Direct Thrombin Inhibitor Argatroban Reduces Stroke Damage in 2 Different Models

Patrick Lyden, MD; Benedict Pereira, BS; Bo Chen, PhD; Lifu Zhao, MD; Jessica Lamb, BS; I-farn Lei, BS; Padmesh Rajput, PhD

Background and Purpose—We showed previously robust neuroprotection with the thrombin inhibitor argatroban and now sought additional support for its neuroprotective potential.

Methods—We used behavioral and histological end points; rigorously blinded the study groups; extended the treatment window to 3 hours after ischemia onset; and used 2 separate models. First, 2-hour filament middle cerebral artery occlusion in 64 male Sprague-Dawley rats was followed by learning and memory testing and quantitative histomorphometry. Randomly assigned treatment was 0.45 mg argatroban, saline, or 0.4 U thrombin. Second, we used the quantal bioassay (n=272) after 2-hour middle cerebral artery occlusion to detect the longest time delay after which therapy failed.

Results—Argatroban powerfully and significantly reversed learning and memory deficits because of focal ischemia compared with saline or thrombin (P<0.03; ANOVA). Argatroban was significantly (P<0.05; t test with Bonferroni) protective when given immediately or after 1, 2, 3, but not 4 hours delay.

Conclusions—We obtained supportive evidence for argatroban protection of the neurovascular unit using behavioral and histological measurements at realistic therapeutic time windows. (Stroke. 2014;45:896-899.)

Key Words: models, animal ■ stroke ■ thrombin ■ treatment outcome

Thrombin causes cell death via the protease-activated receptors, which are found in endothelial cells, astrocytes, and neurons.1 The direct thrombin inhibitor, argatroban, inhibited the effect of thrombin in a dose-dependent manner using histological markers of cell injury.1,2 Many candidate treatments that protect rodent brain in preclinical stroke models, however, failed in clinical trials.3 Recently, several authorities have suggested additional testing requirements for putative neuroprotectants.4,5 We sought additional supportive evidence for argatroban protection of the neurovascular unit using testing standards derived from these published guidelines.

Methods

The protocol was approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center, following all national guidelines for the care of experimental animals. Male Sprague-Dawley rats (n=37; 290–310 g) were assigned randomly: saline or 1.4 U/kg thrombin (Sigma) in 0.2 mL administered intra-arterially for 120 minutes or 0.45 mg argatroban (Axorra) in 0.2 mL administered intravenously for 2 hours.2 We used our published methods for middle cerebral artery occlusion, behavior, and histology.1,6,7

To determine the optimum therapeutic time window for treatment with argatroban, we randomly assigned 272 male Sprague-Dawley rats, as above, to receive saline intravenously or low-dose (10 mg/kg) or high-dose (18 mg/kg) argatroban for 24 hours by Alzet mini-pump (model 2001D, Durect Corp). Treatment effects were assessed using the standard quantal bioassay procedure and 2,3,5-triphenyltetrazolium chloride (TTC) staining.8 Treatment groups were compared using a t test of the respective ED 50s using a Bonferroni correction for multiple comparisons.

Results

Ischemia significantly reduced the learning curve slope (Figure 1A; P<0.05; ANOVA), an effect worsened by thrombin and ameliorated by argatroban (Figure 1A; P<0.05; ANOVA). During the 48-hour retention test, the thrombin-treated subjects spent less time in the correct quadrant (not significant). A spatial probe test (Figure 1B) revealed a significant deficit among the thrombin-treated subjects, an effect ameliorated by argatroban1 (P<0.05; ANOVA). Interestingly, on testing retention of a new location, the thrombin-treated animals remembered as well as other treatment groups, suggesting intact reference memory, consistent with previous data that focal ischemia causes impaired learning with preserved memory.7 Search strategy analysis (Figure 1C) revealed a significant association between treatment and search strategy for the retention test (χ 2; P=0.03) during which argatroban-treated subjects used significantly fewer random searches. After 1, 2, or 3 hours delay after ischemia onset, both high- and low-dose argatroban treatment produced a significant protective effect, illustrated for 3 hours delay in Figure 2A (P<0.05 after t test and Bonferroni correction). After 4-hour delay there was no protective effect (Table). Using TTC exclusion, the drug showed a significant protective effect (Figure 2B). These differences were statistically significantly different using a univariate ANOVA that included a delay time versus treatment interaction term (P=0.01), but only the...
low-dose group was protective and only at 0 or 1 hours delay after stroke onset (Figure 2C).

Discussion

The direct thrombin inhibitor argatroban significantly ameliorates stroke-related behavioral and histological effects in 2 different models. Thrombin-treated subjects performed worse than saline and argatroban-treated subjects in learning/memory tasks (Figure 1). Argatroban-treated subjects showed a steeper learning curve during 4 days of training performing like unlesioned controls (Figure 1A). In the Morris Water maze task, as well as in the Barnes Maze task, acquisition of the escape location during training is considered evidence of working memory and in some reports correlates with hippocampus damage or lesions of the basal nucleus of Meynert.

The spatial probe task emphasizes reference memory and the subjects' must recognize that the escape platform has moved and find the new escape route (Figure 1B). We have shown previously that the spatial probe task is exquisitely sensitive to large lesions after middle cerebral artery occlusion.
The argatroban-treated subjects clearly used a more efficient search strategy during the retention test (Figure 1C). During the probe task, although the argatroban animals solved the problem more quickly (Figure 1B), they used less efficient search strategies (Figure 1C), suggesting improved ability to recognize and solve the new escape problem because they retained an imprint of the task paradigm.

The quantal bioassay uses a different behavioral outcome, is simpler, and is well suited to studies of the therapeutic time window.8,13 We confirmed (Figure 2; Table) that argatroban is highly neuroprotective ≤3 hours, a clinically relevant delay.4,14 Correspondingly, argatroban given ≤1 hour after ischemia showed significant benefit on 48-hour lesion volumes (Figure 2). Stroke Treatment and Academic Roundtable (STAIR) and other guidelines have emphasized behavioral outcomes as the more clinically relevant4 and our data support the conclusion that argatroban therapy is effective as measured with histological or behavioral outcomes.

There are limitations to the studies presented here. Of necessity, the quantal bioassays and TTC lesion volumes were measured after 48 hours, whereas in separate groups behavior testing and histomorphometry were performed after several weeks. Thus, the results cannot be directly compared. Neither approach considers physiological effects, such as cortical spreading depression, that could mediate behavioral impairments. Further studies using serial functional or blood flow studies could shed light on these phenomena. Traditional limitations of animal studies—lack of blinding and randomization—were avoided in these studies.

We confirmed that the direct thrombin inhibitor argatroban benefits several measures of outcome after middle cerebral artery occlusion, likely as a result of thrombin inhibition because intra-arterial thrombin worsened outcomes. Future studies will be needed to determine whether this effect generalizes to include other thrombin inhibitors.

**Sources of Funding**
This work was supported by the National Institute of Neurological Disorders and Stroke, R01 NS075930 (Dr Lyden).
Table. Summary of Quantal Bioassay Results

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Dose</th>
<th>Delay, h</th>
<th>$P_{	ext{ed}50}$±SE</th>
<th>$t$ Test Compared With Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>20</td>
<td>...</td>
<td>None</td>
<td>19.4±3.7</td>
<td>...</td>
</tr>
<tr>
<td>Argatroban</td>
<td>20</td>
<td>Low</td>
<td>None</td>
<td>56.4±11.5</td>
<td>$P&lt;0.01$</td>
</tr>
<tr>
<td>Argatroban</td>
<td>20</td>
<td>High</td>
<td>None</td>
<td>32.7±4.5</td>
<td>$P&lt;0.05$</td>
</tr>
<tr>
<td>Saline</td>
<td>19</td>
<td>...</td>
<td>1</td>
<td>24.0±3.8</td>
<td>...</td>
</tr>
<tr>
<td>Argatroban</td>
<td>19</td>
<td>Low</td>
<td>1</td>
<td>46.3±7.8</td>
<td>$P&lt;0.02$</td>
</tr>
<tr>
<td>Argatroban</td>
<td>19</td>
<td>High</td>
<td>1</td>
<td>40.5±4.9</td>
<td>$P&lt;0.02$</td>
</tr>
<tr>
<td>Saline</td>
<td>20</td>
<td>...</td>
<td>2</td>
<td>14.7±3.2</td>
<td>...</td>
</tr>
<tr>
<td>Argatroban</td>
<td>20</td>
<td>Low</td>
<td>2</td>
<td>37.0±4.4</td>
<td>$P&lt;0.01$</td>
</tr>
<tr>
<td>Argatroban</td>
<td>20</td>
<td>High</td>
<td>2</td>
<td>37.7±3.8</td>
<td>$P&lt;0.02$</td>
</tr>
<tr>
<td>Saline</td>
<td>20</td>
<td>...</td>
<td>3</td>
<td>16.2±3.9</td>
<td>...</td>
</tr>
<tr>
<td>Argatroban</td>
<td>20</td>
<td>Low</td>
<td>3</td>
<td>30.1±5.6</td>
<td>$P&lt;0.05$</td>
</tr>
<tr>
<td>Argatroban</td>
<td>20</td>
<td>High</td>
<td>3</td>
<td>30.1±3.7</td>
<td>$P&lt;0.05$</td>
</tr>
<tr>
<td>Saline</td>
<td>20</td>
<td>...</td>
<td>4</td>
<td>15.5±3.3</td>
<td>...</td>
</tr>
<tr>
<td>Argatroban</td>
<td>20</td>
<td>Low</td>
<td>4</td>
<td>21.9±4.8</td>
<td>Not significant</td>
</tr>
</tbody>
</table>

High- or low-dose argatroban was compared with saline treatment at various delay times. Each $ED_{50}$ is shown with a SE; the $P$ value shown is after correction for multiple comparisons. Argatroban was effective if given immediately or after a 1-, 2-, or 3-hour delay. After a 4-hour delay, the beneficial effect was weak, and not statistically significant.

Disclosures
Dr Lyden discloses a consulting relationship with ZZ Biotech, LLC, as well as a research grant from the National Institutes of Health that supported this research. The other authors report no conflicts.

References
Direct Thrombin Inhibitor Argatroban Reduces Stroke Damage in 2 Different Models
Patrick Lyden, Benedict Pereira, Bo Chen, Lifu Zhao, Jessica Lamb, I-farn Lei and Padmesh Rajput

Stroke. 2014;45:896-899; originally published online January 28, 2014;
doi: 10.1161/STROKEAHA.113.004488

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/45/3/896