethanol is very similar to choline, we hypothesize that prodrug **1** might be a substrate of the choline transporters located at the BBB. After entering the brain, prodrug **1** was hydrolyzed by esterases, resulting in the release of dexibuprofen. In addition to prodrug **1**, prodrug **2** also exhibited a high transport efficiency. The $(C_{brain}/C_{plasma})_{dexibuprofen}$ ratio of prodrug **2** was 15.7-fold higher than that of free dexibuprofen 10 min after administration (Figure 3, $P<0.01$). This result implies that the small alkyl groups disubstituted onto the amino group of ethanolamine seem to facilitate the recognition and transport of these compounds by specific transporters at the BBB.

Our results revealed that at 5 min after the administration of prodrug **1**, the $(C_{brain}/C_{plasma})_{dexibuprofen}$ ratio reached levels as high as 0.829 (Figure 5). Subsequently, the high concentration of dexibuprofen in the brain decreased rapidly. Because dexibuprofen is negatively charged at physiological pH, preventing it from passively penetrating through the BBB, the rapid efflux of dexibuprofen was probably facilitated by efflux transporters located at the BBB. A sustained-release drug delivery system for prodrug **1** designed to maintain a steady concentration of dexibuprofen in the CNS will be investigated in future studies. A more stable ester bond could also be designed to allow prodrugs to be slowly hydrolyzed by brain esterases after entering the brain.

Conclusions

In this study, we designed and synthesized a series of dexibuprofen derivatives that were modified with ethanolamine-related structures. This is the first time that ethanolamine-related structures have been utilized in the modification of compounds for brain-targeting delivery. The distribution and pharmacokinetic studies revealed that all of the prepared derivatives exhibited enhanced brain distribution compared with dexibuprofen ($P<0.01$). These results imply that ethanolamine-related structures may play an important role in transport across the BBB. The mechanism responsible for this enhancement will likely be further elucidated in the near future.

Experimental

(2*S*)-2-(4-isobutylphenyl)propionic acid 2-dimethylaminoethyl ester (1)

N,N-dimethylaminoethanol (1.30 g, 14.6 mmol) and TEA (5 mL, 36 mmol) were dissolved in anhydrous CH_2Cl_2 (15 mL). Dexibuprofen chloride (2.24 g, 10.0 mmol) was added dropwise under an ice bath. The reaction mixture was stirred continuously at room temperature for 4 h. The mixture was then extracted with CH_2Cl_2. The organic layer was combined and sequentially washed with sodium bicarbonate solution (*aq*) and water. It was then dried over anhydrous sodium sulfate and filtered. The resulting filtrate was concentrated under reduced pressure. The residue was then subjected to silica gel chromatography (dichloromethane/methanol, 20:1) to yield prodrug **1** as a colorless oil (2.32 g, 83.7%).

$[\alpha]^{20}_D$+36.3° (c 1, CH_3OH).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.178 (d, 2H, J=8.0 Hz, 2′-H, 6′-H), 7.059 (d, 2H, J=8.0 Hz, 3′-H, 5′-H), 4.191–4.107 (m, 2H, $CH_2C\underline{H}_2OCO$), 3.660–3.720 (m, 1H, $COC\underline{H}CH_3$), 2.503 (t, 2H, J=11.2 Hz, $NC\underline{H}_2CH_2$), 2.418 [d, 2H, J=7.2 Hz, $C\underline{H}_2CH(CH_3)_2$], 2.182 [s, 6H, $N(C\underline{H}_3)_2$], 1.850–1.783 [m, 1H, $CH_2C\underline{H}(CH_3)_2$], 1.459 (d, 3H, J=6.8 Hz, $COCHC\underline{H}_3$), 0.870 [d, 6H, J=6.4 Hz, $CH_2CH(CH_3)_2$].

IR (KBr cm^{-1}): 3089, 2955, 2869, 2821, 1735, 1513, 1460, 1160, 1067.

HRMS for $[M+H]^+$: *m/z* calculated 278.2120; found 278.2115.

(2*S*)-2-(4-isobutylphenyl)propionic acid 2-diethylaminoethyl ester (2)

N,N-diethylaminoethanol (0.88 g, 7.51 mmol) and TEA (2 mL, 14.4 mmol) were dissolved in anhydrous CH_2Cl_2 (10 mL). Dexibuprofen chloride (1.12 g, 5.0 mmol) was added dropwise under an ice bath. The reaction mixture was stirred continuously at room temperature for 2.5 h. The mixture was then extracted with CH_2Cl_2. The organic layer was combined and sequentially washed with sodium bicarbonate solution (*aq*) and water. It was then dried over anhydrous sodium sulfate and filtered. The resulting filtrate was concentrated under reduced pressure. The residue was subjected to silica gel chromatography (dichloromethane/methanol, 20:1) to yield prodrug **2** as a yellow-tinted oil (1.10 g, 72.4%).

$[\alpha]^{20}_D$+39.4° (c 1, CH_3OH).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.197 (d, 2H, J=8.0 Hz, 2′-H, 6′-H), 7.083 (d, 2H, J=8.0 Hz, 3′-H, 5′-H), 4.145 (t, 2H, J=6.0 Hz, $CH_2C\underline{H}_2OCO$), 3.695 (q, 1H, J=7.2 Hz, $COC\underline{H}CH_3$), 2.675 (t, 2H, J=6.0 Hz, $NC\underline{H}_2CH_2$), 2.505 [q, 4H, J=7.2 Hz, $N(C\underline{H}_2CH_3)_2$], 2.440 [d, 2H, J=6.8 Hz, $C\underline{H}_2CH(CH_3)_2$], 1.786–1.888 [m, 1H, $CH_2C\underline{H}(CH_3)_2$], 1.482 (d, 3H, J=6.8 Hz, $COCHC\underline{H}_3$), 0.976 [t, 6H, J=7.2 Hz, $(CH_2C\underline{H}_3)_2$], 0.891 [d, 6H, J=6.8 Hz, $CH_2CH(C\underline{H}_3)_2$].

IR (KBr cm^{-1}): 2967, 2872, 2808, 1736, 1513, 1461, 1380, 1164, 1069.

HRMS for $[M+H]^+$: *m/z* calcd 306.2433; found 306.2436.

N-tert-butoxycarbonyl-2-methylaminoethanol (3a)

2-(methylamino)ethanol (0.50 g, 7.0 mmol) and sodium hydroxide (0.28 g, 7.0 mmol) were dissolved in methanol (10 mL). Di-tert-butyldicarbonate (1.90 g, 8.6 mmol) was then added. The mixture was then stirred at room temperature for 5 h. The mixture was concentrated under reduced pressure, and the residue was then subjected to silica gel chromatography (dichloromethane/methanol, 20:1) to yield prodrug **3a** as a colorless oil (0.86 g, 71.1%).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 3.733 (s, 2H, $HOC\underline{H}_2CH_2$), 3.382 (s, 2H, $NC\underline{H}_2CH_2$), 2.907 (s, 3H, $CH_2NC\underline{H}_3$), 1.452 [s, 9H, $COOC(C\underline{H}_3)_3$].

(2*S*)-2-(4-isobutylphenyl)propionic acid 2-(*N*-tert-butoxycarbonyl-*N*-methylamino) ethyl ester (3b)

Prodrug **3a** (0.70 g, 4.1 mmol) was dissolved in CH_2Cl_2 (10 mL) with added TEA (1 mL, 7.2 mmol). Dexibuprofen chloride (1.10 g, 4.9 mmol) was added dropwise under an ice bath. The mixture was stirred at room temperature for 4 h. The mixture was concentrated, and the residue was subjected to silica gel chromatography (petroleum ether/ethyl acetate, 7:1) to yield prodrug **3b** as a colorless oil (1.16 g, 78.5%).

$[\alpha]^{20}_D$+16.0° (c 1, CH_3OH).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.186 (d, 2H, J=8.0 Hz, 2′-H, 6′-H), 7.086 (d, 2H, J=8.0 Hz, 3′-H, 5′-H), 4.165 (s, 2H, $CH_2C\underline{H}_2OCO$), 3.657–3.750 (m, 1H, $COC\underline{H}CH_3$), 3.368 (m, 2H, $NC\underline{H}_2CH_2$), 2.674–2.722 (m, 3H, $CH_2NC\underline{H}_3$), 2.438 [d, 2H, J=7.2 Hz, $C\underline{H}_2CH(CH_3)_2$], 1.799–1.875 [m, 1H, $CH_2C\underline{H}(CH_3)_2$], 1.482–1.515 (m, 3H, $COCHC\underline{H}_3$), 1.437 [s, 9H, $COOC(C\underline{H}_3)_3$], 0.879–0.901 [m, 6H, J=6.8 Hz, $CH_2CH(C\underline{H}_3)_2$].

(2*S*)-2-(4-isobutylphenyl)propionic acid 2-methylaminoethyl ester (3)

Concentrated hydrochloric acid (0.1 mL) was added to a solution of compound **3b** (1.0 g, 2.7 mmol) in ethyl acetate (10 mL). The mixture was stirred at room temperature for 14 h. The solution was then concentrated, and the residue was subjected to silica gel chromatography (dichloromethane/methanol, 10:1) to yield prodrug **3** as a white solid (0.48 g, 57.2%).

mp 103–105 °C.

$[\alpha]^{20}_D$+9.0° (c 1, CH_3OH).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.203 (d, 2H, J=8.0 Hz, 2′-H, 6′-H), 7.085 (d, 2H, J=8.0 Hz, 3′-H, 5′-H), 4.508–4.549 (m, 1H, $CH_2C\underline{H}_2OCO$), 4.317–4.341 (m, 1H, $CH_2C\underline{H}_2OCO$), 3.847–3.898 (m, 1H, $COC\underline{H}CH_3$), 3.053–3.146 (m, 2H, $NC\underline{H}_2CH_2$), 2.517 (s, 3H, $CH_2NC\underline{H}_3$), 2.433 [d, 2H, J=7.2 Hz, $C\underline{H}_2CH(CH_3)_2$], 1.796–1.863 [m, 1H, $CH_2C\underline{H}(CH_3)_2$], 1.508 (d, 3H, J=6.8 Hz, $COCHC\underline{H}_3$), 0.886 [d, 6H, J=6.8 Hz, $CH_2CH(C\underline{H}_3)_2$].

IR (KBr cm^{-1}): 3391, 2955, 2869, 2845, 1735, 1510, 1464, 1163, 1073.

HRMS for $[M+H]^+$: *m/z* calcd 264.1964; found 2764.1960.

N-tert-butoxycarbonyl-ethanolamine (4a)

Ethanolamine (0.60 g, 10.0 mmol) and sodium hydroxide (0.40 g, 10.0 mmol) were dissolved in water (10 mL) and dioxane (10 mL). Di-tert-butyldicarbonate (2.7 g, 12.0 mmol) was then added, and the mixture was stirred at room temperature for 3.5 h. The mixture was concentrated, and the residue was subjected to silica gel chromatography (petroleum ether/

ethyl acetate, 1:1) to yield prodrug **4a** as a colorless oil (1.54 g, 95.1%).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 3.650 (t, 2H, J=5.2 Hz, HOC$\underline{H}_2$CH_2), 3.241 (t, 2H, J=5.2 Hz, NC$\underline{H}_2$CH_2), 1.415 [s, 9H, COOC(C$\underline{H}_3$)$_3$].

(2*S*)-2-(4-isobutylphenyl)propionic acid 2-(*N*-tert-butoxycarbonyl-amino) ethyl ester (4b)

Compound **4a** (0.7 g, 4.3 mmol) was dissolved in CH_2Cl_2 (10 mL) with added TEA (1 mL, 7.2 mmol). Dexibuprofen chloride (1.44 g, 6.45 mmol) was added dropwise under an ice bath. The mixture was stirred at room temperature for 3.5 h. The mixture was then concentrated, and the residue was subjected to silica gel chromatography (petroleum ether/ethyl acetate, 10:1) to yield prodrug **4b** as a colorless oil (1.38 g, 92.1%).

$[\alpha]^{20}_D$+23.1° (c 1, CH_3OH).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.190 (d, 2H, J=7.6 Hz, 2′-H, 6′-H), 7.088 (d, 2H, J=7.6 Hz, 3′-H, 5′-H), 4.102 (s, 2H, $CH_2C\underline{H}_2OCO$), 3.672–3.725 (m, 1H, COC$\underline{H}$CH_3), 3.288 (s, 2H, NC$\underline{H}_2$CH_2), 2.439 [d, 2H, J=7.2 Hz, C$\underline{H}_2$CH(CH_3)$_2$], 1.793–1.891 [m, 1H, $CH_2C\underline{H}$(CH_3)$_2$], 1.478 (d, 3H, J=6.8 Hz, COCHC$\underline{H}_3$), 1.421 [s, 9H, COOC(C$\underline{H}_3$)$_3$], 0.890 [d, 6H, J=6.0 Hz, CH_2CH(C$\underline{H}_3$)$_2$].

(2*S*)-2-(4-isobutylphenyl)propionic acid 2-aminoethyl ester (4)

Concentrated hydrochloric acid (0.1 mL) was added to a solution of compound **4b** (1.0 g, 2.8 mmol) in ethyl acetate (10 mL). The mixture was stirred at room temperature for 14 h. The solution was then concentrated, and the residue was subjected to silica gel chromatography (dichloromethane/methanol, 10:1) to yield compound **4** as a white solid (0.36 g, 43.5%).

mp 117–120°C.

$[\alpha]^{20}_D$+14.0° (c 1, CH_3OH).

^{1}H-NMR (DMSO-d, 400 MHz): δ 7.214 (d, 2H, J=8.0 Hz, 2′-H, 6′-H), 7.116 (d, 2H, J=8.0 Hz, 3′-H, 5′-H), 4.278–4.335 (m, 1H, $CH_2C\underline{H}_2OCO$), 4.044–4.100 (m, 1H, $CH_2C\underline{H}_2OCO$), 3.781–3.835 (m, 1H, COC$\underline{H}$CH_3), 3.041–3.054 (m, 2H, NC$\underline{H}_2$CH_2), 2.413 [d, 2H, J=7.2 Hz, C$\underline{H}_2$CH(CH_3)$_2$], 1.770–1.837 [m, 1H, $CH_2C\underline{H}$(CH_3)$_2$], 1.408 (d, 3H, J=6.8 Hz, COCHC$\underline{H}_3$), 0.853 [d, 6H, J=6.4 Hz, CH_2CH(C$\underline{H}_3$)$_2$].

IR (KBr cm^{-1}): 3395, 2956, 2871, 1737, 1512, 1378, 1162, 1075.

HRMS for $[M+H]^+$: *m/z* calcd 250.1807; found 250.1801.

N-acetyl-2-aminoethanol (5a)

Sodium hydride (1.44 g, 60 mmol) was added at 0 °C to a stirred solution of ethanolamine (1.84 g, 30 mmol) in 20 mL dioxane, followed by the dropwise addition of acetyl chloride (1.56 g, 20 mmol). The mixture was stirred at room temperature for 2.5 h. The mixture was then extracted with CH_2Cl_2. The organic layer was combined and washed with water. It was then dried over anhydrous sodium sulfate and filtered. The filtrate was subsequently concentrated under reduced pressure. The resulting residue was subjected to silica gel chromatography (dichloromethane/methanol, 8:1) to yield compound **5a** as a colorless oil (1.27 g, 62.1%).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.990 (s, 3H, C$\underline{H}_3$CO), 3.326–3.390 (m, 2H, C$\underline{H}_2$NCO), 3.682 (t, 2H, C$\underline{H}_2$O)

(2*S*)-2-(4-isobutylphenyl)propionic acid 2-(acetylamino)ethyl ester (5)

Compound **5a** (0.5 g, 4.85 mmol) and TEA (2 mL, 14.4 mmol) was dissolved in anhydrous tetrahydrofuran (20 mL). Dexibuprofen chloride (1.31 g, 5.84 mmol) was added dropwise under an ice bath. The reaction mixture was then stirred continuously at room temperature for 2 h. The mixture was concentrated, and the residue was subjected to silica gel chromatography (petroleum ether/ethyl acetate, 1:1) to yield compound **5** as a colorless oil (0.94 g, 66.2%).

$[\alpha]^{20}_D$+17.8° (c 1, CH_3OH).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.111 (d, 2H, J=8.0 Hz, 3′-H,5′-H), 7.200 (d, 2H, J=8.4 Hz, 2′-H, 6′-H), 4.174–4.228 (m, 1H, C$\underline{H}_2$O), 4.056–4.109 (m, 1H, C$\underline{H}_2$O), 3.685–3.737 (m, 1H, COC$\underline{H}$CH_3), 3.397–3.436 (m, 2H, C$\underline{H}_2$NCO), 2.444 [d, 2H, J=7.2 Hz, C$\underline{H}_2$CH(CH_3)$_2$], 1.826–1.872 [m, 4H, C$\underline{H}_3$CO, $CH_2C\underline{H}$(CH_3)$_2$], 1.492 (d, 3H, J=7.2 Hz, COCHC$\underline{H}_3$), 0.892 [d, 6H, J=6.4 Hz, CH_2CH(C$\underline{H}_3$)$_2$].

IR (KBr cm^{-1}): 3294, 3085, 2956, 2871, 1736, 1657, 1553, 1515, 1459, 1373, 1292, 1166, 1070, 799.

HRMS for $[M+H]^+$: *m/z* calcd 292.1913; found 292.1916.

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Author contribution

Xuan ZHANG, Zhi-rong ZHANG, Xing LIU, Tao GONG, and Xun SUN designed the research strategy; Xuan ZHANG and Xing LIU performed the research; Xuan ZHANG and Xing LIU analyzed the data; and Xuan ZHANG wrote the paper.

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