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NSL-26628; No. of Pages 4
ARTICLE IN PRESS
Neuroscience Letters xxx (2009) xxx–xxx

Contents lists available at ScienceDirect
Neuroscience Letters
journal homepage: www.elsevier.com/locate/neulet

A novel animal model to evaluate the ability of a drug delivery system to promote the passage through the BBB

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
16 November 2009
Accepted 20 November 2009

Keywords:
Animal model
BBB drug passage
Colloidal carrier
Drug delivery systems
Nanoparticles
Poly-L-lactid acid
Lithium–tacrine model
Electrocorticogram

ABSTRACT

The purpose of this investigation was to explore the potentiality of a novel animal model to be used for the *in vivo* evaluation of the ability of a drug delivery system to promote the passage through the blood–brain barrier (BBB) and/or to improve the brain localization of a bioactive compound. A Tween 80®-coated poly-L-lactid acid nanoparticles was used as a model of colloidal drug delivery system, able to trespass the BBB. Tacrine, administered in LiCl pre-treated rats, induces electrocorticographic seizures and delayed hippocampal damage. The toxic effects of tacrine-loaded poly-L-lactid acid nanoparticles (5 mg/kg), a saline solution of tacrine (5 mg/kg) and an empty colloidal nanoparticle suspension were compared following i.p. administration in LiCl-pre-treated Wistar rats. All the animals treated with tacrine-loaded nanoparticles showed an earlier outcome of CNS adverse symptoms, i.e. epileptic onset, with respect to those animals treated with the free compound (10 min vs. 22 min respectively). In addition, tacrine-loaded nanoparticles administration induced damage of neuronal cells in CA1 field of the hippocampus in all treated animals, while the saline solution of tacrine only in 60% of animals. Empty nanoparticles provided similar results to control (saline-treated) group of animals. In conclusion, the evaluation of time-to-onset of symptoms and the severity of neurodegenerative processes induced by the tacrine–lithium model of epilepsy in the rat, could be used to evaluate preliminarily the capability of a drug delivery system to trespass (or not) the BBB *in vivo*.

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Central nervous system (CNS)-acting drugs must trepass the blood–brain barrier (BBB) before reaching their target [7]. The BBB possess a selective permeability for some substances to the CNS; lipophilic compounds can diffuse through the endothelial cell membranes and passively enter the CNS with respect to hydrophilic molecules that penetrate more difficulty into the brain [16]. BBB has an important function for the protection of the brain from fluctuations of plasma composition and from circulating agents, such as neurotransmitters and xenobiotics which are able to disturb neural function [1]. Therefore, the role of BBB is crucial for limiting the access of the potentially damaging xenobiotics and metabolites to the CNS by either blocking their access or actively removing them from the brain [8].

The BBB and the protective epithelial layer of the choroid plexus, i.e. the blood–cerebrospinal fluid barrier (BCSFB), represent insurmountable obstacle for the brain penetration of a large number of pharmacologically active compounds aimed to treat CNS-based disturbs, i.e. depression, schizophrenia, epilepsy, multiple sclerosis, and neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease [23]. In clinical development, candidate CNS-acting drugs have the poorest success rate due to the fact that more than 98% of such potential drugs have to be discontinued because of poor permeability across the BBB [6]. This is a real problem for basic research and pharmaceutical industry, making the accent on the necessity to develop – in parallel with new molecules that possess central action(s) – new methods useful for an early assessment of the ability of drugs candidate to penetrate the CNS [17], an ability that could also be increased by enhancing drug delivery into the CNS [9].

A successful strategy to increase drug penetration into the brain is the use of polymeric nanoparticles [13]. The advantage of their use is due to their small size, that allows nanoparticles to

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doi:10.1016/j.neulet.2009.11.051

penetrate into small capillaries and to be taken up within cells, thus allowing an efficient drug accumulation in the target sites and a sustained drug release over a period of days or even weeks after administration [10,18,22].

The administration of 1,2,3,4-tetrahydro-9-amino-acridine (tacrine), a cholinesterase inhibitor, in lithium chloride (LiCl) pre-treated rats, produces motor and electrocorticographic (ECoG) seizures and delayed hippocampal damage [3,4], thus providing an useful tool for the study of epilepsy. This model is very well defined in terms of time-to-onset of ECoG seizures, behavioural and morphological changes occurring in CNS after LiCl/tacrine treatment. It was demonstrated [5] that the effects of tacrine in LiCl pre-treated rats are directly related to its entrance in CNS, follow a dose-dependent profile and no peripheral action regarding to CNS is recognised for tacrine. Therefore, the presence of significant variations in neurological effects of tacrine (i.e. time-to-onset of ECoG seizures, behavioural and morphological changes) eventually occurring in animals treated with the same dose of tacrine administered by different formulations, could be used as a parameter to evaluate the penetration rate of this drug into CNS.

The aim of this investigation was the evaluation, in LiCl/pretereated rats, of the modifications in the characteristic epileptogenic response eventually induced from the administration of tacrine-loaded poly-L-lactid acid (PLA) nanoparticles with respect to the non-encapsulated molecule, to exploit the possibility to propose this experimental set as a relevant and reliable experimental model useful to predict the efficacy of a drug delivery system to enhance the penetration of a CNS-acting drug through the BBB.

PLA nanoparticles were prepared according to the emulsification and diffusion technique with same variation [20]. Briefly, tacrine chloridrate (~4 mg) was dissolved in 1 ml of saline solution (NaCl 0.9%, w/v) and added to an organic phase (3 ml) made up of chloroform/dichloromethane (2:1, v/v) containing PLA (0.012 g) and Span 80 (0.1 g). The obtained mixture was maintained under mechanical agitation at 24,000 rpm for 1 min (Ultraturrax T25, IKA® Werke) forming the primary W/O emulsion. Successively, the primary W/O emulsion was added to a secondary aqueous phase (W^I), containing water (2 ml) and Tween 80® (0.2 g), to obtain the final $W/O/W^I$ emulsion. Finally, the formulation was kept under stirring for 5 h to favour the evaporation of the organic solvent. The PLA nanoparticles were purified through centrifugation at 37,000 rpm for 1 h at 4 °C (Beckman Avanti™ 30, Fullerton, CA) and, after removal of the supernatant, they were re-suspended in saline solution.

Mean size and polydispersity index of nanoparticle systems were evaluated by dynamic light-scattering experiments. The dimensional analysis was carried out by photocorrelation spectroscopy (PCS) (Nanosizer Nano ZS, Malvern Instruments Ltd., Spring Lane South, Worcs, England) using a 4.5 mW laser operating at 670 nm. Experiments were carried out at a back-scattering angle of 173°. A third-order cumulant fitting correlation function was performed by a Malvern PCS sub-micron particle analyzer. Samples were suitably diluted with a filtered (Sartorius membrane filters 0.22 μm) saline to avoid multiscattering phenomena and placed in a quartz cuvette. Experiments were carried out at room temperature. As reported in Table 1, the mean size of PLA nanoparticles was 220 nm and the presence of tacrine did not induce any destabilization phenomenon in the nanoparticle colloidal suspension. The polidispersity index was ~0.1 both in the presence and the absence of tacrine, thus evidencing a suitable narrow nanoparticle size distribution.

The untrapped drug removed by centrifugation, was spectrophotometrically determined at the tacrine λ_{max} 323 nm by a PerkinElmer Lambda 20 UV-Vis spectrophotometer (Uberlingen, Germany) using a PerkinElmer UV WinLab™ ver. 2.8 acquisition software. The amount of tacrine entrapped within PLA nanoparticles was 45%, it was calculated by difference from the drug amount used for the preparation and expressed as a percentage with respect to the drug originally added.

Table 1
Mean sizes and polydispersion index of PLA nanoparticles prepared both in the presence and in the absence of tacrine systems[a]. PLA nanoparticles were coated by Tween 80®.

Sample	Mean sizes (nm)	Polydispersion index
Empty PLA nanoparticles	241.3 ± 7.1	0.124 ± 0.011
Tacrine-loaded PLA nanoparticles	258 ± 6.4	0.138 ± 0.029

[a] Each value represents the average of three different experiments ± standard deviation coming from three different PLA nanoparticle batches.

The tacrine release was evaluated through the dialysis method by using cellulose acetate dialysis tubing (Spectra/Por with molecular cut-off 12,000–14,000 by Spectrum Laboratories Inc., Netherlands) sealed at both ends with clips [21]. An isotonic pH 7.4 phosphate buffer solution was used as the release fluid under constant stirring and it was warmed (GR 150 thermostat, Grant Instruments Ltd., Cambridge, UK) to 37.0 ± 0.1 °C throughout the release experiments. Before dialysis, the tubing was kept overnight in the buffer solution to allow the complete wetting of the membrane. Tacrine-loaded PLA nanoparticle suspension (1 ml) was poured into dialysis bag, which was then transferred into a beaker containing 200 ml of the release buffer thus following sink conditions for 24 h experiments. A sample of release fluid (1 ml) was withdrawn at predetermined time intervals and replaced with the same volume of fresh fluid. Release fluid samples were spectrophotometrically analyzed at tacrine λ_{max} 323 nm. No interference was observed from the components of the PLA nanoparticle formulation. The release studies were carried out in triplicate. As shown in Fig. 1, the release profile of tacrine was characterised by a rapid leakage during the first 5 h (up to 30%), probably due to the rapid diffusion of the compound from the outer part of the nanoparticles followed by a slower release up to 24 h with a released aliquot of ~44%. These features are related to both physico-chemical properties of the drug as well as the morphological and technological characteristics of the colloidal drug carrier.

To evaluate the ability of the polymeric carrier to effectively transport tacrine at level of the CNS, *in vivo* experiments on an animal model of neurotoxicity were carried out. All *in vivo* experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986

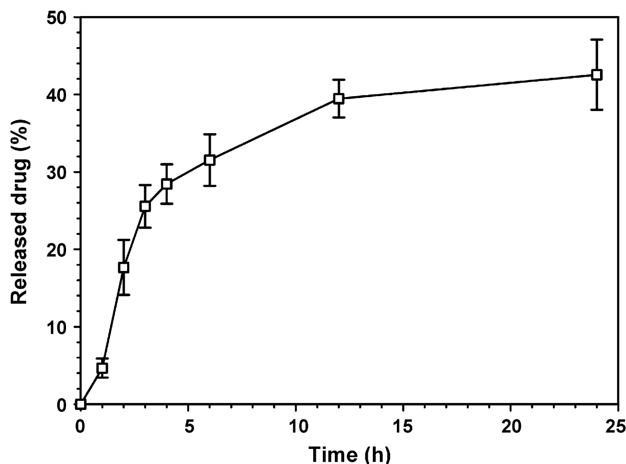

Fig. 1. Release profile of tacrine from Tween 80®-coated PLA nanoparticles. Experiments were carried out at room temperature. Values represent the average of three different experiments ± standard deviation.

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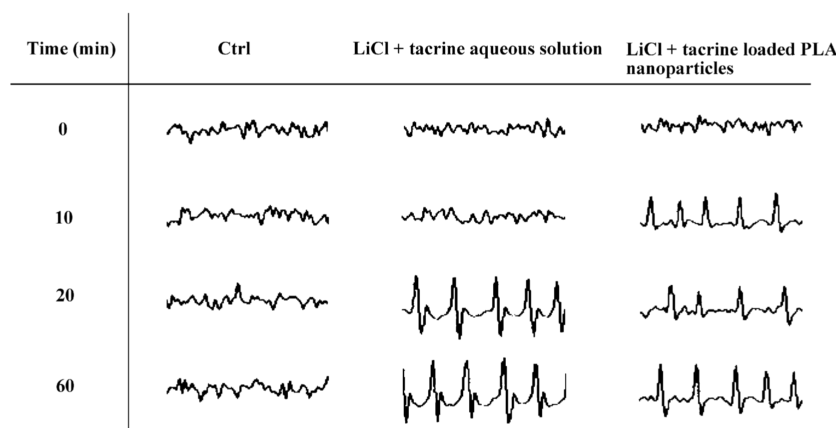

Fig. 2. EcoG traces of Wistar rats pre-treated with LiCl (12 mequiv./kg) and after 24 h submitted to treatment with saline solution (NaCl 0.9%, w/v), free tacrine (5 mg/kg) and tacrine-loaded Tween 80®-coated PLA nanoparticles (5 mg/kg).

(86/609/EEC). Adult male Wistar rats, weighing 300–350 g (Harlan, San Pietro al Natisone, Udine), were maintained at standard conditions of both temperature (20 ± 2 °C) and humidity (65%) with 12 h light/12 h dark cycle (light on 8:00 a.m.) and food and water *ad libitum*. One week before experiments, animals were anaesthetized with chloral hydrate (400 mg/kg i.p.) and six recording electrodes were chronically implanted on the epidural surface. The electrodes were stereotaxically positioned (D. Kopf Instruments) on the cerebral cortex of both hemispheres, according to the coordinates of the brain atlas of Paxinos and Watson [19] and anchored to the skull with dental acrylic cement [11]. Rats were allowed one-week recovery and no alterations of posture or motor activity change was observed over this period. Rats were then submitted to the treatment with lithium chloride (12 mequiv./kg) and after 24 h, they were treated intraperitoneally with tacrine (free or encapsulated at a dose of 5 mg/kg), with empty PLA nanoparticles or saline solution (NaCl 0.9%, w/v). The same volume of various formulations was always administered. Control group was pre-treated with LiCl but submitted to no treatment. The cortical electrodes were connected to the EEG instrument ESAOTE Biomedica (Firenze) 1 h before the experiments to record the electrocorticogram (ECoG). The evaluation of the ECoG spectra (0.25–16 Hz) was carried out during the hour before the treatment and during the last two hour. The postural and locomotor changes were observed and recorded by two independent observers that were unaware to the treatment. 24 h after the experiment, rats were anaesthetized with chloral hydrate (400 mg/kg i.p.), transcardially perfused with 4% (w/v) buffered formaldehyde (pH 7.4) after a brief rinse with saline and heparin (0.1%, w/v) and the brain was removed, dehydrated in alcohol and embedded in paraffin. Serial section (10 μm) was sliced using a microtome (Leitz). Slices were positioned on polylysined glasses (26 mm × 76 mm, Bio-Optica), moisturized, stained with Nissl method (Cresyl-violet) and examined through an optical microscope (Leitz Aristoplan). The morphological analysis was carried out by an image analysis system IM500 (Leitz).

The treatment of animals (n = 10) with empty nanoparticles 24 h after the administration of LiCl induced no behavioural modifications or ECoG variations during the observation period (ECoG profiles were similar to the control group, Fig. 2). The histological examination of the brain of these animals did not show the presence of neurodegeneration or other tissue damage (Fig. 3). Similar findings were achieved for the animal group treated with saline. On the contrary, the animals (n = 10) treated with free tacrine showed clear behavioural modifications with ptialism, wet shake dog and tonic–clonic convulsions 22.3 ± 2 min after the administration (Table 2). At the same time it was possible to observe modifications of the ECoG traces thus showing trains of short duration epilectogenic spikes that were repeated during the entire observation period (Fig. 2). The histological examination of the brain of the animals treated with free tacrine evidenced a marked degeneration of hippocampal cellular area CA1 in six subjects on ten (Table 2

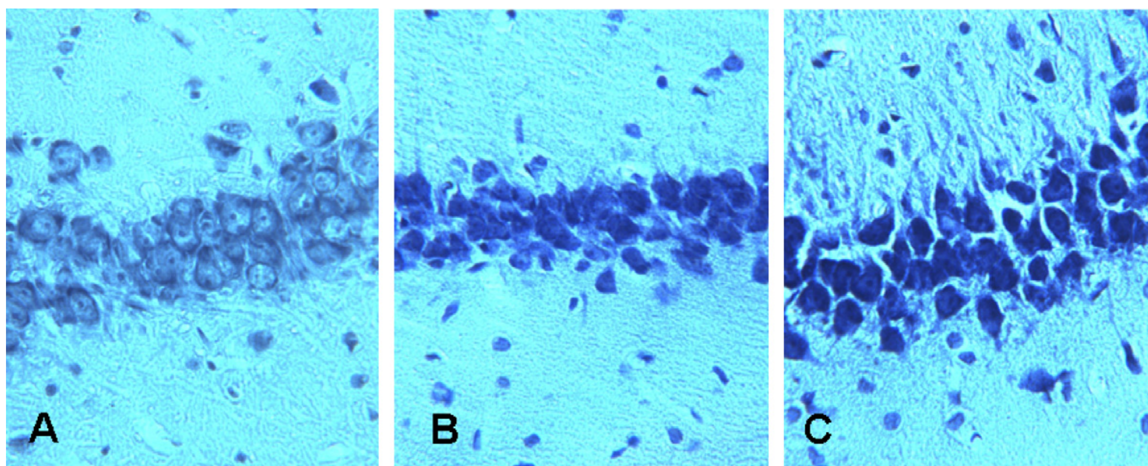

Fig. 3. Hippocampal morphology (CA1 area) from animals treated with empty nanoparticles (A), free tacrine (5 mg/kg) (B) and tacrine-loaded PLA nanoparticles (5 mg/kg) (C), 24 h after the administration of LiCl (12 mequiv./kg).

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Table 2
Central nervous system neurotoxicological parameters in LiCl (12 mequiv./kg) pre-treated Wistar rats (300–350 g) submitted to various treatments.

Treatment	Start time of symptoms and ECoG modification (Min ± Sem)[a]	Presence of histological damage (number of animals)[a,b]
Ctrl[c]	–	0/10
Saline	–	0/10
Empty PLA nanoparticles	–	0/10
Free tacrine (5 mg/kg)	22.3 ± 2	6/10
Tacrine-loaded PLA nanoparticles (5 mg/kg)	10.5 ± 1	10/10

[a] Each value represents the average of three different experiments ± standard deviation.
[b] The presence of histological damage was expressed as a rates between the number of animal which present a neurological damage and the total number of animal submitted to the experiment.
[c] Animals pre-treated with LiCl and submitted to no further treatment.

and Fig. 3). All the animals ($n = 10$) submitted to the treatment with tacrine-loaded PLA nanoparticles showed marked behavioural modifications with ptialism, wet shake dog and tonic–clonic convulsions, but they manifested this symptoms 10.5 ± 1 min after the injection of the formulation (Table 2). Also in this case modifications of the ECoG traces, i.e. trains of short duration epilectogenic spikes throughout the duration of the experiment, were observed (Fig. 2). In this treatment group the histological examination of the brain showed a marked degeneration of hippocampal cellular area CA1 in all treated animals (Table 2 and Fig. 3).

The early appearance of toxic effects of tacrine-loaded PLA nanoparticles with respect to those induced by the free drug is essentially due to its faster and greater uptake inside the brain. The rationale of this event is probably related to the presence of Tween 80® along the surface of colloidal nanoparticles that allows them to overcome the BBB through an endocytotic mechanisms [2,14,15,12] mediated by the plasmatic protein apolipoprotein E (apo-E). It is well known that apo-E represents an essential factor in the transport of lipoproteins (e.g. low density lipoprotein) through the BBB because of the great presence of LDL receptors. Tween 80®-coated nanoparticles mimic LDLs after apo-E adsorption on their surfaces acting as “Trojan horses” via LDL receptors [12] determining the different toxicological profile of the free and the encapsulated form of tacrine.

In conclusion, our experimental results demonstrate that the tacrine/LiCl model could be used as an indirect method to evaluate both the extent and the rate of the brain uptake of a drug delivery system *in vivo*.

Acknowledgements

The authors wish to thank Drs Angelo Rocca and Laura Cosco (ARPACal-CERA) for technical assistance and Mr Domenico Apuzzo (CNR-ISN) for administrative assistance.

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