Drug Development and Industrial Pharmacy, 2012; 38(1): 123–128

ISSN 0363-9045 print/ISSN 1520-5762 online
DOI: 10.3109/03639045.2011.592533

informa
healthcare

RESEARCH ARTICLE

Nanoparticle-mediated delivery of Neurotoxin-II to the brain with intranasal administration: an effective strategy to improve antinociceptive activity of Neurotoxin

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Abstract

Background: Neurotoxin-II (NT-II), an analgesic peptide which was separated from the venom of Naja naja atra, is endowed an exceptional specificity of action that block transmission of the nerve impulse by binding to the acetylcholine receptor in the membrane. However, it has limited permeability across the blood–brain barrier (BBB) after intravenously (i.v.) injection.

Methods: In this study, we explored the potential application of nanoparticles overcoated with polysorbate 80 (P-80-NP) as drug carrier system for the nasal delivery of NT and the antinociceptive properties of NT-loaded P-80-NP (NT-P-NP) were also evaluated.

Results: The brain delivery of NT-II could be enhanced with nanoparticles coated with polysorbate-80 through intranasally (i.n.) administration. Compared with NT-II solution, NT-P-NP exhibited sustained release *in vitro* and higher concentrations of NT-II in the brain. The antinociceptive animal testing also revealed that intranasal delivery of NT-loaded nanoparticle coated with polysorbate-80 were able to promote better biodistribution of the drug into the brain.

Conclusion: The nanoparticles overcoated with polysorbate-80 were capable of transporting the loaded drug across the BBB after intranasal administration.

Keywords: Polysorbate-80, nasal delivery, blood–brain barrier, central nervous system, drug loading

Introduction

The blood–brain barrier (BBB) is an active interface between the circulation and the central nervous system (CNS) which restricts the free movement of different substances between the two compartments and plays a crucial role in the maintenance of the homeostasis of the CNS. It restricts the transport of potentially toxic or harmful substances from the blood to the brain. On the other hand, due to the relative impermeability of the barrier, many drugs are unable to reach the CNS in therapeutically relevant concentration, making the BBB one of the major impediments in the treatment of CNS disorders[1]. In the past few years, a number of different approaches have been developed for drugs to overcome this barrier[2–5]. One possibility of delivering drugs to the brain is by the use of nanoparticles. Nanoparticles for pharmaceutical and chemical use are defined as polymeric particles made of natural or artificial polymers ranging in size between about 10 and 1000 nm[6]. Drugs may be bound in the form of a solid solution or dispersion, or adsorbed to the surface or chemically attached. On one hand, nanoparticles can protect the entrapped drugs from degradation so as to increase the drug concentration at the brain. It has been reported that nanoparticles overcoated with polysorbate 80 were capable of transporting the loaded drugs across the BBB after administration.

The use of a delivery system for nasal administration (i.n.) is a promising alternative to overcome BBB[7]. The

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(Received 24 April 2011; revised 21 May 2011; accepted 24 May 2011)

effectiveness of this route for enhancing the brain delivery of large molecules, including proteins or peptides, suggested that the nasal pathway functions for the direct delivery of drugs into the brain[8,9].

Snake venoms are composed mainly of proteins and peptides, which possess a variety of biological activities. Some snake venoms have demonstrated antinociceptive activity, and certain isolated neurotoxins have demonstrated significant analgesia in animal models[10,11]. Neurotoxin-II (NT-II), an analgesic peptide which was separated from the venom of Naja naja atra[12,13], is endowed an exceptional specificity of action that block transmission of the nerve impulse by binding to the α-subunit of the nicotinic acetylcholine receptor in the membrane[14,15,16]. However, it has limited permeability across the BBB after intravenously (i.v.) injection [17,18]. In order to get better antinociceptive activity and reduce related toxicity and side effects, it is necessary to identify the most efficient way to achieve drug targeting for meeting the requirements of modern therapy. Hence, in the present research work, the possibility of targeting delivery of NT-II with polysorbate-80 coated polylactide homopolymers (PLA) nanoparticles (NT-P-NP) into the brain was investigated.

Materials and methods

Chemicals

NT-II was provided by Kunming Institute of Zoology, the Chinese Academy of Sciences. Polysorbate-80 was products from Huadong Medicine Group Co. Polylactide homopolymers (PLA) (m.w.of 11KDa) was purchased from Sigma. Other reagents were analytical grade.

Animals

Kunming strain of Swiss mice weighing 20–22 g, were purchased from the Experimental Animal Center, Zhejiang University. The female and male mice were maintained at room temperature under alternating natural light/dark photoperiod, and had access to standard laboratory food and fresh water *ad libitum.* This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals. Care was taken to minimize discomfort, distress, and pain to the animals.

Preparation of nanoparticles

The procedures used for preparing the nanoparticles were performed according to the method introduced by Qiaoyuan Cheng[19]. Briefly, a 50 μL of solution (pH 2.5 adjusted with 0.1 N HCl) containing 56μg NT-II was emulsified in 1ml of PLA in ethyl acetate (50mg/ml) by sonication on an ice-bath for 30 s (40w). Then, 2ml of aqueous sodium cholate solution (1%, w/v) was added and the resulting (w/o)/w emulsion was sonicated for 20 s (40 w). The double emulsion was diluted in 100 ml aqueous sodium cholate solution (0.3%, w/v) and the solvent was rapidly eliminated by evaporation under vacuum. Finally, the nanoparticles were isolated by centrifugation (22000 × g, 30 min), washed three times with water, and lyophilized. Polysorbate 80 was added at a ratio of 1:1 (w/w) to reconstitute nanoparticles and incubated for 30 min.

Characterization of NT-loaded nanoparticle

NT-loaded nanoparticles were characterized for zeta potential and size using both transmission electron microscopy (TEM) and dynamic light scattering (DLS). Values of the particle sizes and zeta potentials are presented as mean ± standard deviation (*SD*) from three replicate samples.

Determination of drug loading and entrapment efficiency

After the double emulsion was diluted in 100ml aqueous sodium cholate solution, the nanoparticle suspension was centrifuged at 20,000 rpm for 30 min at 4°C. The concentrations of NT-II were measured by high-pressure liquid chromatography (see below). The entrapment efficiency was calculated as the difference between the amount of drug entrapped in the nanoparticles and the total amount of drug.

Evaluation of *in vitro* release

The *in vitro* release was studied using a dialysis bag diffusion technique[20,21]. The NT-loaded nanoparticle suspension (coated and uncoated with polysorbate-80) and NT-II solution was placed in dialysis bags (cutoff 8.0 kDa; Solarbio), which were sealed at both ends, and then the bags were dipped into 200 mL phosphate-buffered saline (pH 6.8), which were maintained at 37°C and constantly stirred at 100 rpm. At regular time intervals, 2 mL of dissolution medium was withdrawn and the same volume of fresh phosphate-buffered saline was added accordingly. The amount of NT in the medium was measured by high-pressure liquid chromatography analysis (see below).

Evaluation of *in vivo* release

The mice were randomly divided into four groups ($n = 10$, each group); Groups 1 and 2 mice were administered intranasally with NT-P-NP (45 mg lyophilized nanoparticles was dissolved in 0.15 ml PBS, 17 mg NT-II /kg) and free NT solution (F-NT, 17 mg NT-II /kg), respectively. Group 3 mice were treated (17 mg NT-II /kg) intravenously with NT-P-NP (IV-NT). Group 4 mice were administered intranasally with distilled water used as controls. After antinociceptive properties test, the animals were anesthesitized with ethyl ether and sacrificed by decapitation[22]. The brain, lungs, heart, liver, spleen, and kidneys were quickly dissected and stored at –20°C. The amount of drug in brain was measured by high-pressure liquid chromatography analysis (see below).

Evaluation of antinociceptive properties of NT-loaded nanoparticle

Formalin test has been used as a model for tonic pain and localized inflammatory pain. 20 μL of a 1%-formalin

solution was injected into the right hind paw of mice, and the licking time was recorded after the first 5 min (1st phase, corresponding to a direct chemical stimulation of nociceptors) and after 20 min (2nd phase, involving inflammation), for 5 min each time [23,24].

Determination of Neurotoxin-II

The procedures used for detecting the NT-II were performed according to the method introduced by Zeyi Yan[25]. Briefly, about 200 mg of brain sample was homogenized and homogenate sample was then mixed with 10 ml of ethanol-water (3:7, v/v), shaken briefly and sonicated for 1 h. The extracts were centrifuged at 15,000 g for 10 min and the supernatant filtered through a 0.45 μm membrane. The solution was derivatized immediately. Samples were derivatized in 0.5 M NaHCO3 within 2 min at ambient temperature by adding 50 mM para-nitrobenzyloxycarbonyl chloride (PNZ-Cl) in acetonitrile. The PNZ-derivatives were quantified by high-pressure liquid chromatography/ultraviolet absorption (LC-10ATvp liquid chromatograph; SHIMADZU) at a wavelength of 260 nm, and a Luna-C18 column (250 × 4.6 mm I.D., 5 μm particle diameter (Phenomenex, USA) was used. SCL-10Avp system control SPD-10Avp UV-vis detector, FRC-10A fraction collector, CTO-2AS thermostat and 7725 injection valve was used in this study. A N3000 workstation (Zhejiang University, China) was used to collect and analyze data.

Data analysis

All data were analyzed by a one-way analysis of variance, and the differences between means were established by Duncan's multiple-range test. The data represents means and standard deviations. The differences between NT-P-NP and IV-NT groups were also analyzed by Paired-Samples T Test. The significant level of 5% ($p < 0.05$) was used as the minimum acceptable probability for the difference between the means.

Results

Physicochemical characteristics of nanoparticles

The physical properties of the nanoparticle formulations, before and after bioconjugation, are usually established using the following techniques: (i) Dynamic light scattering to estimate the dynamic diameter and surface charge of the aqueous dispersed nanoparticles, (ii) transmission electron microscopy (TEM) to determine particle size and size distribution. Figure 1 shows the TEM morphology of NT-P-NP nanoparticles with an average diameter of about 60 nm. Most nanoparticles could be observed to be of spherical shape with a relatively smooth surface. The zeta potential of the nanoparticles was –26.6 ± 2.0mV ($n = 3$). Coinciding with TEM photography, dynamic light scattering results indicated that the average size of the NT-P-NP nanoparticles was 68 ± 2.6 nm ($n = 3$).

Evaluation of entrapment efficiency

NT-II could be encapsulated efficiently in nanoparticles (36.6 ± 3.6% encapsulation efficiency). To prove the NT-loaded nanoparticles stability in the presence of serum and polysorbate-80, nanoparticles were diluted 1:10 with distilled water, 10% mouse serum, 90% mouse serum, and 1% polysorbate 80. Under the same conditions, no significant reduction of the NT-II loading efficiency was observed (Figure 2).

Evaluation of *in vitro* release

The release profiles of NT-II solution, NT-loaded nanoparticle and NT-P-NP nanoparticles were shown in Figure 3. As shown in Figure 3, we observed that approximately 90% of NT-II had been released from NT-II solution after 24 h. However, the total cumulative releases from the NT-loaded nanoparticle and NT-P-NP nanoparticles were 65.22 ± 1.61% and 46.38 ± 2.40%, respectively.

Evaluation of *in vivo* release

Table 1 showed the NT-II concentrations (μg/g) in different organs after mice were administered intranasally with NT-P-NP and free NT-II solution and intravenously with NT-P-NP (IV-NT), respectively. When the mice were given NT-II solution intranasally, the concentration of NT-II in the brain was 0.26 ± 0.03 ng/g. When the mice were treated intravenously with NT-P-NP (IV-NT) the concentration of NT-II in the brain was 2.50 ± 0.21 ng/g. However, when mice were administered intranasally with NT-P-NP, the concentration of NT-II in the brain was 8.66 ± 0.30 ng/g. Paired-Samples T Test revealed that significantly difference in the concentration of NT(peptide)

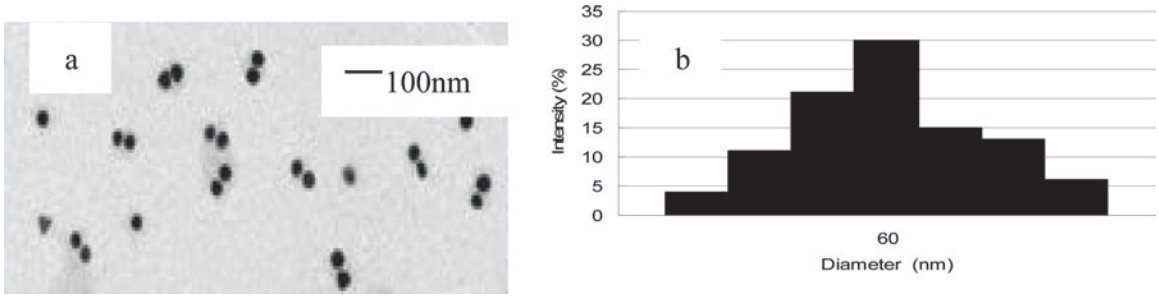

Figure 1. Physical characterization of the NT-P-NP nanobioconjugates. Their size and size-distribution are shown using (A) Transmission electron microscopy (TEM) and (B) dynamic light scattering (DLS).

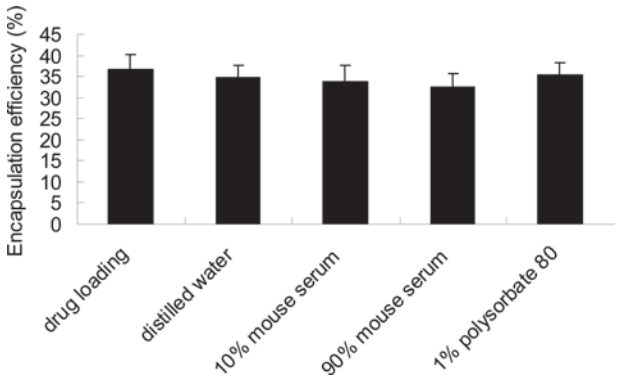

Figure 2. NT-II encapsulation efficiency after drug loading and after purification and dilution with water, 10% mouse serum, 90% mouse serum, and 1% polysorbate 80 (Tween 80), respectively.

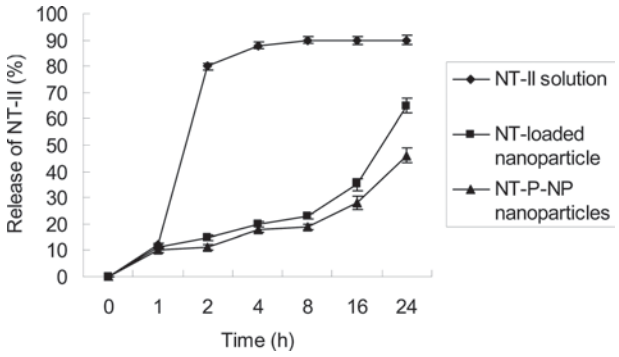

Figure 3. *In vitro* release profile of NT-II solution, NT-loaded nanoparticle and NT-P-NP nanoparticles.

was observed between the NT-P-NP and IV-NT groups ($p = 0.001$).

Evaluation of antinociceptive properties

In the formalin test (Table 2), although both phases of the response were significantly inhibited, the NT-P-NP effect was predominant in Phase 1, causing 66% inhibition of licking time. It indicated that both peripheral analgesic properties and central analgesic effects are the antinociception mechanism of NT-P-NP. On the contrary, the IV-NT effect was predominant in Phase 2, causing 50% inhibition of licking time. It indicated that peripheral analgesic properties are the antinociception mechanism of IV-NT. However, both phases of the response were not significantly inhibited by F-NT.

Discussion

Drug delivery using nanoparticles to target the brain has shown promise in improved drug efficacy and reduced drug toxicity[26,27]. Nanoparticles are able to cross the BBB by mimicking low density lipoprotein (LDL), enabling them to interact with the LDL receptor, resulting in their uptake by brain endothelial cells[26,27]. Due to number of problems related with oral, parenteral, rectal and other routes of drug administration, the interest of pharmaceutical scientists has increased towards

Table 1. Mean NT-II concentration in brain (ng/g).

Groups	Mean NT concentration	*p* value
NT-P-NP	8.66 ± 0.30	
IV-NT	2.50 ± 0.21	$p = 0.002$
F-NT	0.26 ± 0.03	$p = 0.000$

Note: Values are shown as means ± SD. *p* Value versus NT-P-NP group ($n = 10$).

Table 2. Antinociceptive effect of NT-II in mice submitted to the formalin test ($n = 10$).

	Licking time (s)		Inhibition (%)	
Groups	1st phase	2nd phase	1st phase (%)	2nd phase (%)
Control	62.21 ± 3.3	33.3 ± 3.1	—	—
NT-P-NP	25.09 ± 4.9**	21.4 ± 3.3*	59.7	35.7
IV-NT	56.11 ± 3.1	13.7 ± 2.3*	9.80	58.9
F-NT	56.22 ± 3.3	28.9 ± 3.1	9.63	13.2

Note: Values are shown as means ± SD.
*$p < 0.05$ versus control group, **$p < 0.01$ versus control group.

exploring the possibilities of intranasal delivery of various drugs. A drug administered by the nasal route may primarily enter the blood of the general circulation and then penetrate across BBB into the CNS. Efforts have been made to deliver various drugs, especially peptides and proteins, through nasal route for the treatment of local, brain and systemic ailments[28,29,30]. NT-II is an analgesic peptide which has limited permeability across the BBB after intravenously (i.v.) injection. In the present research work, the possibility of intranasal delivery of NT-II with polysorbate-80 coated polylactide homopolymers (PLA) nanoparticles (NT-P-NP) into the brain was investigated.

PLA nanoparticles containing NT-II were prepared by emulsion polymerization and presented a relatively homogeneous shape (Figure 1), with a mean size lower than 100 nm. The entrapment efficiency of the prepared nanoparticles in our study was 36.6 ± 3.6%. These results are in agreement with recently reported data[19]. PLA, the typical biodegradable polyester currently employed in the clinic approved by Food and Drug Agency of USA, was employed to obtain a stable nanoparticle form. The zeta potential was negative (–26.6 ± 2.0 mV), indicating sufficient repulsion between particles to avoid their aggregation and formation of a table nanoparticle suspension. The drug-loading stability experiments revealed a stable drug-loading even in the presence of different concentrations of serum and polysorbate 80.

The first nanoparticle system that was shown to transport molecules across the BBB was polysorbate-80-coated poly (butyl cyanoacrylate) nanoparticles[27]. There are different mechanisms the transport of substances across the BBB is facilitated by polysorbate 80-coated nanoparticles[31,32,33]. The release pattern of NT-loaded nanoparticle coated with polysorbate-80 was noted to be typically biphasic. The initial burst in the first hour indicates that some of the drug molecules could be on the surface of the nanoparticles and the following sustained release might be due to the drug entrapped in

the nanoparticles. According to the *in vivo* results of our experiments, intranasal delivery of NT-loaded nanoparticle coated with polysorbate-80 were able to promote better biodistribution of the drug into the brain, as compared with the free drug solution and intravenously (i.v.) injection drug-loaded nanoparticles.

The antinociceptive animal testing also revealed that intranasal delivery of NT-loaded nanoparticle coated with polysorbate-80 were able to promote better biodistribution of the drug into the brain. The formalin test is different from most models of pain. It is possible to assess the way an animal responds to moderate, continuous pain generated by injured tissue. This model is constituted by two distinct phases. The first phase represents the irritating effects of formalin at the sensorial fibers-C[34]. The second is an inflammatory pain response. Thus, it's possible to appraise the animal's answer to a moderate and continuous pain caused by the tissue lesion as well as the role of pain regulatory endogenous systems. Central acting analgesics, such as morphine, inhibit both phases. Peripheral acting drugs, such as nonsteroid antiinflammatory and corticosteroids, inhibit only the second phase[24]. The present results revealed that both phases of the response were significantly inhibited by the NT-P-NP. However, the IV-NT effect was predominant in Phase 2. This result is consistent with the conclusion that nanoparticles could exert enhanced delivery of NT-II into the brain significantly after in. administration.

Conclusion

The present results demonstrate that the brain delivery of NT-II could be enhanced with PLA nanoparticles coated with polysorbate-80 through i.n. administration. Compared with NT-II solution, NT-P-NP exhibited sustained release *in vitro* and higher concentrations of NT-II in the brain. The antinociceptive animal testing also revealed that intranasal delivery of NT-loaded nanoparticle coated with polysorbate-80 were able to promote better biodistribution of the drug into the brain. It may be concluded that nanoparticles overcoated with polysorbate-80 were capable of transporting the loaded NT-II across the BBB after intranasal administration.

Declaration of interest

This project was supported by Nature Sciences Foundation of Zhejiang (Y2101127). The authors declare that they have no competing interests.

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