Neuroprotective effect of ONO-1078, a leukotriene receptor antagonist, on focal cerebral ischemia in rats

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KEY WORDS cerebral ischemia; cerebral arteries; ONO-1078; leukotriene receptor; blood-brain barrier; brain edema

ABSTRACT

AIM: To determine whether ONO-1078 (pranlukast), a potent leukotriene receptor antagonist, has neuroprotective effect on focal cerebral ischemia in the rat. METHODS: Focal cerebral ischemia was induced by 30 min of middle cerebral artery (MCA) occlusion and followed by 24 h reperfusion. ONO-1078 (0.003-1.0 mg·kg⁻¹) or vehicle (saline 1 mL·kg⁻¹) was ip injected 30 min before MCA occlusion and 2 h after reperfusion. The neurological score, infarct volume, neuron density (in cortex, hippocampus, and striatum), brain edema, and albumin exudation around the vessels were determined 24 h after reperfusion. RESULTS: ONO-1078 slightly improved the neurological deficiency, and dramatically decreased infarct volume and neuron loss which showed a bell shaped dose response effect with highest effect at doses of 0.01-0.3 mg·kg⁻¹. Enlargement of the ischemic hemisphere and albumin exudation were inhibited at doses of 0.01-1.0 mg·kg⁻¹. CONCLUSION: ONO-1078 has the protective effect on focal cerebral ischemia in rats, which is partially attributed to the inhibition of brain edema. This may represent a novel approach to the treatment of acute cerebral ischemia with cysteinyl leukotriene receptor antagonists.

INTRODUCTION

Arachidonic acid (AA) and its 5-lipoxygenase-metabolite leukotriene C₄ (LTC₄), one of the cysteinyl leukotrienes, are generated and released during cerebral ischemia in the brains of both patients¹ and experimental animals².³. Cysteinyl leukotrienes are potent inflammatory mediators involved in various diseases. Intracerebral injection of AA or LTC₄ resulted in rapid breakdown of blood-brain barrier (BBB) followed by vasogenic brain edema⁴.⁵. Therefore, it is possible that accumulation of AA and its products, such as LTC₄, may play a critical role in BBB dysfunction, brain edema, and neuronal death. It was reported that 5-lipoxygenase inhibitors, such as AA-861, nordihydroguaiaretic acid (NDGA), and MK-886, could inhibit brain edema and neurotoxicity after ischemia⁶.⁷. However, whether cysteinyl leukotriene receptor antagonists may also have a neuroprotective effect on cerebral ischemia has not been well explored.

ONO-1078, 4-oxo-8-[p-(4-phenylbutyloxy)benzoyl-amono]-2-(tetrazd-5-yl)-4H-1-benzopyran hemihydrate, is a potent antagonist of cysteinyl leukotrienes (LTC₄, D₄, and E₄) which possesses anti-inflammatory and anti-asthma effects, now clinically

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used for treatment of bronchial asthma as a therapeutic
drug named pranlukast\(^8\,^9\). It dramatically inhibited the
plasma exudation induced by LTC\(_4\), LTD\(_4\), antigen, and
other stimuli in the airway, heart, skin, and nose of guinea
pigs and rats\(^10\,^35\). Kobayashi et al.\(^16\) reported that ONO-
1078 inhibited subarachnoid hemorrhage-induced de-
layed cerebral vasospasm, and we recently found that
this compound protected mice from focal cerebral is-
chemic injury\(^17\). Therefore, this study was to clearly
determine whether and how ONO-1078 protected acute focal cerebral ischemia in the rats with middle cerebral
artery (MCA) occlusion.

**MATERIALS AND METHODS**

**Chemicals** ONO-1078 was kindly gifted by Dr
TSUBOSHIMA Masami (Ono Pharmaceutical Co, Osaka, Japan). Anti-rat-albumin antibody was pur-
chased from Santa Cruz, USA; anti-rabbit-HRP IgG from
Organo Teknika Corp, Japan; 3, 3’-diaminobensidine
tetrahydrochloride (DAB), 2,3,5-triphenyltetrazolium
cloride (TTC), hematoxylin, eosin, and chloral hydrate
were from Sigma Chemical Co, St Louis, USA.

**Focal cerebral ischemia** One hundred and twelve male Sprague-Dawley rats (Grade II), weighing 250 to
350 g, were from Laboratory Animal Center of Zhejiang
Medical University (Certificate 350 g, were from Laboratory Animal Center of Zhejiang
University, Hangzhou, China), and for measuring arterial blood pH,
\(p_{A,co_2}\) and \(p_{A,co_2}\) (Blood Gas Analyzer ABL 330, Leidu
Inc). Rectal (core) temperature was continuously moni-
tored and maintained at 37 °C with a thermostatically
controlled heating lamp during the surgery.

Focal cerebral ischemia was induced by the su-
ture occlusion method\(^18\). A 4-0 nylon monofilament
suture coated with 1 % polylysine\(^19\) was inserted
20- 25 mm to occlude the origins of the anterior cere-
bral artery, the middle cerebral artery (MCA), and the
posterior communication artery, and withdrawn after
30 min of occlusion. In sham-operated animals, the
suture was inserted 5 mm from the incision. ONO-
1078 at doses of 0.003, 0.01, 0.03, 0.1, 0.3, and 1.0
mg·kg\(^{-1}\) or saline (1 mL·kg\(^{-1}\)) were ip injected 30 min
before MCA occlusion and 2 h after reperfusion.

**Evaluation of neurological scores** Twenty-four
hours after reperfusion, the neurological scores were
determined by a modified method described by Longa
et al.\(^18\): 0, no deficit; 1, failure to extend left forepaw
fully; 2, circling to the left; 3, failing to the left; 4, no
spontaneous walking with a depressed level of con-
sciousness.

**Calculation of infarct volumes** After evalua-
tion of neurological scores, the animals were anesthe-
tized with chloral hydrate again and decapitated. The
brains were quickly removed and coronally dissected
into 2 mm thick sections. The brain sections were in-
cubated for 30 min in an isotonic phosphate-buffered
saline containing 2 % TTC at 37 °C, then fixed by im-
ersion in a 10 % buffered formalin solution. All of
the brain sections with the caudal face upward were
recorded with a digital camera (C-1400, Olympus,
Japan), then the imaging data were transferred to a com-
puter and analyzed using an image analyzer (AnalyPower
1.0, Zhejiang University, Hangzhou, China). The areas
of both hemispheres and the areas of the infarcted tis-
uce were calculated. The corrected total infarct area
was calculated according to Lin et al.\(^20\). The left (non-
ischemic) stained area (AL) and right (ischemic) stained
area (AR) were measured. AL minus AR was defined
as the total infarct area of this brain section. Infarct
area times the thickness of the section (2 mm) was the
infarct volume. Total infarct volume was the sum of the
infarct volumes from all of the sections. Subcorti-
ナル infarct areas were traced manually on the images,
and the infarct volume was calculated by computer.
Cortical infarct volume was calculated by subtracting
the subcortical infarct volume from the total infarct
volume. The areas of the right and the left hemispheres
in a section at the level of 3.8 to 4.0 mm caudal from
bregma were manually traced and calculated. Brain
edema was evaluated indirectly as percentage increase
of the ischemic hemisphere area, using the following
formula: \((A_i - A_n)/A_i \times 100\ %\). Here, \(A_i=\)ischemic hemi-
sphere area, and \(A_n=\)non-ischemic hemisphere area.

**Histopathology** In another series, animals were
anesthetized with chloral hydrate and decapitated 24 h
after reperfusion, and the brains were quickly removed.
Serial coronal sections (10 mm) were obtained by cryoul-
tricromicrotomy, fixed in 10 % buffered formalin for 10
min, then stained with hematoxylin and eosin. The nor-
mal neurons in the hippocampal CA1 region, temporoparietal cortex III, IV layers (3.8 to 4.0 mm caudal to
bregma), and striatum (0.4 to 0.2 mm rostral from
bregma) using an image analyzer described above.

**Assessment of albumin exudation** The albumin immunohistochemistry was performed on coronal sections (10 μm) at the two levels described above. Anti-rat-albumin antibody (1:500), anti-rabbit-HRP IgG (1:1000), and 3, 3’-diaminobenzidine (DAB) were added respectively and successively. An area that contained 2-3 small vessels was selected in cortex and striatum. Then the gray scales were detected with the image analyzer (AnalyPowe 1.0). The albumin exudation was evaluated as percentage increase of the gray scales of ischemic cortex or striatum, using the following formula: 
\[
\frac{(G_i - G_n)}{G_n} \times 100 \%
\]
Here, \(G_i\) = gray scale of ischemic region, and \(G_n\) = gray scales of non-ischemic region.

**Statistical analysis** All values are presented as mean±SD. One-way ANOVA or independent-sample t-test was used for statistical analysis of the differences between groups, and paired-sample t-test was used for differences of neuron density between two hemispheres of same brain sections (SPSS 6.0 for Windows, 1993, SPSS inc, USA). \(P<0.05\) was considered statistically significant.

**RESULTS**

There were no significant differences in body weight, rectal temperature, mean arterial blood pressure, arterial blood pH, \(p_{Aco_2}, p_{Aco_2}\), and blood glucose before and during MCA occlusion, and 30 min after reperfusion among the groups (Tab 1).

ONO-1078 attenuated the injuries of cerebral ischemia at doses from 0.01 to 1.0 mg·kg\(^{-1}\). The neurological scores showed a tendency of attenuation in the rats treated with ONO-1078, but the significant difference was only observed at 0.03 mg·kg\(^{-1}\) (Tab 2). ONO-1078 (0.01-0.3 mg·kg\(^{-1}\)) significantly reduced the total infarct volumes with the maximum reduction observed at doses 0.03 and 0.1 mg·kg\(^{-1}\) (84.4 % and 83.0 %, respectively). At 0.03 and 0.1 mg·kg\(^{-1}\), cortical infarct volumes were reduced by 81.6 % and 78.8 %, and sub-cortical infarct volumes by 94.6 % and 96.5 %, respectively (Fig 1). In the saline controls, the ischemic hemisphere areas increased after ischemia, which

Tab 1. Summary of selected physiological parameters before, during and 30 min after MCAO. Mean±SD. MAP: mean arterial pressure; MCAO: middle cerebral artery occlusion.

<table>
<thead>
<tr>
<th>Values</th>
<th>Sham Operation</th>
<th>Saline (Control)</th>
<th>0.003</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1.0</th>
</tr>
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<tbody>
<tr>
<td>n</td>
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<td>9</td>
<td>8</td>
<td>8</td>
<td>8</td>
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<td>8</td>
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<tr>
<td>Body weight/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP/mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>294±18</td>
<td>298±33</td>
<td>295±14</td>
<td>293±34</td>
<td>298±28</td>
<td>285±25</td>
<td>314±31</td>
<td>291±31</td>
</tr>
<tr>
<td>MCAO</td>
<td>84±3</td>
<td>90±9</td>
<td>86±14</td>
<td>86±14</td>
<td>83±10</td>
<td>88±11</td>
<td>90±9</td>
<td>89±7</td>
</tr>
<tr>
<td>30 min after</td>
<td>86±10</td>
<td>90±12</td>
<td>94±9</td>
<td>91±18</td>
<td>86±14</td>
<td>93±6</td>
<td>95±8</td>
<td>91±13</td>
</tr>
<tr>
<td>MCAO Baseline</td>
<td>85±7</td>
<td>88±15</td>
<td>89±11</td>
<td>86±16</td>
<td>91±9</td>
<td>87±8</td>
<td>90±8</td>
<td>89±8</td>
</tr>
<tr>
<td>30 min after reperfusion</td>
<td>88±5</td>
<td>88±16</td>
<td>97±12</td>
<td>84±19</td>
<td>84±9</td>
<td>90±8</td>
<td>90±16</td>
<td>89±6</td>
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<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>7.276±0.019</td>
<td>7.30±0.04</td>
<td>7.29±0.03</td>
<td>7.28±0.04</td>
<td>7.27±0.03</td>
<td>7.28±0.04</td>
<td>7.29±0.04</td>
<td>7.28±0.04</td>
</tr>
<tr>
<td>30 min after reperfusion</td>
<td>7.273±0.022</td>
<td>7.28±0.03</td>
<td>7.28±0.03</td>
<td>7.29±0.03</td>
<td>7.27±0.03</td>
<td>7.28±0.03</td>
<td>7.29±0.03</td>
<td>7.28±0.04</td>
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<tr>
<td>(p_{Aco_2}/mmHg)</td>
<td>BaseLine</td>
<td>38.9±1.6</td>
<td>41±5</td>
<td>40.8±2.5</td>
<td>42±5</td>
<td>41±3</td>
<td>42±5</td>
<td>42±6</td>
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<tr>
<td>30 min after reperfusion</td>
<td>40.1±2.5</td>
<td>41±3</td>
<td>41±3</td>
<td>40±4</td>
<td>41±4</td>
<td>41.3±1.4</td>
<td>40.4±1.1</td>
<td>42±7</td>
</tr>
<tr>
<td>(p_{Aco_2}/mmHg)</td>
<td>BaseLine</td>
<td>94±3</td>
<td>90±10</td>
<td>88±8</td>
<td>92±6</td>
<td>86±12</td>
<td>89±18</td>
<td>87±12</td>
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<tr>
<td>30 min after reperfusion</td>
<td>97±7</td>
<td>96±10</td>
<td>86±12</td>
<td>90±12</td>
<td>96±18</td>
<td>100±6</td>
<td>90±8</td>
<td>88±9</td>
</tr>
<tr>
<td>Glucose/g·L(^{-1})</td>
<td>BaseLine</td>
<td>1.41±0.20</td>
<td>1.2±0.4</td>
<td>1.3±0.4</td>
<td>1.28±0.28</td>
<td>1.24±0.4</td>
<td>1.17±0.28</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>30 min after reperfusion</td>
<td>1.43±0.18</td>
<td>1.2±0.27</td>
<td>1.4±0.3</td>
<td>1.16±0.20</td>
<td>1.4±0.4</td>
<td>1.1±0.3</td>
<td>1.4±0.4</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>Temperature/°C</td>
<td>BaseLine</td>
<td>36.9±0.4</td>
<td>37.0±0.3</td>
<td>37.1±0.3</td>
<td>37.0±0.3</td>
<td>37.1±0.3</td>
<td>37.0±0.3</td>
<td>37.0±0.3</td>
</tr>
<tr>
<td>30 min after reperfusion</td>
<td>36.9±0.4</td>
<td>37.1±0.3</td>
<td>37.1±0.3</td>
<td>37.0±0.3</td>
<td>37.0±0.3</td>
<td>36.9±0.3</td>
<td>37.1±0.3</td>
<td>37.0±0.3</td>
</tr>
</tbody>
</table>
represented the brain edema. ONO-1078, at 0.01-1.0 mg·kg⁻¹, significantly inhibited the enlargement of ischämic hemispheres (Fig 2).

ONO-1078 (0.03-0.3 mg·kg⁻¹) also showed protective effect at different brain regions as evaluated by neuron density. ONO-1078 significantly reduced ischemia-induced neuron death at 0.03 and 0.1 mg·kg⁻¹ in the cortex, at 0.03 mg·kg⁻¹ in the hippocampus, and at 0.1-0.3 mg·kg⁻¹ in the striatum. On the contrary, 0.003, 0.01, and 1.0 mg·kg⁻¹ of ONO-1078 had no significant inhibiting effect (Tab 3). ONO-1078 (0.01-1.0 mg·kg⁻¹) significantly inhibited the increased albumin exudation both in ischemic cortex and striatum, but no dose-dependence was observed (Fig 3).

**DISCUSSION**

Our results indicated that ONO-1078 possessed the neuroprotective effect on focal brain ischemia of acute phase in rats. This was supported by the evidence that ONO-1078 improved neurological deficiency, decreased infarct volumes both in cortical and subcortical regions, inhibited neuron death, and reduced brain edema and inhibited albumin exudation from cerebral vessels. These findings are in agreement with the protective effect of ONO-1078 on focal cerebral ischemia in mice as we reported [17] and other reports that showed the protective effects of 5-lipoxygenase inhibitors [2,3,6,7].

The dose-effect relationship of neuroprotection by ONO-1078 is not a typical one. Except at the lowest dose (0.003 mg·kg⁻¹), ONO-1078 showed two types of the dose-effect relationship at the doses from 0.01 to 1.0 mg·kg⁻¹. A typical one was observed in inhibiting brain edema and albumin exudation. But there was no significant difference among the larger doses. Another was a bell-shaped relationship in improving neurological deficiency, inhibiting infarct volumes and the reduction of neuron density, with maximal effect at 0.03 and 0.1 mg·kg⁻¹. However, we can not explain
why ONO-1078 is ineffective at higher doses (0.3 and 1.0 mg·kg\(^{-1}\)). This bell-shaped dose-effect relationship is often found in neuroprotective agents against brain ischemia, such as GP683 (an adenosine kinase inhibitor)\(^{21}\), \(N^0\)-nitro-L-arginine (a NOS inhibitor)\(^{21}\), flunarizine (a calcium antagonist)\(^{23}\), YM90K [an \((\pm)\)-\(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist]\(^{24}\) in \textit{in vivo} and \textit{in vitro} models of cerebral ischemia. The exact reasons for this phenomenon are unclear although it has been proposed that higher doses might cause cerebral vasoconstriction, reduction of blood pressure, and hypoglycemia\(^{25}\).

In the present study, we detected plasma albumin exudation (which normally cannot exude from vessels) to observe the effect of ONO-1078 on BBB dysfunction. We found that ONO-1078 effectively inhibited albumin exudation at a wide dose range from 0.01 to 1.0 mg·kg\(^{-1}\). This suggests that ONO-1078 may inhibit vasogenic brain edema, and thus produce other neuroprotective effects. However, the action of ONO-1078 on BBB dysfunction may not fully explain its neuroprotection, especially for the different effects of larger dose of ONO-1078 (1.0 mg·kg\(^{-1}\)). At larger dose, ONO-1078 effectively inhibited brain edema and albumin exudation, but failed to decrease infarct volume and neuron death. Therefore, other mechanisms of ONO-1078 in protecting the ischemic brain need to be further investigated.

In summary, we found that ONO-1078 protected focal cerebral ischemia of rats, which at least partly attributed to the inhibition of dysfunction of BBB. These findings suggest a novel approach to treat acute brain ischemia via antagonism of leukotriene receptors. ONO-1078 may also act on other disorders associated with BBB dysfunction and brain edema, such as brain trauma and inflammation.

**ACKNOWLEDGMENTS** We thank Dr TSUBOSHIMA Masami, Ono Pharmaceutical Co Ltd, Osaka, Japan, for the supply of ONO-1078, and Dr SHEN Jian-Zhong for helpful discussion.

**Tab 3. Effect of ONO-1078 on neuron density (1×10^3 cells per mm^2) in hippocampus (CA1), cortex, and striatum.** Mean±SD. \(^b\)P<0.05, \(^c\)P<0.01 vs saline control. \(^e\)P<0.05, \(^f\)P<0.01 vs sham operation, one-way ANOVA. \(^h\)P<0.05, \(^i\)P<0.01 vs non-ischemic hemisphere, paired t-test.

<table>
<thead>
<tr>
<th>Drugs/mg·kg(^{-1})</th>
<th>n</th>
<th>Cortex Non-ischemic</th>
<th>Ischemic</th>
<th>Hippocampus Non-ischemic</th>
<th>Ischemic</th>
<th>Striatum Non-ischemic</th>
<th>Ischemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>5</td>
<td>0.71±0.13</td>
<td>0.66±0.13</td>
<td>0.63±0.11</td>
<td>0.67±0.11</td>
<td>0.57±0.13</td>
<td>0.55±0.11</td>
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<tr>
<td>Saline control</td>
<td>8</td>
<td>0.77±0.17</td>
<td>0.33±0.05(^e)</td>
<td>0.62±0.11</td>
<td>0.44±0.11(^h)</td>
<td>0.56±0.14</td>
<td>0.31±0.05(^g)</td>
</tr>
<tr>
<td>ONO-1078 0.003</td>
<td>7</td>
<td>0.71±0.08</td>
<td>0.39±0.13(^e)</td>
<td>0.62±0.11</td>
<td>0.46±0.08(^e)</td>
<td>0.52±0.08</td>
<td>0.30±0.08(^e)</td>
</tr>
<tr>
<td>0.01</td>
<td>7</td>
<td>0.62±0.05</td>
<td>0.45±0.13(^e)</td>
<td>0.67±0.08</td>
<td>0.56±0.08(^b)</td>
<td>0.54±0.05</td>
<td>0.39±0.11(^b)</td>
</tr>
<tr>
<td>0.03</td>
<td>6</td>
<td>0.64±0.07</td>
<td>0.60±0.10(^f)</td>
<td>0.62±0.07</td>
<td>0.59±0.17(^b)</td>
<td>0.56±0.10</td>
<td>0.46±0.17(^b)</td>
</tr>
<tr>
<td>0.1</td>
<td>7</td>
<td>0.63±0.08</td>
<td>0.56±0.11(^c)</td>
<td>0.63±0.19</td>
<td>0.57±0.05(^h)</td>
<td>0.54±0.11</td>
<td>0.51±0.13(^b)</td>
</tr>
<tr>
<td>0.3</td>
<td>6</td>
<td>0.63±0.10</td>
<td>0.50±0.10(^b)</td>
<td>0.65±0.07</td>
<td>0.58±0.05(^b)</td>
<td>0.65±0.12</td>
<td>0.55±0.12(^c)</td>
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<tr>
<td>1.0</td>
<td>7</td>
<td>0.68±0.13</td>
<td>0.49±0.03(^b)</td>
<td>0.64±0.08</td>
<td>0.54±0.08(^b)</td>
<td>0.52±0.08</td>
<td>0.38±0.13(^c)</td>
</tr>
</tbody>
</table>

Fig 3. Inhibiting effect of ONO-1078 on the percentage increase albumin exudation in ischemic cortex and striatum in rats. n=5-8 per group. Mean±SD. \(^e\)P<0.05, \(^f\)P<0.01 vs control (0, saline). \(^h\)P<0.05, \(^i\)P<0.01 vs sham operation, one-way ANOVA.
REFERENCES


