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Synthesis and preliminary biological evaluation of naproxen-probenecid conjugate for central nervous system (CNS) delivery

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Abstract: Naproxen, known as the most potent non-steroid anti-inflammatory drugs, is vitally crucial in the treatment of neurodegenerative diseases. However, due to the poor brain penetration ability, it causes serious adverse effects with the therapeutic doses. Predictably, these unfavorable factors have hindered its further application. In this study, a novel brain-targeting conjugate, Nap-Pro, was designed and synthesized. The chemical stability and metabolic stability of this conjugate were determined in phosphate buffer, blood serum and brain homogenate, respectively. The cytotoxicity of Nap-Pro was evaluated in b End. 3 cells. In addition, the brain targeting capacity of Nap-Pro was also investigated *in vivo*. Importantly, Nap-Pro showed excellent capacity to cross the brain-blood barrier (BBB), suggesting probenecid was a super carrier that enhanced the delivery of drugs into brain. Collectively, the probenecid modification was a promising strategy to develop the novel drug delivery systems for brain targeting.

Keywords: Brain targeting, brain-blood barrier, probenecid, drug delivery, conjugate.

INTRODUCTION

Recently, non-steroidal anti-inflammatory drugs (NSAIDs) have been used as neuroprotective agents in the treatment of central nervous system (CNS) diseases (Zhao *et al*., 2014). In particular, these drugs are extensively used to combat Alzheimer diseases (AD), a neurodegenerative disorder that tends to occur in elderly patients (Azimi *et al*., 2020). The neuropatho logical hallmarks of AD include the deposition of amyloid-β (Aβ) plaque and neuro inflammation (Haupt *et al*., 2021; Yang *et al*., 2021). Naproxen delays the onset of CNS diseases and reduces the risk of developing these diseases, and it therefore becomes a most potent drug for the treatment of CNS disorder (Karami *et al*., 2019). Due to the low permeability of naproxen, the possibility of its effective accumulation decreases. Besides, its distribution in the brain becomes extremely limitation due to a physiological barrier (Blood-brain barrier, BBB) that separates the brain from its blood supply (Zhao *et al*., 2018). The barrier restricts the flow of all macromolecular drugs and over 98% of micro molecular drugs cross over the BBB and pass into the human brain. To improve the naproxen concentration in human brain, doctors may prescribe naproxen in higher doses, which can lead to stronger side effects and toxicity in patients. Therefore, scientists are trying to develop novel strategies so as to effectively deliver naproxen into the brain of patients with CNS diseases.

In patients with CNS diseases, a bottleneck effect is drug induced by BBB when systemic administration is used for delivery. This situation is true for hydrophilic drugs. Although several strategies are used to promote the drugs through the BBB (Diao and Polli, 2011; Wang *et al*., 2017), the condition of CNS patients could be sufficiently improved with the brain-targeting delivery systems. In addition, higher dosages of these drugs cause severe toxicity and side effects.

Carrier-mediated transporter (CMT) system is a potential effective method to specifically increase the drug concentration in the brain, which is due to the high affinity between transporter and the drugs. Several physiological transport systems, such as monocarboxylic acid transporter 1 (MCT1) (Fan *et al*., 2009), sodium-dependent vitamin C transporter 2 (SVCT2) (Zhao *et al*., 2014), and glucose transporter (GLUT1) (Zhao *et al*., 2018), *et al*, are introduced into the cerebral capillary of endothelial cells, which are widely used in the transportation of nutrients and endogenous compounds. Probenecid is one of the competitive inhibitors of MCT1 (Jones *et al*., 2017), indicating that probenecid may serve as a good carrier for drugs to target brain.

We have been developing brain-targeting drugs in recent years (Zhao *et al*., 2014; Zhao *et al*., 2018; Zhao *et al*., 2021). Herein, our aim was to construct a conjugate that couples with naproxen and probenecid to enhance the permeability of naproxen in the human brain (fig. 1). The physicochemical stability, cellular cytotoxicity, as well as targeting efficiency of Nap-Pro, were tested *in vivo* and compared with naked naproxen. The probenecid modification was also a promising strategy to develop the

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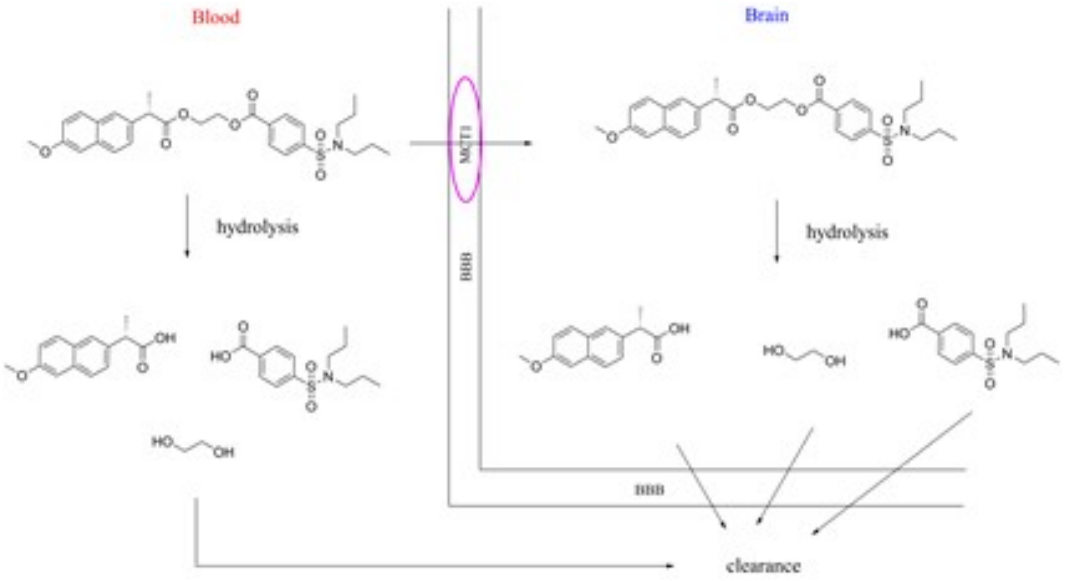

Fig. 1: The structure of Nap-Pro and its sequential metabolism pathway in brain.

novel drug delivery systems for brain targeting.

MATERIALS AND METHODS

Materials

All the starting materials, unless otherwise specified, were used as received. ^{1}H-NMR spectra were taken on a Varian INOVA 400 or 600 (Varian, Palo Alto, CA) using CD_3Cl as solvent. Mass spectroscopy data of the product were collected on a Waters Micro mass GCT or a Bruker Apex IV FTMS instrument. Kunming mice were purchased from Chengdu Dashuo Experimental Animal Co., Ltd (Chengdu, China). In this study, all the animal procedures were performed after being approved by the Experiment Animal Administrative Committee of Sichuan Nursing Vocational College (P.R. China). The animals was kept in a 20˚C environment with 40-60% humidity and a 12-h light/dark cycle. Animals had free access to food and water and clean and hygienic feeding conditions were maintained.

Chemistry

Synthesis of compound 2

The naproxen 1 (0.12g, 0.52mmol) was dissolved in dichloromethane (10mL), following adding dicyclohexyl-carbodiimide (0.16g, 0.78mmol) and cat. Dimethylami-nopyridine. After the mixture was stirred for 30 min, glycol (0.32g, 5.20mmol) in dichloromethane (10mL) was added and the mixture was further stirred for 6 h at the ambient temperature. Subsequently, the filtrate was concentrated after filtration. Subsequently the residue was purified by column chromatography to get compound 2 (0.11g, 76%) as white waxy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.58-1.60 (m, 4H), 3.75 (t, 2H, *J*=4 Hz), 3.89-3.91 (m, 4H), 4.19-4.22 (m, 2H), 7.11-7.71 (m, 6H). ESI-MS (m/z): calculated for $C_{16}H_{18}O_4Na$ $[M+Na]^+$ 297.11, found 297.25. Elemental Analysis: C, 70.06; H, 6.61, found C, 70.21; H, 6.52.

Synthesis of conjugate Nap-Pro

The probenecid (0.14g, 0.50mmol) was dissolved in dichloromethane (10mL), following adding dicyclohexyl-carbodiimide (0.15g, 0.75mmol) and cat. Dimethylami-nopyridine. After the mixture was stirred for 30 min, compound 2 (0.14g, 0.50mmol) in dichloromethane (10mL) was added and the mixture was further stirred for 6h at the ambient temperature. Subsequently, the filtrate was concentrated after filtration and the residue was purified by column chromatography to yield the conjugate Nap-Pro (0.24 g, 87%) as white waxy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.87 (t, 6H, *J*=8 Hz), 1.48-1.56 (m, 4H), 1.58 (d, 3H, *J*=3.6 Hz), 3.06 (t, 4H, *J*=8 Hz), 3.87-3.91 (m, 1H), 3.93 (s, 3H), 4.37-4.49 (m, 4H), 7.08-7.81 (m, 10H). ESI-MS (m/z): calculated for $C_{29}H_{35}$ NO_7SNa $[M+Na]^+$ 564.20, found 564.64. Elemental Analysis: C, 64.31; H, 6.51; N, 2.59; S, 5.92, found C, 64.25; H, 6.64; N, 2.40; S, 5.79.

Stability of Nap-Pro in phosphate buffer (Wang et al., 2018)

Reversed-phase chromatography performed on a Kromasil RP18 column (250mm × 4.6mm, 5μm), analysis was carried out using the high-performance liquid chromatography (HPLC) system (Waters, America), thermo stated at 25°C. The solution of methanol/water (73:27) was used as the mobile phase at a flow rate of 1.0mL/min and the UV detector was set to monitor the signal at 238 nm wavelength.

The chemical stability of conjugate Nap-Pro *in vitro* was determined by culturing Nap-Pro in pH 2.21, 5.86, 7.33, and 7.88 phosphate buffer solutions, respectively. In

general, the Nap-Pro in CH_3OH (1mL) was added into different buffer (4mL) with the final concentration at 200 μM. The mixture was shaken continuously at 45 rpm at 37°C. The medium sample (200μL) were taken at the predetermined time points (0, 1, 2, 3, 4, 6, 12, 24, 36 and 48h) and replaced with the same amount of fresh medium. These samples were further analyzed by the aforementioned HPLC method.

Metabolic stability in plasma and brain homogenate (Zhao et al., 2018)

To detect the stability of Nap-Pro in plasma and brain homogenate, the conjugate was incubated in either plasma or brain homogenate. In brief, the Nap-Pro in CH_3OH (1mL, 100μg/mL) was added into plasma or brain homogenate (4mL). The mixture was shaken continuously at 45 rpm at 37°C. The medium sample (200μL) were taken at the predetermined time points (0, 1, 2, 3, 4, 6, 12, 24, 36 and 48 h) and replaced with the same amount of fresh plasma or brain homogenate. These samples were analyzed by the aforementioned HPLC method.

MTT assay (Wang et al., 2018)

The bEnd.3 cells maintained in our lab were cultured in Dulbecco's Modified Eagles Medium (DMEM) medium, containing 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. To measure the cytotoxicity of Nap-Pro, naproxen, and probenecid in bEnd.3 cells, we performed the (3,4,5-dimethylthiazol-yl)-2,5-diphenyl-tetrazolium (MTT) assay (Zhao *et al*., 2019). 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 approximately 48 h. Then, these drugs were diluted to pre-determined concentrations (10, 20, 50, 100, 200 and 500 μM) with phosphate-buffered saline (PBS). These diluted solutions were then added. After 24h incubation, the cells were treated with MTT (20μL, 5.0mg/mL) for 4h. Then, they were thrice washed with PBS. These cells were then isolated using 200μL DMSO. The OD value of the samples was measured at 490 nm wavelength.

Brain targeting and biodistribution in vivo (Wang et al., 2018)

The Kunming mice (20-22g) were randomly divided into two groups (n=3). All animal experiments were performed after being approved by the Sichuan Nursing Vocational College Animal Ethical Experimentation Committee. The mice were kept in well-spaced ventilated cages, and fed a healthy and balanced diet. The mainly tissue distribution study was conducted in the mice to evaluate and compare the brain-targeting potential of the conjugate Nap-Pro and naproxen. Naproxen or Nap-Pro solutions (10mg/kg, calculated as naproxen) were injected into the tail vein of each mice.

At the indicated time-points (2_{th}, 6_{th} and 12_{th} h), we collected plasma samples from the eye socket. Subsequently, the mice were sacrificed and their organs (brain, liver and kidney) were removed and rinsed with saline. Then, the organ tissues were dried on a filter paper. These tissues were homogenized with twice the amount of saline, and the mixture was vortexed with an equal volume of methanol for 5min. Then, 20μL of NaOH solution (6 M) was added into the homogenate (200μL). After carrying out hydrolysis for 10 min, naproxen was released from the conjugate. Thereafter, 20μL of 6M HCl was treated to neutralize NaOH solution. After 5 min, the mixture was centrifuged at 13000 rpm for 10 min. The supernatant was concentrated at 40°C under N_2 flow. The residues were dispersed again in 200μL of methanol and centrifuged for 10min at 13000rpm. Then, 20μL of the supernatant was injected into the HPLC system for analysis.

STATISTICAL ANALYSIS

GraphPad 5.0 (GraphPad Software, Inc.) was used for statistical analysis. Three repeats were performed for each experiment. Data were presented as the mean ± standard deviation (SD). Student's t-test was used for statistical comparisons between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Chemistry

The synthetic pathway of conjugate Nap-Pro was outlined in fig. 2. Briefly, naproxen, the starting material, was couple with glycol in the presence of DCC/DMAP to generate compound 2, which was then conjugated with probenecid to yield the designed conjugate Nap-Pro. The final yield of these two steps was about 66%. The structures of the intermediate and the target product were characterized by the following analytical techniques: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS).

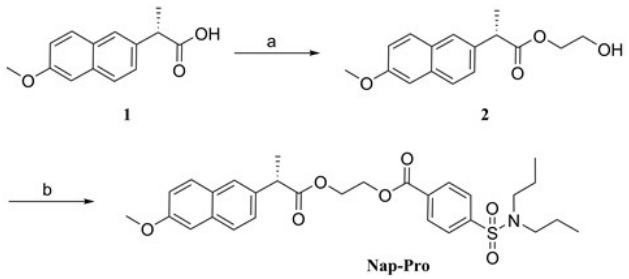

Fig. 2: The synthesis of conjugate Nap-Pro. Reagents and conditions: (a) Glycol, dicyclohexylcarbodiimide, dimethylaminopyridine, dichloromethane, r.t., 6h. (b) Probenecid, dicyclohexylcarbodiimide, dimethylaminopyridine, dichloromethane, r.t., 6 h.

Stability of Nap-Pro in phosphate buffer, and plasma and brain homogenate

Stability studies of the conjugate Nap-Pro was performed in various phosphate buffers to simulate different

physiological environment. As shown in table 1, the pseudo first order rate constants (K_{disapp}) and half-lives ($t_{1/2}$) of Nap-Pro in PBS were calculated by linear regression of the peak area against time. The results indicate that the conjugate was stable in the PBS. Furthermore, the conjugate was highly stable at pH 7.88, wherein its $t_{1/2}$ value was more than 30h. Owing to the slow hydrolysis of Nap-Pro, the naproxen could be released completely in the physiological environment.

Table 1: Stability of Nap-Pro at 37°C

	K_{disapp} (h^{-1})	$t_{1/2}$ (h)
pH 2.21	3.45 × 10-2	20.1
pH 5.86	2.68 × 10-2	25.9
pH 7.33	2.15 × 10-2	32.3
pH 7.88	3.77 × 10-2	18.4
Plasma	13.6 × 10-2	5.1
Brain homogenate	18.2 × 10-2	3.8

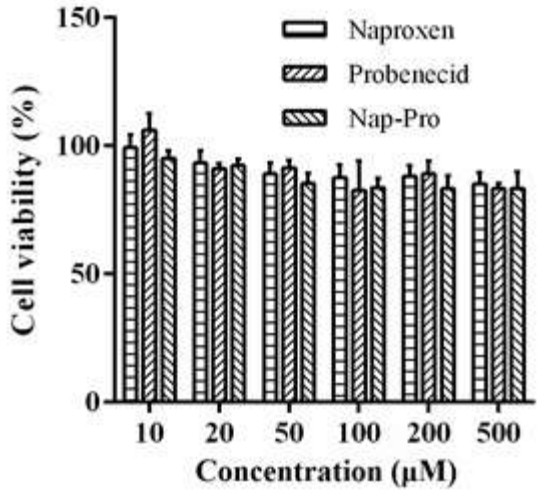

Fig. 3: After the addition of Nap-Pro, cell viability of bEnd.3 cells was determined by MTT assay over an incubation period of 24 h. Cell viability was expressed as a percentage of the control cell culture. The values were expressed as mean ± SD (n=3).

We investigated the stability of Nap-Pro in plasma and brain homogenate at 37°C. The values of the constants K_{disapp} and $t_{1/2}$ were determined by plotting linear regression of the peak area against time and the results were presented in table 1. Interestingly, the conjugate Nap-Pro exhibited considerable stability in mice plasma, wherein its $t_{1/2}$ value was about 5.1h. Thus, $t_{1/2}$ value of the conjugate was high, so the prod rug Nap-Pro had plenty of time to get into brain before being metabolized. However, the Nap-Pro conjugate showed a faster hydrolysis in brain homogenate with a high metabolic rate than that in plasma. This is because the brain had abundant esterase that degraded the conjugate and released the drug naproxen. Thus, aggregation effects were prevented and the naproxen concentration was improved in the brain. All the results indicate that the conjugate Nap-Pro had superior physicochemical properties.

MTT assay

As shown in fig. 3, the cytotoxicity of the conjugate Nap-Pro was much lower than those of the other two groups. At a dose level of 10-500μM, Nap-Pro hardly exhibited any toxicity on bEnd.3 cells. These data indicate that the conjugate Nap-Pro had limited toxicity.

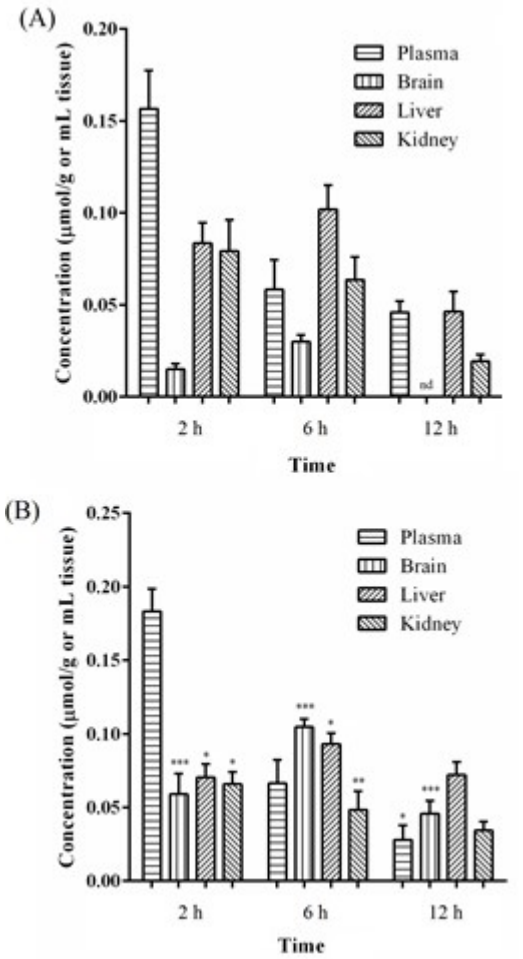

Fig. 4: The concentration of naproxen 2h, 6h and 12h after administering an injection of naproxen (A) or Nap-Pro (B) in the plasma, brain, liver, and kidney. * Values are expressed as mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 versus naproxen group. The term 'nd' is the short form for 'not detected' at 12h after being injected with naproxen.

Brain targeting and bio distribution in vivo

The pharmacokinetic and bio distribution study was conducted *in vivo* to evaluate the brain-targeting ability of Nap-Pro. The conjugate in tissues was hydrolyzed to release naproxen by additional base to get convenience for detection and accuracy of HPLC due to that Nap-Pro could only partial release parent drug naproxen in biological matrix within limited period. As shown in fig. 4,

the concentration of Nap-Pro slowly declined in plasma and the cycle time got extended. Therefore, the conjugate had plenty of time to get into brain before being metabolized.

To further determine whether Nap-Pro could be transported across BBB, the tissue distribution of naproxen and its conjugate Nap-Pro were detected at fixed time intervals after injection. The results indicate that naproxen successfully crossed the BBB to reach the brain. However, the concentration of naproxen could not be detected after 12h. Compared to free naproxen, the conjugate Nap-Pro significantly increased the naproxen concentration in the brain. Therefore, the concentration of naproxen in the experimental group was about four times greater than that of the control group. This effect was attributed to the peculiarity of probenecid, a competitive inhibitor of MCT1, which could be effectively transported into the brain. Furthermore, both the groups showed a significant uptake in the liver and kidney. This is because these organs had high metabolic capability (Yang *et al*., 2020; Zhao *et al*., 2020).

DISCUSSION

AD, as a main type of brain diseases, is also the most common neurodegenerative disease. The incidence of AD has increased rapidly worldwide (Wen *et al*., 2021). It is one of the most senile dementia and characterized by chronic inflammation, amyloid plaque deposits, and nerve fiber damage. This disease has affected about 50 million people worldwide (Jiang *et al*., 2020). Recently, numerous studies have reported that long-term use of naproxen effectively reduces the risk of AD or even delays its onset (Wang *et al*., 2018). Additionally, these studies have also elaborated the mechanism through which naproxen acts as a neuroprotective agent. NSAIDs act as neuroprotective agents by inhibiting the activity of cyclooxygenase (COX), further inhibiting the synthesis of prostaglandin (dos Santos *et al*., 2020). In our previous studies, naproxen significantly relieved the damage caused to the brain ischemia model *in vivo* (Wang *et al.,* 2018).

Only a handful of treatments are presently available as the efficacy of most drugs is restricted by BBB, which is mediated by the endothelial tight junctions existed in the brain microvasculature. The barrier also prevents the drug naproxen from entering the brain. Owing to the poor permeability of naproxen, it is prescribed in higher doses to achieve satisfactory therapeutic effects. Thus, it causes some severe side-effects and toxicity. Previous study showed contradictory results about using the naproxen in preventing some neurodegenerative diseases, and the clinical trials had failed to reach the efficacy end points (Ozben and Ozben, 2019; Kazberuk *et al.,* 2020). In addition, the significant peripheral side effects of naproxen could restrict their use (Chiu *et al.,* 2020). Nonetheless, there is a strong therapeutic rationale for using naproxen in restoring the disorders of glial cells characteristic of the neurodegenerative processes. Therefore, the improved delivery of naproxen across the BBB and specifically into the activated microglia and astrocytes could also increase the pharmacological effects in neurodegenerative diseases. New promising strategies must be developed to precisely deliver drugs into the brain and combat neurodegenerative diseases. For instance, Montaser *et al*. designed and prepared a naoproxen prod rug to deliver the drug into brain to battle with the neuro inflammation (Montaser *et al*., 2020).

Transporter-mediated brain delivery via a prodrug approach constitutes a promising way to deliver drugs into the brain (Williams *et al*., 2020). The improved brain delivery of the MCT1-utilizing prodrugs is evident and reported in several studies (Fan *et al*., 2009; Sun *et al*., 2020). However, the released amount of the parent drugs from the prodrugs in the brain is also limited. Therefore, we, for example, study the probenecid-naproxen conjugate, as it is highly likely that probenecid analogue can bind to MCT1. Here we developed and subsequently synthesized a novel brain-targeting conjugate Nap-Pro, which had superior properties, such as moderate stability in PBS, plasma, as well as brain homogenate. Owing to the stabilization of this conjugate, the drug naproxen had plenty of time to reach the targeted brain before being hydrolyzed. Also, we found that Nap-Pro could significantly enhance penetrate the BBB and improve the uptake of naproxen *in vivo*. The results indicate that probenecid remarkably increased the concentrations of naproxen in brain as compared to that observed in the free naproxen group. It may be because of the high transport efficiency of MCT1 that transports the analogue probenecid, which is one of the most competitive inhibitors of MCT1.

Interestingly, this work proves that probenecid is a superior carrier and enhances the delivery of CNS drugs into the brain. The conjugate Nap-Pro indeed had the potential to improve the clinical therapeutic effects of AD. In addition, the findings from this study can also be applied to the other neuroprotective and immunomodulatory drugs. 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 brain drug delivery via a targeting function has gained wide attention. This strategy not only enhances the potency of drugs but also diminishes their side effects and toxicity. In the present study, a biodegradable

conjugate Nap-Pro was designed and synthesized for brain-targeting effects. The results of biodistribution *in vivo* showed that the concentrations of naproxen in the brain were obviously higher when the conjugate Nap-pro was injected into the mice. This concentration of naproxen was higher than that observed when naked naproxen of the same dose was administered. This indicates that the conjugate Nap-pro effectively penetrate the BBB due to the transport ability of probenecid. Therefore, the modification induced by probenecid is a promising strategy for brain-targeting drug delivery systems in future.

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