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Single injection of ONO-1301-loaded PLGA microspheres directly after ischaemia reduces ischaemic damage in rats subjected to middle cerebral artery occlusion

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oedema; MCAO; neuroprotective effect; PLGA microspheres; prostacyclin

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Abstract

Objectives ONO-1301 was developed as a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity. In this study, we investigated the therapeutic time window of oral ONO-1301 and the effect of a single subcutaneous injection of ONO-1301-loaded poly(lactide-co-glycolide) (PLGA) microspheres (ONO-1301 PLGA MS) on infarction volume, functional deficits and plasma ONO-1301 levels following a 1 h middle cerebral artery occlusion (MCAO) in rats.

Methods Rats were treated with ONO-1301 (3 mg/kg) orally twice-daily starting 1 (directly), 6 or 24 h after MCAO. Rats received a single subcutaneous injection of ONO-1301 PLGA MS (10 mg/kg) directly after MCAO. Neurological scores were evaluated directly after, 1 and 6 h, 1, 2, and 3 days after MCAO. Infarct volume, oedema and plasma ONO-1301 levels were measured three days after MCAO.

Key findings Neurological scores, oedema and infarct volume were all significantly improved in rats repeatedly treated with oral ONO-1301 and subcutaneous ONO-1301 PLGA MS directly after MCAO. Plasma ONO-1301 levels were significantly lower in rats treated directly after MCAO (either with ONO-1301 or ONO-1301 PLGA MS) than in rats treated 6 h or 24 h after MCAO.

Conclusions ONO-1301 PLGA MS subcutaneous treatment directly after MCAO showed a neuroprotective effect as well as oral ONO-1301. This treatment should be clinically more convenient than ONO-1301 oral administration since it is delivered as a single treatment after MCAO.

Introduction

ONO-1301 is a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity. Its inhibitory effect on thromboxane synthetase is mediated by binding of thromboxane synthase to the 3-pyridin moiety and a carboxylic acid group in ONO-1301.[1]ONO-1301 has been reported to improve pulmonary hypertension[2,3] and pulmonary fibrosis.[4] However, the neuroprotective effect of ONO-1301 against transient brain ischaemia has not previously been investigated in detail. In this rat study, ONO-1301 was shown to be capable of reducing brain damage after ischaemic trauma if given shortly after the ischaemic insult. Furthermore, the duration of action of prostacyclin analogues is so short that they need to be continuously infused or frequently administered.[5–8] Prostacyclin has also been shown to cause episodes of hypotension[9] and a sudden unheralded sinus bradycardia[10] due to its vasodilatory effects. Beneficial effects of low-dose prostacyclin infusion (0.5 ng/kg/min) have also been reported following severe traumatic brain injury.[11,12]

Prevention of the ischaemic penumbra becoming part of the ischaemic core is the most important aim following focal ischaemia. Immediately after the onset of focal ischaemia, the penumbra is evanescent but it progressively deteriorates over just a few hours, eventually becoming subsumed within the ischaemic core.[13] The middle cerebral artery occlusion (MCAO) model has been used to examine the evolution of the ischaemic penumbra as an exceptionally reproducible model of the events following transient ischaemia.[14]

The purpose of this study was to investigate the neuroprotective effects of repeated treatment with ONO-1301 from 1, 6 and 24 h after MCAO. The neuroprotective effect of a single subcutaneous injection of ONO-1301 poly(lactide-co-glycolide) microspheres (ONO-1301 PLGA MS) was

compared with low-dose repeated oral treatment with ONO-1301 from 1 h after MCAO to evaluate the merit of single treatment with ONO-1301 PLGA MS. The neuroprotective effect was measured using a neurological deficit score, infarct volume and degree of oedema. ONO-1301 plasma levels were measured on the third day after MCAO.

In this study, the neuroprotective effect of ONO-1301 in rats subjected to middle cerebral artery occlusion was newly confirmed when ONO-1301 powder or ONO-1301 PLGA MS was administered from 1 h after ischaemia. Furthermore, single treatment with ONO-1301 PLGA MS suppressing the initial burst was more effective than repeated treatment with ONO-1301 powder in the stand-point of neuroprotective effect and inhibition of side effects.

Materials and Methods

Materials

ONO-1301 was generously supplied by ONO Pharmaceutical Co., Ltd (Osaka, Japan). PLGA (average molecular weight 50 000, co-polymer ratio of D,L-lactide to glycolide: 50/50, PLGA 5050; Wako Pure Chemical Industries Ltd, Osaka, Japan) was used as a substrate in the microspheres. Polyvinyl alcohol (PVA; Nacalai Tesque Ltd, Kyoto, Japan) and Tween 80 (Nacalai Tesque Ltd, Kyoto, Japan) were used as dispersants in the production of PLGA microspheres. D-Mannitol (Wako Pure Chemical Industries Ltd, Osaka, Japan) was used as addition agent in the freeze-drying process. High-reagent-grade acetone, acetonitrile, methanol and ethanol were used as good solvents for PLGA; Japanese Pharmacopoeia-grade purified water was used as the poor solvent.

Animals

Male Wistar rats, 250–300 g, were obtained from Charles River Japan Inc. (Hino, Japan), and were housed in groups of 4–5 rats per cage in a temperature-controlled room (23 ± 2°C) at a relative humidity of 60 ± 10%. The lights were on from 0700 to 1900 h. The rats had free access to food (CRF-1, Oriental Yeast Co., Ltd, Tokyo, Japan) and water in their home cage. Experiments were conducted between 0900 and 1700 h. The study protocols were performed according to the Guideline for the Care and Use of laboratory Animals approved by Mukogawa Women's University (permission number 100035). All efforts were made to minimize the number of animals used for experiments and their suffering.

Transient focal cerebral ischaemia

Focal cerebral ischaemia was induced according to the method described in a previous study.[15] Rats were anaesthetized with 2% halothane and anaesthesia was maintained thereafter with 1% halothane (Flosen; Takeda Chemical Industries, Osaka, Japan). After a midline neck incision, the left common and external carotid arteries were isolated and ligated. A nylon monofilament (4-0, Ethilon; Johnson & Johnson, Tokyo, Japan) coated with silicon resin (Xantopren; Heleus Dental Material, Osaka, Japan) was introduced through a small incision into the common carotid artery and advanced to a position 20–21 mm distal to the carotid bifurcation for occlusion of the middle cerebral artery. One hour after occlusion, the rats were re-anaesthetized with halothane and reperfusion was established by withdrawal of the filament.

Rats were randomly divided into five groups: group (a) received a single oral treatment with 0.5% w/v carboxymethyl cellulose solution immediately after MCAO (vehicle-ischaemia group); group (b) received ONO-1301 3 mg/kg orally twice daily starting directly (1 h) after MCAO; group (c) received ONO-1301 3 mg/kg orally twice daily from 6 h after MCAO; group (d) received ONO-1301 3 mg/kg orally twice daily from 24 h after MCAO; and group (e) received a single subcutaneous injection of ONO-1301 PLGA MS, 10 mg/kg, directly after MCAO.

Preparation of ONO-1301 PLGA MS

A sustained-release formulation of ONO-1301 was prepared by loading ONO-1301 onto poly(lactide-co-glycolide) (PLGA) microspheres using an oil-in-water emulsion/solvent evaporation method described in a previous report.[16] The preparative procedures can be summarized as follows: an aqueous solution of polyvinyl alcohol (a poor solvent of PLGA) was placed in a glass vessel. A methanol–dichloromethane solution containing PLGA and ONO-1301 was dropped into the vessel, while stirring, to form an oil-in-water emulsion. Dichloromethane was then evaporated off by stirring at room temperature. After centrifugation and washing, ONO-1301 PLGA MS were isolated by lyophilization.

Particle size and morphology

The volume diameters of the wet particles sampled before lyophilization were measured using a Multisizer3 Coulter Counter (Beckman Coulter, Inc., Japan). The volume diameters of the wet particles were considered as geometric diameters. When the particle size was measured after lyophilization, the dry particles were resuspended in buffer using a vortex mixer and analysed with the particle size analyser.

Scanning electron microscopy (SEM) was used to characterize the surface and cross section of the beads with a razor-blade. The sample was coated for 70 s under an argon atmosphere with gold-palladium (Pelco Model 3 sputter coater; Fuji Electric FA Components & System Co., Ltd, Tokyo, Japan) and examined with a scanning electron microscope (JSM-6360; Jeol Ltd, Tokyo, Japan).

Encapsulation efficiency

Acetonitrile containing n-propyl 4-hydrobenzoate served as an internal control to obtain the encapsulation efficiency, and this solution was homogenized by a sonicator. The concentration of ONO-1301 in this solution was analysed by high-performance liquid chromatography (HPLC). The encapsulation efficiency was calculated as follows:[3]

$$\text{Encapsulation efficiency (EE \%)} = (\text{measure value} / \text{theoretical value}) \times 100.$$

Drug release from ONO-1301 PLGA MS

ONO-1301 PLGA MS were suspended in phosphate-buffered saline (0.067 mol/l salt concentration, pH 6.8) containing 0.2% Tween-80 and the concentration adjusted to 100 μg ONO-1301/ml. Volumes of 1 ml were incubated at 37°C. At various time intervals (1, 4, 7, 14, 21 and 28 days) thereafter, the liquid phase from one sample was discarded, the pellet dissolved in dimethyl sulfoxide, and the ONO-1301 level analysed by HPLC.

Neurological deficit score

The neuroprotective effects of ONO-1301 and ONO-1301 PLGA MS were evaluated at 1 and 6 h, 1, 2 and 3 days after MCAO, using a neurological deficit score based on three signs: the first sign was abnormal posture when the rats were lifted by their tails; the second sign was the reduction of traction power in their right hindlimbs; and the third sign was the degree of hemiplegia in their right forelimbs when the rats were lifted by their tails. Each sign was scored using the following criteria: 0, no abnormality; 1, mild abnormality; and 2, severe abnormality. The neurological deficit was expressed as the sum of the three scores,[17] and could range between 0 and 6.

Assay of plasma levels of ONO-1301

Plasma levels of ONO-1301 were measured after repeated oral treatment with 3 mg/kg ONO-1301 and a single subcutaneous treatment with 10 mg/kg ONO-1301 PLGA MS. Blood was drawn from the inferior vena cava of 15–20 rats per group on the third day after MCAO (12 h after the final oral administration of 3 mg/kg ONO-1301). The blood was immediately transferred to chilled glass tubes containing 1 mg/ml disodium ethylenediaminetetraacetic acid and 500 U/ml aprotinin, and centrifuged. Plasma ONO-1301 levels were measured by liquid chromatography with tandem mass spectrometry assay.

Measurement of infarct areas and observation of cerebral oedema

In total, 17 rats received 0.5 w/v% carboxymethyl cellulose solution directly after MCAO, 20 rats received 3 mg/kg of ONO-1301 orally twice daily starting 1 h after MCAO, 15 rats received 3 mg/kg of ONO-1301 orally twice daily starting 6 h after MCAO, 18 rats received 3 mg/kg of ONO-1301 orally twice daily starting 24 h after MCAO and 18 rats received a single subcutaneous injection of 10 mg/kg ONO-1301 PLGA MS 1 h after MCAO. Three days after MCAO all 88 rats were decapitated and their brains fixed in a 35% formaldehyde solution. The hindbrain was detached by cutting through the midbrain, and the cerebral hemispheres were cut into five coronal slices, each 2 mm thick.[18] Sections, 7–8 μm thick, were stained using hemalum and eosin by a method combining crystal violet and Luxol fast blue. Each section was photographed through the light microscope, and infarct size was measured using an Avionics Japan TVIP-2000 image analyser by a researcher blind to the treatment given. The total area of infarct was analysed by image analysis system, and the ratio of infarction was determined (total area of ipsilateral cerebral hemisphere divided by infarcted area[19]). The degree of cerebral oedema was measured by calculating the ratio of the areas of the two cerebral hemispheres.[20]

Statistical analysis

Property of ONO-1301 PLGA MS and the data of drug release study are expressed as means ± SD. In-vivo data are expressed as means ± SE. Data analysis of the neurological scores, infarct volume and degree of oedema were evaluated for statistical significance using Dunnett's *t*-test. ONO-1301 plasma levels were evaluated for statistical significance using Student–Newman–Keuls (SNK) test; $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Property of ONO-1301 PLGA microspheres prepared in this study

ONO-1301 PLGA MS containing PLGA 5050 used in the in-vivo study are shown in Figure 1. The average diameter of the prepared microspheres was 33.1 ± 11.8 μm. The size distribution of particle is shown in Figure 1a. The drug encapsulation efficiency of prepared microspheres was 74.3 ± 0.2%. The SEM image of the prepared microsphere was spherical in shape with a smooth surface (Figure 1b). The drug residual ratio after 1, 4, 7, 14, 21 and 28 days was 84.6 ± 1.6, 75.7 ± 0.4, 71.6 ± 0.6, 40.4 ± 4.1, 10.3 ± 2.7 and 6.9 ± 0.7%, respectively (Figure 1c). ONO-1301 PLGA MS prepared in this study was spherical in shape with smooth surface, good drug encapsulation efficiency was confirmed, and the drug release from PLGA MS lasted about for 3 weeks.

This particular type of formulation used in this study is the same as formulation No. 1 in our previous report,[16] and the obtained data was essentially the same.[16] The obtained data in the previous study in Ref 16 was as follows: the drug

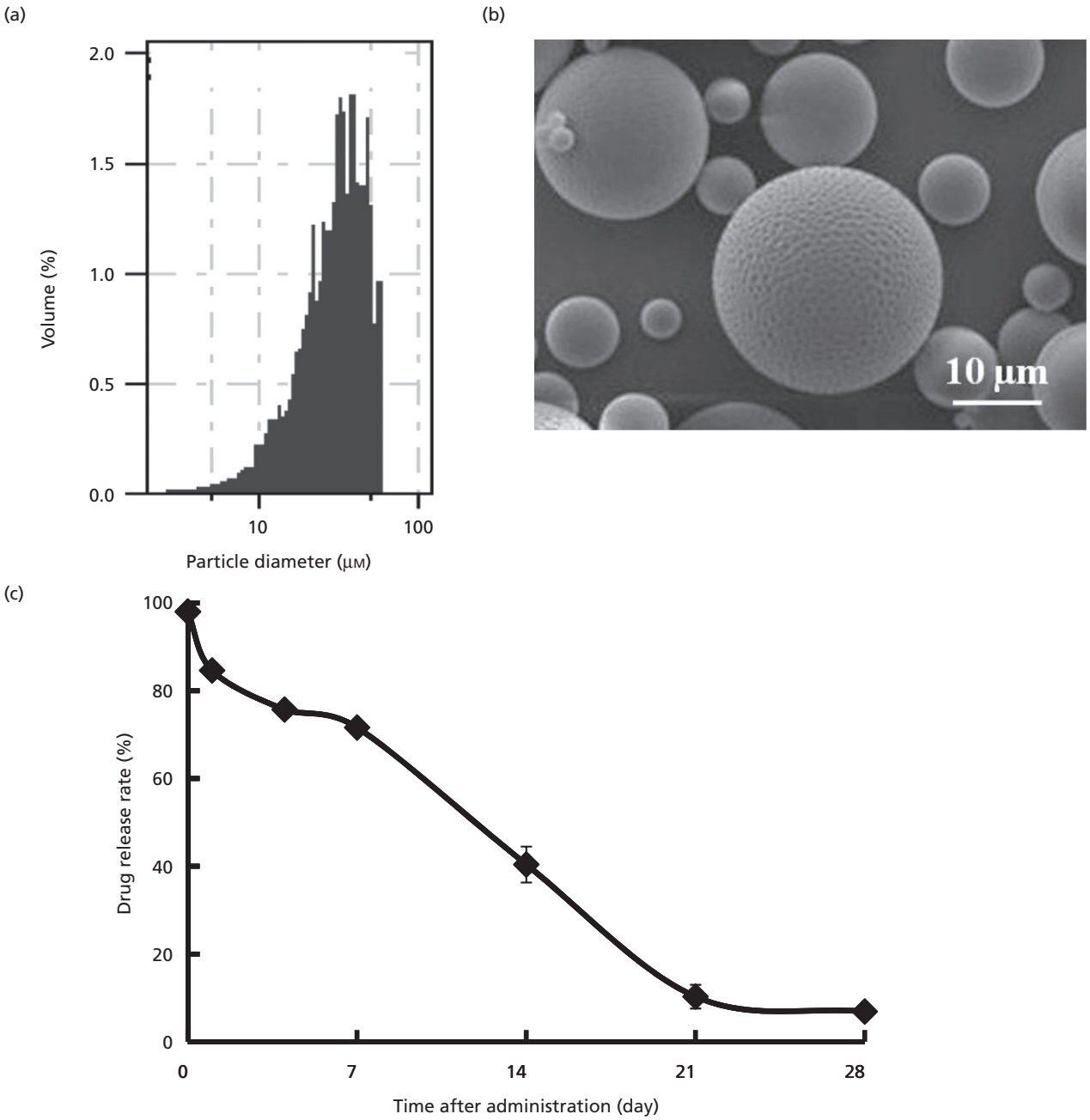

Figure 1 Property of ONO-1301 PLGA MS prepared in this study. The distribution of particle diameter (a) and scanning electron micrographs (b) of ONO-1301 PLGA MS prepared in this study is displayed. The drug release study of ONO-1301 from ONO-1301 PLGA MS was displayed (c). Values represent the mean ± SD (n = 3).

encapsulation efficiency of prepared microspheres was 74.3 ± 1.8%; the average diameter of the prepared microspheres was 30.3 ± 11.2 μm.

With the glucocorticoid soft drug loteprednol etabonate (LE)-loaded PLA and PLGA microsphere it has been shown that the approach is able to customize slow release and to reduce systemic side effects, since LE has high activity.[21] In our previous study, ONO-1301 PLGA MS showed a slow release profile, without side effects, and a customized release profile.[16] In this study, we needed to choose the formulation that exhibited slow release and reduced side effects to investigate neuroprotective effects in the acute phase after MCAO. To protect against neuronal damage in the acute phase, defined as 3 weeks after ischaemic attack,[22] in occupational therapy and drug therapy was the most important to reduce after effects induced by cerebral ischaemia. Thus, formulation

Table 1 Effect of ONO-1301 administration on neurological deficit score following middle cerebral artery occlusion in rats

Group	Time after MCAO				
	1 h	6 h	1 day	2 days	3 days
(a)	6.0 ± 0.0	5.8 ± 0.1	5.8 ± 0.1	5.6 ± 0.1	5.6 ± 0.2
(b)	6.0 ± 0.0	5.6 ± 0.1	3.9 ± 0.5**	2.8 ± 0.5**	2.1 ± 0.5**
(c)	6.0 ± 0.0	5.7 ± 0.1	5.3 ± 0.2	4.8 ± 0.4	4.2 ± 0.5*
(d)	6.0 ± 0.0	5.8 ± 0.1	5.3 ± 0.2	4.8 ± 0.3	3.7 ± 0.4*
(e)	6.0 ± 0.0	5.7 ± 0.1	3.1 ± 0.5**	2.4 ± 0.5**	1.8 ± 0.4**

Group (a) vehicle-treated (n = 17). Group (b) repeated oral treatment with 3 mg/kg ONO-1301 from 1 h after MCAO (n = 20). Group (c) repeated oral treatment with 3 mg/kg ONO-1301 from 6 h after MCAO (n = 15). Group (d) repeated oral treatment with 3 mg/kg ONO-1301 from 24 h after MCAO (n = 18). Group (e) single subcutaneous treatment with 10 mg/kg ONO-1301 PLGA MS at 1 h after MCAO (n = 18). *P < 0.05, **P < 0.01 vs vehicle-treated group (Dunnett's t-test). Values represent the mean ± SE of 15–20 rats.

No.1 described in our previous report[16] was used in this study since the drug release profile was shown for 3 weeks.

Neurological deficit score

Neurological deficit scores are shown in Table 1. Scores were assessed at five time-points: 1 and 6 h and 1, 2 and 3 days after MCAO. At the first time-point, 1 h after MCAO, the neurological deficit score was 6 in all groups. By 1 and 2 days after MCAO, the score in the MS-treated group was significantly improved compared with the vehicle-treated group (group (a) versus (e), $P < 0.05$). At the last time point, 3 days after MCAO, scores were significantly improved in all groups compared with vehicle (groups (b) and (e) $P < 0.01$, (c) and (d) $P < 0.05$).

The most effective protection against brain damage following MCAO was achieved when oral ONO-1301 treatment was started directly (1 h) after the ischaemic insult. This protection was more effective than when oral dosing was not started until 6 h or 24 h after MCAO.

Formation of cerebral infarcts and oedema

Treatment with oral ONO-1301 (3 mg/kg twice daily) and subcutaneous ONO-1301 PLGA MS (10 mg/kg) reduced the volume of infarction and degree of brain oedema compared with vehicle by 3 days after MCAO. The infarct volume and degree of brain oedema in groups (b) and (e) were significantly decreased compared with group (a) ($P < 0.01$; Figures 2 and 3, respectively). Figure 3b shows the degrees of oedema in the different brain slices. The left side of the brain from the vehicle-treated group (a) in particular (area of ipsilateral cerebral hemisphere marked) was more swollen than the right side. The degree of oedema was correlated with the volume of infarction.

It has been reported that neurological impairment and degree of oedema are correlated with the size of the infarct area in the brain.[23] Our results were considered with conclusion shown in this previous article. One of the most important considerations when treating acute stroke patients is to establish whether potentially salvageable (penumbral) tissue is still present within the brain. It has previously been demonstrated that penumbral lesions were still present 6 h after MCAO in rats.[24] It is therefore postulated that the neuroprotective effect of ONO-1301 can be attributed to prevention of the ischaemic penumbra becoming subsumed within the ischaemic core.

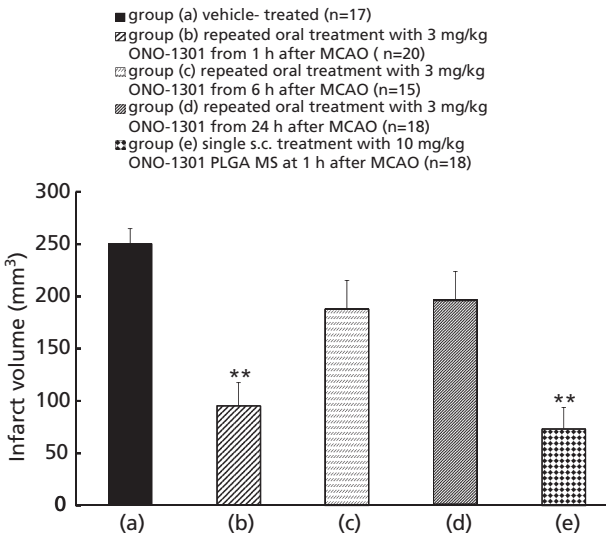

Figure 2 Effect of ONO-1301 administration on cerebral infarction induced by MCAO in rats. Brain damage was evaluated as infarct volume in five groups of rats, as shown. **P < 0.01 vs vehicle-treated group (Dunnett's t-test). Values represent the mean ± SE of 15–20 rats.

When we compared the neuroprotective effects of ONO-1301 PLGA MS with oral ONO-1301 treatment, we found that a single subcutaneous injection of ONO-1301 PLGA MS directly (1 h) after MCAO was as effective as repeated twice-daily oral administration of ONO-1301 starting directly (1 h) after MCAO in reducing ischaemic brain damage.

These results suggest that ONO-1301, either given orally or by a single subcutaneous administration of ONO-1301 PLGA MS, directly after ischaemic insult, can limit neurological damage caused by transient ischaemia in rats.

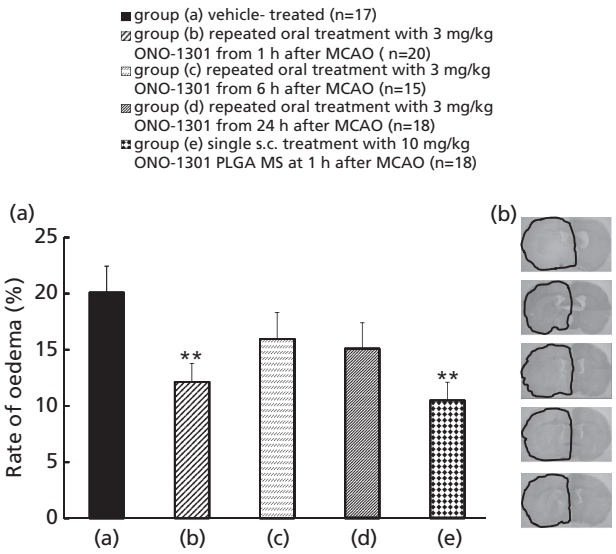

Figure 3 Effect of ONO-1301 administration on degree of brain oedema and cerebral infarction induced by MCAO in rats. Rats received oral ONO-1301 or subcutaneous ONO-1301 PLGA MS following MCAO. (a) Brain damage was evaluated as the degree of edema (%) in each of the five groups. $*P < 0.05$, $**P < 0.01$ vs vehicle-treated group (Dunnett's *t*-test). Values represent the mean ± SE of 15–19 rats. (b) Photographs of the brain (bregma, 1.3-mm section) in each of the five groups. The left side of the brain was marked with solid line as swollen area compared with the right side.

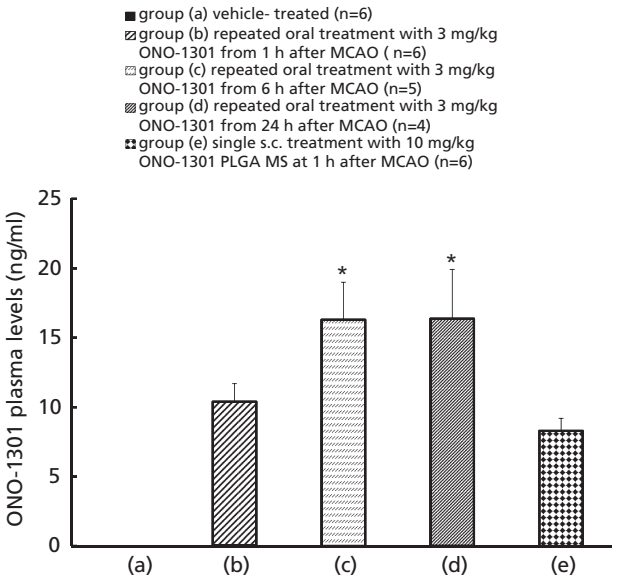

Figure 4 ONO-1301 plasma levels after administration of oral ONO-1301 and ONO-1301 PLGA MS following MCAO in rats. ONO-1301 plasma levels were evaluated on the third day after MCAO in five groups of rats, as shown. $*P < 0.05$ vs MS-treated group (Student–Newman–Keuls test). The data are given as mean ± SE The number of rats used was 4–6/group.

Plasma levels of ONO-1301 after administration to rats subjected to middle cerebral artery occlusion

Plasma ONO-1301 concentrations were measured 3 days after MCAO in all groups.

Blood was collected from the vehicle group (e) and the three groups receiving oral treatment 12 h after the final dose. In the group receiving ONO-1301 PLGA MS, blood was collected three days after injection. ONO-1301 plasma levels increased in a time-dependent manner in all treated groups. The ONO-1301 plasma levels of groups (c) and (d) were both significantly higher than those of group (e) ($P < 0.05$), while those in group (b) were not significantly different from group (e), both being around 10 ng/ml (Figure 4). From these results, it can be suggested that the optimal therapeutic dose of ONO-1301 lies around 10 ng/ml.

In a previous study, it was reported that continuous intravenous infusion of low-dose prostacyclin (0.5–1.0 ng/kg/min) may prevent secondary damage following focal brain trauma.[25] The constant plasma level of around 10 ng/ml ONO-1301, provided by ONO-1301 PLGA MS administered directly after the ischaemic insult, seemed to exert sufficient neuroprotective effect to prevent secondary damage. It has been reported that the drug metabolism of mice subjected to MCAO is decreased, due to cell death in brain tissue affected by ischaemia.[26] Thus, a delay in treatment following ischaemic damage can lead to alterations in the rate of drug metabolism, which may in turn cause variations in drug plasma levels such that the therapeutic range may be exceeded. We postulate that delaying the administration of ONO-1301 until 6 to 24 h after MCAO does not prevent this alteration of the rate of drug metabolism, and may therefore result in plasma levels which exceed the therapeutic range.

ONO-1301 was developed as a new type of prostacyclin agonist, which has long-lasting prostacyclin activity and inhibitory effect on thromboxane synthase. The long-shelf life of ONO-1301 indicates that ONO-1301 exhibits chemical and biologic stability comparable with that of conventional prostacyclin and its analogues. ONO-1301 does not contain prostanoid structures, such as a five-membered ring and allylic alchol, which are subject to metabolism by 15-hydroxyprostaglandin dehydrogenase.[2] This is the reason for the long-lasting activity of ONO-1301 and its stability might be retained even when ONO-1301 is released from ONO-1301 PLGA microspheres.

Conclusions

The primary finding of this study was that repeated oral treatment with low-dose ONO-1301 (3 mg/kg twice daily) starting 1 h after ischaemic insult, can reduce brain damage in rats subjected to MCAO. If treatment is delayed by 6 h or 24 h, the neuroprotective effect is not significant.

Secondly, a single subcutaneous treatment with ONO-1301 PLGA MS, a formulation developed to reduce the acute systemic side-effects of ONO-1301 and provide sustained low levels of drug release, was found to exert a similar neuroprotective effect after cerebral ischaemic injury. A single treatment with ONO-1301 PLGA MS is likely to be clinically more convenient to administer than repeated twice-daily treatment with oral ONO-1301.

Declarations

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Tanouchi T *et al.* Highly selective inhibitors of thromboxane synthetase. 2 Pyridine derivatives. *J Med Chem* 1981; 24: 1149–1155.
2. Kataoka M *et al*. A long-acting prostacyclin agonist with thromboxane inhibitory activity for pulmonary hypertension. *Am J Respir Crit Care Med* 2005; 172: 1575–1580.
3. Obata H *et al*. Single injection of a sustained-release prostacyclin analog improves pulmonary hypertension in rats. *Am J Respir Crit Care Med* 2008; 177: 195–201.
4. Murakami S *et al*. Prostacyclin agonist with thromboxane synthase inhibitory activity (ONO-1301) attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 2006; 290: 59–65.
5. Okano Y *et al*. Orally active prostacyclin analogue in primary pulmonary hypertension. *Lancet* 1997; 349: 1365.
6. Nagaya N *et al.* Effect of orally active prostacyclin analogue on survival of outpatients with primary pulmonary hypertension. *J Am Coll Cardiol* 1999; 34: 1188–1192.
7. Olschewski H *et al*. Inhaled iloprost for severe pulmonary hypertension. *N Engl J Med* 2002; 347: 322–329.
8. Simonneau G *et al*. Continuous subcutaneous infusion of treprostinil, a prostacyclin analogue, in patients with pulmonary hypertension: a double-blind randomized controlled trial. *Am J Respir Crit Care Med* 2002; 165: 800–804.
9. Ike O *et al*. Biodegradation and antitumour effect of adriamycin-contapoly (L-lactic acid) microspheres. *Biomaterials* 1991; 12: 757–762.
10. Umeki N *et al*. Preparation and evaluation of biodegradable microspheres containing a new potent osteogenic compound and new synthetic polymers for sustained release. *Int J Pharm* 2010; 392: 42–50.
11. Grände PO *et al*. Low-dose prostacyclin in treatment of severe brain trauma evaluated with microdialysis and jugular bulb oxygen measurements. *Acta Anaesthesiol Scand* 2000; 44: 886–894.
12. Naredi S *et al*. An outcome study of severe traumatic head injury using the 'Lund therapy' with low-dose prostacyclin. *Acta Anaethesiol. Scand.* 2001; 45: 402–426.
13. Ginsberg MD. Adventures in the pathophysiology of brain ischemia: penumbra, gene expression, neuroprotection: the 2002 Thomas Willis Lecture. *Stroke* 2003; 34: 214–223.
14. Belayev L *et al*. Middle cerebral artery occlusion in the rat by intraluminal suture: neurological and pathological evaluation of an improved model. *Stroke* 1996; 27: 1616–1623.
15. Hayakawa K *et al*. Delayed treatment with minocycline ameliorates neurologic impairment through activated microglia expressing a high-mobility group box1-inhibiting mechanism. *Stroke* 2008; 39: 951–958.
16. Hazekawa M *et al*. Preparation of ONO-1301-loaded PLGA microspheres and their effect on nerve conduction velocity. *J Pharm Pharmacol* 2011; 63: 362–368.
17. Yamamoto M *et al*. Behavioral changes after focal cerebral ischemia by left middle cerebral artery occlusion in rats. *Brain Res* 1988; 452: 323–328.
18. Tamura A *et al*. Focal cerebral ischemia in the rat. 1: Description of technique and early neuropathological consequences following middle cerebral artery occlusion. *J Cereb Blood Flow Metab* 1981; 1: 53–60.
19. Choi JM *et al*. Neuroprotective effect of cilostazol against focal cerebral ischemia via antiapoptotic action in rats. *J Pharmacol Exp Ther* 2002; 300: 787–793.
20. Xing B *et al*. Ischemic postconditioning protects brain and reduces inflammation in a rat model of focal cerebral ischemia/reperfusion. *J Neurochem* 2008; 105: 1737–1745.
21. Pinto E *et al*. Feasibility of localized immunosuppressioon: 2. PLA microspheres for the sustained local delivery of a soft immunosuppressant. *Phatmazie* 2010; 65: 429–435.
22. Yoshikawa N. Relationship between standing balance and symmetry measurements in patients following recent strokes (3 weeks) or older strokes (6 months). *Rigaku Ryoho Janaru* 2005; 39: 199–199.
23. Chao X *et al*. Neuroprotective effect of osthole against acute ischemic stroke on middle cerebral ischemia occlusion in rats. *Brain Res* 2010; 1363: 206–211.
24. McCabe C *et al*. Differences in the evolution of the ischemic penumbra in stroke-prone spontaneously hypertensive and Wistar-Kyoto rats. *Stroke* 2009; 40: 3864–3868.
25. Reinstrup P, Nordström CH. Prostacyclin infusion may prevent secondary damage in precontusional brain tissue. *Neurocrit Care* 2011; 14: 441–446.
26. Lipton P. Ischemic cell death in brain neurons. *Physiol Rev* 1999; 79: 1431–1568.

