Direct Thrombin Inhibitor Argatroban Reduces Stroke Damage in Two Different Models

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Abstract

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 endpoints; rigorously blinded the study groups; extended the treatment window to 3 hours following ischemia onset; and used 2 separate models. First, 2-h filament MCAo in 64 male Sprague-Dawley rats was followed by learning and memory testing and quantitative histomporphometry. Randomly assigned treatment was 0.45mg argatroban, saline, or 0.4U thrombin. Second, we used the quantal bioassay (n=272) after 2-hour MCAo to detect the longest time delay after which therapy failed.

Results—Argatroban powerfully and significantly reversed learning and memory deficits due to focal ischemia compared to 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.

Introduction

Thrombin causes cell death via the protease activated receptors (PARs), 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 pre-clinical stroke models, however, failed in clinical trials[3]. Recently a number of 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 IACUC at Cedars-Sinai Medical Center, following all national guidelines for the care of experimental animals. Male Sprague Dawley rats (n=37, 290g to 310g) were assigned randomly: saline or 1.4units/kg thrombin (Sigma) in 0.2ml administered intra-arterially (IA) over 120min, or 0.45mg argatroban (Axxora) in 0.2ml administered intravenously (IV) for 2 hours[2]. We used our published methods for middle cerebral artery occlusion (MCAo), behavior, and histology[6,1,7].

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

Results

Ischemia significantly reduced the learning curve slope (Fig. 1a, $p<0.05$, ANOVA), an effect worsened by thrombin and ameliorated by argatroban (Fig. 1a, ANOVA, $p<0.05$). During the 48 hour retention test, the thrombin treated subjects spent less time in the correct quadrant(NS). A spatial probe test (Fig. 1b) revealed a significant deficit among the thrombin-treated subjects, an effect ameliorated by argatroban[1] ($p<0.05$, ANOVA). Interestingly, upon 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 (Fig. 1c) revealed a significant association between treatment and search strategy for the retention test (Chi Square, $p=0.03$) during which argatroban treated subjects used significantly fewer random searches.

After 1, 2, or 3 hours delay following ischemia onset, both high and low dose argatroban treatment produced a significant protective effect, illustrated for 3 hours delay in Fig. 2a ($p<0.05$ after t-test and Bonferroni correction). After 4 hours delay there was no protective effect (Table). Using TTC exclusion, the drug showed a significant protective effect (Fig. 2b). These differences were statistically significantly different using a univariate ANOVA that included a delay time vs. 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 (Fig. 2c).

Discussion

The direct thrombin inhibitor argatroban significantly ameliorates stroke-related behavioral and histological effects in two different models. Thrombin treated subjects performed worse than saline and argatroban treated subjects in learning/memory tasks (Fig. 1). Argatroban treated subjects showed a steeper learning curve during 4 days of training performing like unlesioned controls (Fig. 1a)[1]. 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[9-11]. The spatial probe task emphasizes reference memory and the subjects' must recognize that the escape platform has moved and find the new escape route (Fig. 1b). We have previously shown that the spatial probe task is exquisitely sensitive to large lesions following MCAo[12]. The argatroban treated subjects clearly used a more efficient search strategy during the retention test (Fig. 1c). During the probe task, although the argatroban animals solved the problem more quickly (Fig. 1b), they used less efficient search strategies (Fig. 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[13,8]. We confirmed (Fig. 2 and Table) that argatroban is highly neuroprotective up to 3 hours, a clinically relevant delay[4, 14]. Correspondingly, argatroban given up to 1 hour after ischemia showed significant benefit on 48-hour lesion volumes (Fig. 2). STAIR and other guidelines have emphasized behavioral outcomes as the more clinically relevant[4] and our data supports that 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, while 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 MCAo, likely as a result of thrombin inhibition since intra-arterial thrombin worsened outcomes. Future studies will be needed to determine if this effect generalizes to include other thrombin inhibitors.

Acknowledgments

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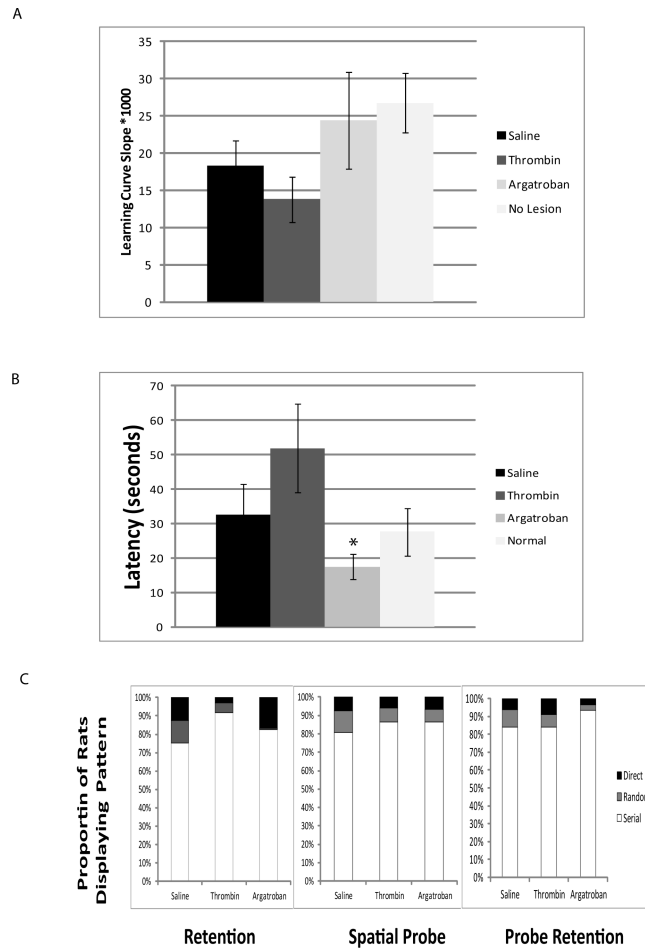
A

B

C

Retention   Spatial Probe   Probe Retention

Figure 1.

A. Learning Curves. Over 5 days in the Barnes Maze, each animal learned the location of the escape hole. The linear learning curve—the inverse of the mean latency-to-escape on each day—was highest in the 'no lesion' controls and slowest in the thrombin group (p=0.03, ANOVA, Tukey's). B. Spatial Probe Escape Latency. The spatial probe assesses the time to locate a new escape location. Saline and thrombin prolonged the time to locate the new escape location[1]. Argatroban ameliorated this effect (* p<0.05 compared to thrombin treatment). C. Search Strategy. The proportion of searches characterized as direct, serial or random are shown during the retention, spatial probe, and probe retention tests, without error bars for clarity. Argatroban treated subjects used fewer random searches in the retention task (p<0.03, Chi Square).
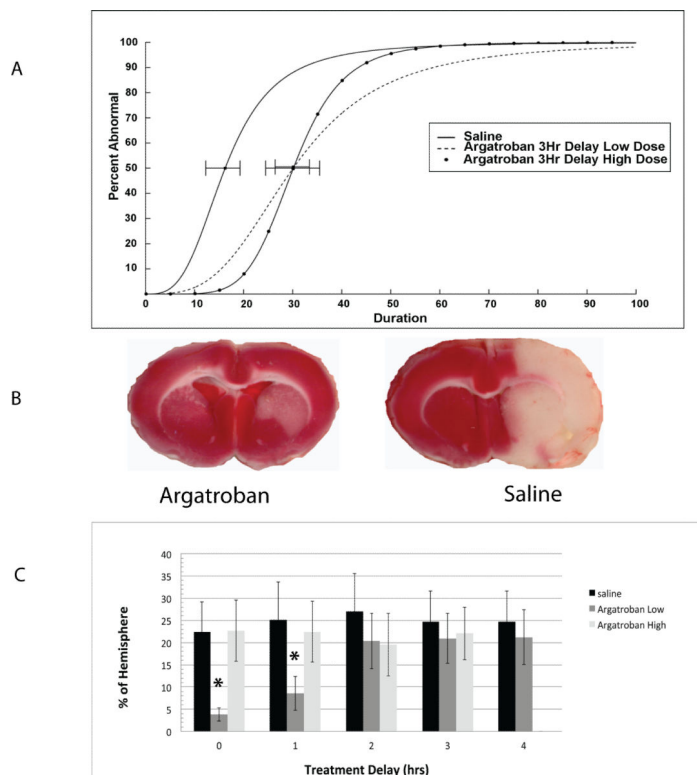
Figure 2.

A. Quantal Bioassays. Each bioassay describes the effect of low dose or high dose argatroban given at various delay time points, compared to saline[8]. The group tolerance to ischemia is characterized by the ED_{50}, minutes of MCAo that renders 50% of the subjects abnormal. The ED_{50} of each treated group is compared to the saline group after a Bonferroni correction for multiple comparisons, illustrated with curves for those subjects treated after a 3-hour delay. Both low and high dose argatroban were significantly more protective than saline ($p<0.05$, t-test after Bonferroni). B. Sections after 48 hours with TTC staining. Representative sections from saline and argatroban treated subjects illustrating the pale, non-metabolizing area of lesioned cerebrum reduced by argatroban. Subjects received treatment 3 hours after onset of 2-hour MCAo, and were sacrificed 48 hours later. C. Lesion volume. Mean±SD total lesion volume is shown for each group at the relevant time delays. The volume of metabolic impairment is significantly reduced (improved) by low dose argatroban given immediately or after a 1-hour delay. ** $p<0.01$, ANOVA with Tukey's.

Table

Summary of Quantal Bioassay Results

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

Group	N	Dose	Delay	$P_{50}\pm SE$	t-test compared to Saline
Saline	20		None	19.4±3.7	
argatroban	20	Low	None	56.4±11.5	P<0.01
argatroban	20	High	None	32.74±4.5	P<0.05
Saline	19		1 hour	24.0±3.8	
argatroban	19	Low	1 hour	46.3±7.8	P<0.02
argatroban	19	High	1 hour	40.5±4.9	P<0.02
Saline	20		2 hours	14.7±3.2	
argatroban	20	Low	2 hours	37.0±4.4	P<0.01
argatroban	20	High	2 hours	37.7±3.8	P<0.02
Saline	20		3 hours	16.2±3.9	
argatroban	20	Low	3 hours	30.1±5.6	P<0.05
argatroban	20	High	3 hours	30.1±3.7	P<0.05
Saline	20		4 hours	15.5±3.3	
	20	Low	4 hours	21.9±4.8	NS