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## PDGFR- $\alpha$ Inhibition Preserves Blood-Brain Barrier after Intracerebral Hemorrhage

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### Abstract

**Objective**—Perihematomal edema results from disruption of the blood-brain barrier (BBB) by key mediators, such as thrombin, following intracerebral hemorrhage (ICH). Platelet derived growth factor receptor alpha (PDGFR- $\alpha$ ), a tyrosine kinase receptor, was found in previous studies to play a role in orchestrating BBB impairment. In the present study, we investigated the role of PDGFR- $\alpha$  following ICH-induced brain injury in mice, specifically investigating its effect on BBB disruption.

**Methods**—Brain injury was induced by autologous arterial blood (30  $\mu$ l) or thrombin (5 U)-injection into mice brains. A PDGFR antagonist (Gleevec) or agonist (PDGF-AA) was administered following ICH. PDGF-AA was injected with a thrombin inhibitor, hirudin in ICH mice. Thrombin-injected mice were given Gleevec or PDGF-AA neutralizing antibody. A p38 MAPK inhibitor, SB203580 was delivered with PDGF-AA in naïve animals. Post-assessment included neurological function tests, brain edema measurement, Evans blue extravasation, immunoprecipitation, western blot and immunohistology assay.

**Results**—PDGFR- $\alpha$  suppression prevented neurological deficits, brain edema and Evans blue extravasation at 24–72 hours following ICH. PDGFR- $\alpha$  activation led to BBB impairment and this was reversed by SB203580 in naïve mice. Thrombin inhibition suppressed PDGFR- $\alpha$  activation and exogenous PDGF-AA increased PDGFR- $\alpha$  activation, regardless of thrombin inhibition. Animals receiving a PDGF-AA neutralizing antibody or Gleevec showed minimized thrombin injection-induced BBB impairment.

**Interpretation**—PDGFR- $\alpha$  signaling may contribute to BBB impairment via p38 MAPK mediated MMP activation/expression following ICH and thrombin may be the key upstream orchestrator. The therapeutic interventions targeting the PDGFR- $\alpha$  signaling may be a novel strategy to prevent thrombin-induced BBB impairment following ICH.

### Introduction

Spontaneous intracerebral hemorrhage (ICH) is the result of small vessel bleeds within the brain parenchyma and the subsequent formation and expansion of the hematoma. This process represents the deadliest and least treatable stroke subtype, accounting for close to 15–20% of all strokes<sup>1</sup>. One of the main reasons for its devastating nature is the formation

of perihematomal cerebral edema, a consequence that occurs from disruption of the blood-brain barrier (BBB). To this date, many factors have been implicated in orchestrating the disruption including thrombin, inflammatory mediators, hemoglobin degradation products<sup>2</sup>, and matrix metalloproteinases (MMPs)<sup>3</sup>. Yet the mechanism to explain how the process is carried out still remains to be elucidated.

Platelet derived growth factor receptors (PDGFRs) are a subfamily of tyrosine kinase receptors including two members, PDGFR- $\alpha$  and PDGFR- $\beta$ , expressed throughout various cell-types in the brain, including astrocytes, neurons<sup>4</sup>, and capillary endothelial cells<sup>5</sup>. These receptors have extracellular domains which ligands, platelet derived growth factors (PDGFs) can bind to initiate downstream signaling pathways. Recently, several lines of evidence have suggested that PDGFRs, especially PDGFR- $\alpha$  may be involved in the stroke process, specifically orchestrating the disruption of the BBB<sup>6-7</sup>. In one study the authors observed that PDGFR- $\alpha$  agonists injection into the CSF of naïve mice significantly increased Evans blue extravasation compared to just PBS injected animals<sup>6</sup>.

As a result in the present study, we investigated the role of the PDGFR- $\alpha$  following an ICH-induced brain injury in mice, specifically investigating its position as a key orchestrator of BBB disruption. We hypothesize that PDGFR- $\alpha$  signal may contribute to BBB impairment via a p38 MAPK pathway mediated MMPs activation/expression following ICH injury and thrombin, an established mediator of BBB injury in ICH, may be the upstream regulator of PDGFR- $\alpha$  activation. In order to test this aim, first we investigated the expression of PDGFR- $\alpha$  and its ligand, PDGF-AA in brain following ICH. We next used both a PDGFR antagonist (Gleevec) and agonist (PDGF-AA) to manipulate PDGFR- $\alpha$  activation, and measured the phosphorylation level of the PDGFR- $\alpha$  while observing the pre-determined outcomes. We also gave a p38 MAPK inhibitor known as SB 203580 hydrochloride, to potentially reverse the BBB disruption induced by PDGFR- $\alpha$  activation. Because of our hypothesis that thrombin may act as the key upstream orchestrator, hirudin, a thrombin specific inhibitor was also administered into animals with or without PDGFR- $\alpha$  agonist injection following ICH. Furthermore, in an established thrombin injection model, PDGFR- $\alpha$  antagonist or PDGF-AA neutralizing antibody was introduced to determine the role of thrombin in activating and/or inhibiting the PDGFR- $\alpha$  pathway.

## Materials and Methods

### Animals

All procedures for this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Loma Linda University. Please see details in Supplementary Text.

### Intracerebral Hemorrhage Mouse Model

ICH was induced using the autologous arterial blood injection model (bICH) which was modified as previously described<sup>8</sup>. Please see details in Supplementary Text.

### Injection of Thrombin into Basal Ganglia

Animals were fixed in the same manner as the autologous blood injection model described above with the same coordinates used. Thrombin (Sigma) was dissolved in sterilized PBS and delivered into the right basal ganglia (5 U/5  $\mu$ l per mouse). Control animals were given 5  $\mu$ l of PBS.

### Experimental Design

Four separate experiments were conducted (Fig 1, experiment 1–4) in two models. Experiment 1: Gleevec was administered (intraperitoneal injection) at three doses 1 hour

following bICH. Post-assessment included western blot, zymography (6 hours), neurological deficits, brain edema and Evans blue extravasation (24 and 72 hours).

Experiment 2: PDGF-AA was co-injected with blood into right basal ganglia. Neurological deficits and brain edema were determined at 24 hours; PDGF-AA was injected with or without a p38 MAPK inhibitor into right basal ganglia in naïve animals. Evans blue extravasation was detected at 1 and 24 hours.

Experiment 3: The thrombin specific inhibitor, hirudin with or without PDGF-AA was injected with blood into right basal ganglia. Post-assessment included western blot (6 hours), neurological deficits and Evans blue extravasation (24 hours).

Experiment 4: Gleevec was administered (intraperitoneal injection) 1 hour following thrombin injection. PDGF-AA antibody was co-injected with thrombin into right basal ganglia. Post-assessment included western blot, zymography (6 hours) and Evans blue extravasation (24 hours). Please see details in Supplementary Text.

### **Neurobehavioral Function Test**

Neurobehavioral functions were evaluated by modified Garcia test<sup>9-10</sup> and corner turn test<sup>11</sup>. Please see details in Supplementary Text.

### **Brain Water Content Measurement**

Please see details in Supplementary Text.

### **BBB Permeability**

BBB permeability was evaluated with Evans blue staining (250  $\mu$ l of 4% solution in saline) as previously described<sup>12</sup>. Please see details in Supplementary Text.

### **Immunoprecipitation**

Please see details in Supplementary Text.

### **Western Blotting**

Please see details in Supplementary Text.

### **Gelatin Zymography**

MMP-2/9 activity was measured by gelatin zymography modified from previous study<sup>13</sup>. Please see details in Supplementary Text.

### **Immunofluorescence**

Please see details in Supplementary Text.

### **Statistics**

Data was expressed as mean  $\pm$  standard error of the mean. Analysis was performed using GraphPad Prism software. For the rating scale data (modified Garcia test), data were expressed as median  $\pm$  25th–75th percentiles. Please see details in Supplementary Text.

## Results

### PDGFR- $\alpha$ and PDGF-AA were upregulated following bICH injury

Western blot was performed to determine the profile of PDGFR- $\alpha$  at 3, 6, 12, 24 and 72 hours and endogenous PDGF-AA level at 6 hours following bICH. Western blot results revealed that PDGFR- $\alpha$  level (Fig 2A, B) was increased 3 hours post bICH and reached a peak around 6 hours in which the PDGFR- $\alpha$  level was almost six times more than sham animals ( $p < 0.05$ ). Following this peak, the level of PDGFR- $\alpha$  declined at 12 hours ( $p < 0.05$ ) and 24 hours, returning close to normal level by 72 hours. Endogenous PDGF-AA (Fig 2C, D), a specific PDGFR- $\alpha$  ligand/agonist was significantly increased in the ipsilateral hemisphere (Ipsi) 6 hours post bICH compared to both contralateral (Contra) hemisphere ( $p < 0.05$ ) and sham animals ( $p < 0.05$ ). The double immunofluorescence staining revealed that the PDGFR- $\alpha$  immunoreactivity was mainly found on the neurovascular structure, including perivascular related astrocytes and the endothelial cells (Fig 2E).

### PDGFR- $\alpha$ suppression improved neurobehavioral functions, reduced brain edema, and preserved BBB integrity

A PDGFR- $\alpha$  antagonist, Gleevec was administered at three doses (30, 60, and 120 mg/kg) by intraperitoneal injection 1 hour following bICH. Neurobehavioral functions, brain edema and BBB permeability were evaluated at 24 and 72 hours following bICH. The results at 24 hours revealed that vehicle animals demonstrated severe deficits compared to sham animals in both modified Garcia test ( $p < 0.01$ ; Fig 3A) and corner turn test ( $p < 0.01$ ; Fig 3B). Following Gleevec administration at medium (60 mg/kg;  $p < 0.01$ ) and high doses ( $p < 0.05$ ; 120 mg/kg), there was a significant improvement in neurological score in modified Garcia test. With regards to corner turn test, the medium dose (60 mg/kg) significantly improved neurobehavioral function compared to vehicle animals ( $p < 0.05$ ). We also evaluated neurobehavioral function at the delayed stage (72 hours) post bICH using the medium dose Gleevec treatment. The results demonstrated that the medium dose treatment could significantly improve neurobehavioral function following both modified Garcia test and corner turn test at 72 hours compared to vehicle animals ( $p < 0.05$ ).

At 24 hours post bICH, the medium (60 mg/kg) and high-dose (120 mg/kg) treatment significantly decreased brain edema in the ipsilateral basal ganglia (ipsi-BG) compared to vehicle group (ipsi-BG: 60 mg/kg,  $80.82 \pm 0.30$  vs vehicle,  $81.88 \pm 0.23$ ,  $p < 0.05$ ; 120 mg/kg,  $80.92 \pm 0.34$  vs vehicle,  $81.88 \pm 0.23$ ,  $p < 0.05$ ; Fig 3C). In the ipsilateral cortex (ipsi-CX), brain edema was significantly increased in the vehicle group compared to sham group (ipsi-CX; vehicle,  $80.22 \pm 0.26$  vs sham,  $79.12 \pm 0.21$ ,  $p < 0.05$ ). Although following Gleevec treatment the brain edema showed a trend towards reduction, there was no statistical significance reached. With regards to the 72 hours post bICH medium-dose (60 mg/kg) treatment, we found a significant reduction in brain edema in the ipsilateral basal ganglia compared to the vehicle group (ipsi-BG: 60 mg/kg,  $81.75 \pm 0.20$  vs vehicle,  $83.29 \pm 0.23$ ,  $p < 0.05$ ; Fig 3D). Evans blue extravasation (Fig 3E) was significantly increased at both 24 hours and 72 hours compared to sham groups ( $p < 0.01$ ), and significantly reduced after medium-dose (60 mg/kg) Gleevec treatment ( $p < 0.05$ ).

### PDGFR- $\alpha$ suppression inhibited MMP activity and MMP-10/13 expression through orchestration of the p38 MAPK pathway post bICH

Phosphorylated PDGFR- $\alpha$  (Fig 4A, B) was significantly increased compared to sham animals (about seven times;  $p < 0.05$ ) while Gleevec treatment (60 mg/kg) significantly reduced PDGFR- $\alpha$  phosphorylation level ( $p < 0.05$ ) 6 hours post bICH. Gleevec treatment (60 mg/kg) also significantly reduced the active MMP-9 level ( $p < 0.05$ ) but not MMP-2 compared to vehicle animals (Fig 4C–E), and reduced MMP-10 ( $p < 0.05$ ; Fig 4F, G) and

MMP-13 expression ( $p < 0.05$ ; Fig 4H, I). The results also revealed that phosphorylated p38 MAPK was significantly reduced following Gleevec treatment ( $p < 0.05$ ) yet, did not reduce the phosphorylation level of Erk1/2 and JNK1/2 (Fig 4J, K). Additionally, we also found that phosphorylated ATF-2, the substrate of p38 was also significantly reduced ( $p < 0.05$ ; Fig 4L, M). Additionally, the cellular localization of PDGFR- $\alpha$  downstream mediators was determined by double immunofluorescence staining. Similar to the PDGFR- $\alpha$ , MMP-9, MMP-13 and phosphor-p38 immunoreactivity were mainly found in the neurovascular structure, including astrocytes and the endothelial cells, and MMP-10 was only found in the endothelial cells (Supplemental Fig 1).

### **PDGFR- $\alpha$ activation increased brain edema post bICH**

At 24 hours post PDGF-AA delivery, neurobehavioral deficits were evaluated using modified Garcia test (Supplemental Fig 2A) and corner turn test (Supplemental Fig 2B). Our results revealed no difference in deficit severity compared to vehicle treatment animals, although two out of nine animals with PDGF-AA injection died in 24 hours. We also found that the brain edema in the ipsilateral basal ganglia was significantly increased compared to vehicle animals (ipsi-BG: PDGF-AA,  $82.59 \pm 0.24$  vs Vehicle,  $81.87 \pm 0.23$ ,  $p < 0.05$ ; Supplemental Fig 2C) 24 hours after PDGF-AA delivery.

### **PDGFR- $\alpha$ activation impaired BBB integrity but was reversed using a p38 MAPK inhibitor in naïve mice**

At 24 hours following PDGF-AA injection, Evans blue extravasation was significantly increased in the ipsilateral hemisphere compared to just PBS injection mice ( $p < 0.01$ ). BBB permeability was also detected 1 hour following PDGF-AA injection. The results showed that the Evans blue extravasation was also increased compared to just PBS injection ( $p < 0.05$ ; Fig 5A). A p38 MAPK inhibitor, SB 203580 hydrochloride was co-injected with PDGF-AA into the right basal ganglia of naïve mice. 24 hours later, we found that the Evans blue extravasation was significantly diminished compared to PDGF-AA injection animals ( $p < 0.05$ ; Fig 5B).

### **Thrombin inhibition preserved BBB integrity, while suppressing PDGFR- $\alpha$ activation and PDGF-AA expression post bICH**

Thrombin inhibitor, hirudin was co-injected with autologous arterial blood into the right basal ganglia of mice. 24 hours following hirudin injection, Evans blue extravasation (Fig 6A) was significantly reduced in hirudin injected animals compared to vehicle animals ( $p < 0.05$ ). Hirudin treatment also significantly improved neurological scores following modified Garcia test ( $p < 0.05$ ; Supplemental Fig 3A), but failed to show improvement with corner turn test (Supplemental Fig 3B). Our results demonstrated that level of phosphorylated PDGFR- $\alpha$  (Fig 6B, C) and PDGF-AA (Fig 6D, E) were both significantly decreased in hirudin treated animals compared to vehicle animals ( $p < 0.05$ ) 6 hours post bICH.

### **PDGFR- $\alpha$ activation reversed the protective effects of thrombin inhibition on BBB integrity post bICH**

Our results demonstrated that Evans blue extravasation was significantly increased compared to only hirudin treated mice ( $p < 0.05$ ) 24 hours following hirudin and PDGF-AA co-injection (Fig 7A). The protection asserted by hirudin on neurobehavioral function was reversed following PDGF-AA administration in modified Garcia test ( $p < 0.05$ ; Supplemental Fig 4A) but not in corner turn test (Supplemental Fig 4B) 24 hours after injection. Additionally, we also observed that the level of phosphorylation of PDGFR- $\alpha$  significantly increased by PDGF-AA compared to just hirudin treated mice ( $p < 0.05$ ) 6 hours after injection (Fig 7B, C).

### **PDGFR- $\alpha$ suppression reduced thrombin-induced BBB impairment through the PDGFR- $\alpha$ /p38/MMPs pathway**

Our results showed that Gleevec treatment significantly diminished Evans blue extravasation compared to thrombin injected animals ( $p < 0.05$ ; Fig 8A). Phosphorylated PDGFR- $\alpha$  was significantly increased 6 hours following thrombin injection and significantly reduced in the Gleevec treated mice compared to just thrombin injected mice ( $p < 0.05$ ; Fig 8B, C). Gleevec treatment significantly reduced MMP-9 level ( $p < 0.05$ ) but not MMP-2 (Supplemental Fig 5A–C) 6 hours following thrombin injection. Similarly, MMP-10 (Supplemental Fig 5D, E) and MMP-13 (Supplemental Fig 5F, G) expression were also significantly reduced after treatment ( $p < 0.05$ ). Additionally, Gleevec treatment significantly diminished the phosphorylation level of p38 MAPK ( $p < 0.05$ ; Supplemental Fig 5H, I) as well as p38 MAPK substrate, ATF2 ( $p < 0.05$ ; Supplemental Fig 5J, K) compared to just thrombin injected mice.

### **Neutralization of PDGF-AA with anti-PDGF-AA antibody reduced thrombin-induced BBB impairment**

PDGF-AA level was significantly increased 6 hours in ipsilateral hemisphere following thrombin injection compared to contralateral hemisphere and sham ( $p < 0.05$ ; Fig 8D, E). 24 hours after PDGF-AA antibody injection, Evans blue extravasation level was significantly diminished compared to either control (Thrombin+inactive antibody) or just thrombin injected mice ( $p < 0.05$ ; Fig 8F).

## **Discussion**

Intracerebral hemorrhage is a fatal stroke subtype that currently has no effective treatment option. Even if patients survive the initial attack, the growing hematoma triggers a series of life threatening events leading to accumulation of cerebral edema, progression of neurobehavioral deficits, and possibly death<sup>14</sup>. In the present study, we investigated the effects of the PDGFR- $\alpha$  and its ability to orchestrate BBB disruption following an ICH injury. Our findings suggest that therapeutic interventions targeting the PDGF-AA/PDGFR- $\alpha$  system may be a novel strategy to prevent BBB impairment and thus attenuate the subsequent accumulation of brain edema responsible for both structural and functional damage following ICH injury.

In order to determine the role of PDGFR- $\alpha$  on BBB disruption in ICH, a PDGFR antagonist, Gleevec was used to suppress PDGFR- $\alpha$  activity, which has showed protective effect on BBB integrity in ischemic stroke model<sup>6</sup>. Gleevec represented a new class of anticancer drugs and has been approved by US Food and Drug Administration for the therapy on chronic myelogenous leukemia and other cancers by inhibition of several tyrosine kinase, including PDGFR- $\alpha$ . It was regarded as a new gold standard for treatment of chronic myeloid leukemia at all stages<sup>15</sup> while some dose-related adverse events have been observed in some patients during Gleevec therapy, such as nausea, vomiting, diarrhea, and fluid retention etc.<sup>16–17</sup>. In our study, we observed a dose-dependent effect of Gleevec treatment on neurological function improvement after ICH. The medium dose (60 mg/kg) significantly improved neurological function while the low dose (30 mg/kg) or high dose (120 mg/kg) did not.

Increased BBB permeability following PDGF administration is not a new concept. Previous study led by Su and colleagues has suggested just that – specifically showing that PDGF injections into the CSF of naïve mice could increase the Evans blue extravasation compared to just PBS injections<sup>6</sup>. Yet another study led by Yao and colleagues recently found that cocaine-induced PDGF could increase vascular permeability and that administration of a

PDGF neutralizing antibody could abolish this effect<sup>7</sup>. Similar to these studies, we found that ICH injury resulted in a transient increase in PDGFR- $\alpha$ /PDGF-AA levels, peaking at 6 hours and returning to baseline by 72 hours. This resulted in a significant increase in brain edema accumulation and BBB disruption which we measured at 24 hours. To our surprise, there was no simultaneous decline in neurological functions with further brain edema accumulation in the bICH with exogenous PDGFR- $\alpha$  agonist group compared to the bICH vehicle group. We attributed this unexpected outcome to the inability of neurological function test to pick up subtle changes in edema accumulation that occurred between the vehicle and agonist group.

With regards to mechanics, we now discuss the potential downstream signaling of PDGF-AA/ PDGFR- $\alpha$  which we hope will explain the mediation of the BBB disruption. MAPK pathway has been established as one of the downstream effectors of PDGFR- $\alpha$  signaling<sup>18</sup>. There are several subfamilies of MAPKs including the extracellular signal-regulated kinases (ERK1/2), ERK5, the Jun amino-terminal kinases (JNK1–3) and the p38 kinases<sup>19</sup>. Generally, p38 and JNK are detrimental in stroke models, with previous research showing that p38/MAPK2 is involved in control of the tight junctional closures among astrocytes<sup>20</sup> and plays a key role in orchestrating BBB disruption and vasogenic edema formation following focal cerebral ischemia and reperfusion<sup>21</sup>. In our study, we found that p38 MAPK level, and not Erk or JNK level, were significantly decreased following Gleevec treatment - and that phosphorylated ATF2 (activating transcription factor 2), a substrate of p38 and JNK, was also markedly decreased. Moreover, the p38 MAPK inhibitor, SB 203580 hydrochloride, which was administered with PDGF-AA in naïve animals, was found to reverse the PDGF-AA induced BBB impairment. These findings suggest that PDGF-AA/ PDGFR- $\alpha$  system may orchestrate the damage to the BBB integrity through a p38 MAPK signaling pathway.

The detrimental role of matrix metalloproteinases, especially MMP-9 and MMP-2 has been well documented in the literature with regards to their effects on BBB integrity following ICH injury<sup>22–24</sup>. Current studies suggested that MMP-10 and 13 were upregulated in both animal and human brain infarcted tissue following ischemic stroke damage<sup>25–26</sup>. Similar to other members in the MMP family, MMP-13 (collagenase-3) can breakdown collagen and gelatin structures and in previous *in vitro* studies has been shown to cleave pro-MMP-9 to active MMP-9<sup>27</sup> –which can occur following MMP-10 as well<sup>28</sup>. With regards to this study, we found that PDGFR- $\alpha$  suppression significantly reduced MMP-9 activity but not MMP-2. We also observed that the expression of MMP-10 and MMP-13 were significantly decreased following PDGFR- $\alpha$  suppression. All of which resulted in preservation of the BBB integrity. In all, taken together MMPs may be the direct downstream proteins of PDGFR- $\alpha$ / p38 pathway and direct mediators of BBB impairment following ICH.

Now that we've discussed downstream orchestrators of PDGFR- $\alpha$  induced BBB damage, we wanted to investigate who was responsible for the upstream regulation of PDGFR- $\alpha$  signaling following ICH injury. Previous literature has alluded to the notion that thrombin regulates the expression of PDGF-AA through a PAR-1 receptor found in endothelial cells<sup>29</sup>. Therefore in the present study, two different mice models were conducted to investigate the potential relationship between thrombin and PDGF-AA. First in the autologous arterial blood-induced ICH model, we found that PDGF-AA expression was significantly down-regulated following the delivery of hirudin, a thrombin specific inhibitor. We also found that the effects of hirudin on BBB preservation were reversed by exogenous PDGF-AA injection. In the thrombin injection model, we first found the increase of phosphorylated PDGFR- $\alpha$  level as well as its downstream signals, p38 MAPK and MMPs, and the diminishment following PDGFR- $\alpha$  suppression by Gleevec treatment. In this case, we also found that the PDGF-AA level was significantly upregulated in the ipsilateral

hemisphere. Additionally, a PDGF-AA neutralizing antibody given with thrombin markedly reduced BBB permeability. Taken together, these findings demonstrated that thrombin is an essential upstream regulator of PDGF-AA/PDGFR- $\alpha$  system.

Why not just block thrombin? The dual role of thrombin in ICH has been well described in previous studies. On one hand, thrombin itself can directly damage the BBB and cause brain edema formation following ICH; while on the other hand, it can act as an essential element in the coagulation cascade to stop bleeding. The concentration of thrombin generated in the brain following ICH has been calculated. Normally, 1 ml of whole blood can provide roughly 260 to 360 units of thrombin from prothrombin. That means that about 15 U of thrombin is generated following a 50  $\mu$ l blood injection (about 30  $\mu$ l plasma)<sup>30</sup>. A number of studies have regarded thrombin generated during blood clotting and hematoma formation as a major cause of brain edema formation<sup>31-32</sup>. One study led by Xi et al. revealed that thrombin is also responsible for prolonged brain edema following ICH<sup>32</sup>.

Mounting evidence that thrombin infusion into the brain produces the same amount of BBB disruption suggested that thrombin could be directly responsible for the breakdown<sup>33</sup>. Moreover, thrombin can cleave its receptors and induce downstream protein production, such as vascular endothelial growth factor (VEGF) which can lead to increased endothelial cell permeability<sup>34</sup>. Therefore, antithrombin therapy using intravenous is considered as a way to prevent brain tissue damage during invasive procedure including surgical removal of hematomas and possibly even direct infusion of thrombin inhibitors into hematomas<sup>35</sup>. Unfortunately, a series of studies showed that the thrombin inhibitors, such as argatroban and hirudins, can provide protective effects in animal models<sup>36-37</sup> but, while in phase 1 clinical trials hemorrhagic transformations and increased hemorrhages were major adverse effects that occurred in patients<sup>35, 38</sup>. As a result, it is very reasonable to develop a therapy strategy that can disrupt downstream thrombin mediators following ICH because they provide fewer side effects than direct thrombin inhibition.

It is also important to note that in addition to PDGF-AA, thrombin also regulates the expression of other PDGFs, such as PDGF-BB<sup>39</sup> which has led to BBB disruption in previous study<sup>6</sup>. Although in our study we hypothesized that PDGFR- $\alpha$  activation may be responsible for BBB impairment, our study cannot rule out the possibility that other PDGFs may be involved in BBB disruption and thus remains one of the main limitations of this study.

Since the PDGF signals expressed transiently and peaked 6 hours after ICH, one of the limitations of our study was the potential narrow therapeutic time window. Previous study showed that BBB permeability in the perihematomal region increased markedly 8 to 12 hours after ICH, and continued to rise for 48 hours<sup>33</sup>. And the early BBB disruption is associated with the thrombin which is generated by the hematoma<sup>31</sup>. Our study was based on the pathophysiology of intracerebral hemorrhage and brain edema formation and may provide insight in understanding the mechanism of BBB disruption and clue on brain edema therapy. In the present study, Gleevec was administered 1 hour after ICH. However, the profile of PDGFR- $\alpha$  expression showed that at 12 hours and 24 hours after ICH, the PDGFR- $\alpha$  level was still 3.08 times and 1.76 times higher than that of sham animals respectively, therefore, a delayed treatment will be conducted in our future study to further establish the therapeutic time window.

In conclusion, our findings suggest that PDGFR- $\alpha$  may contribute to BBB impairment and brain edema formation induced by ICH. Thrombin may in fact be the upstream regulator of PDGFR- $\alpha$  signaling that regulates PDGF-AA expression (potential mechanisms see



Supplemental Fig 5). Targeting the PDGFR- $\alpha$  signaling may provide an alternative treatment to thrombin-induced BBB injury following ICH.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

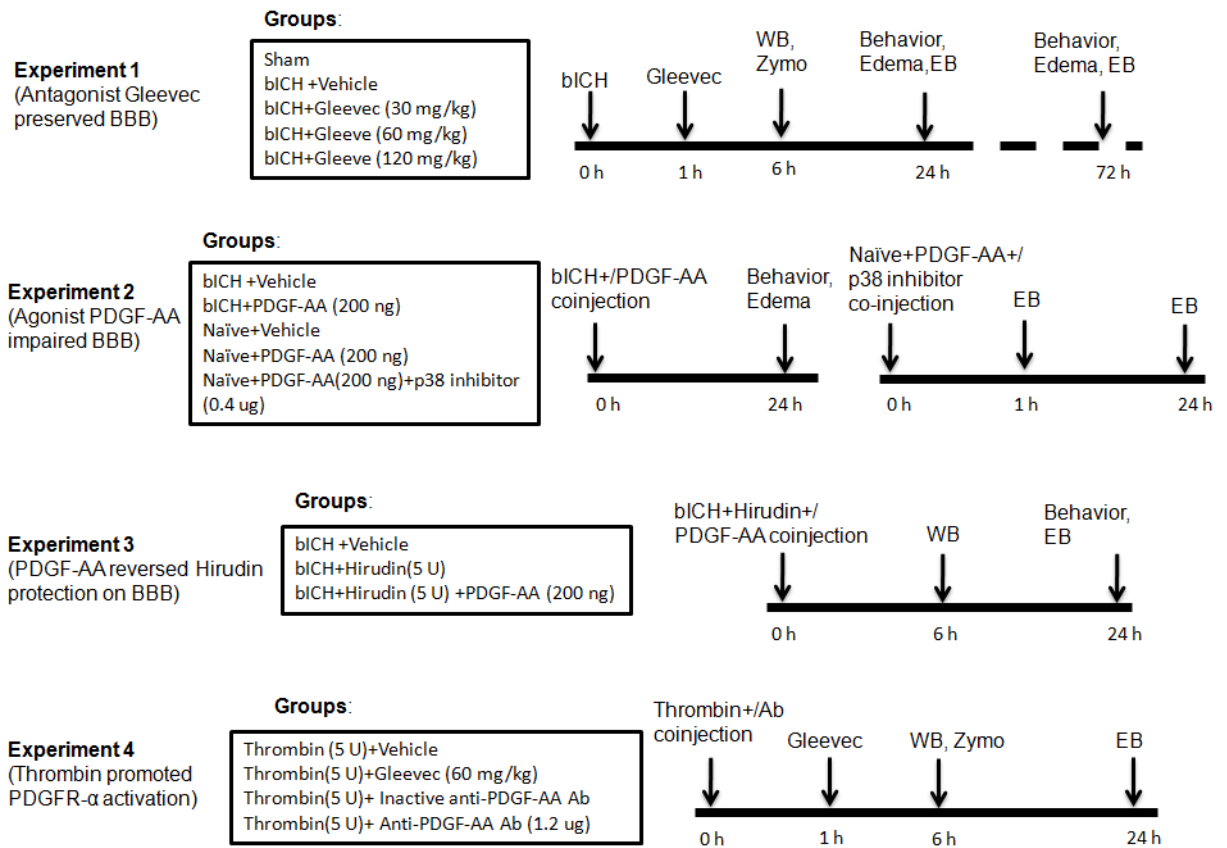
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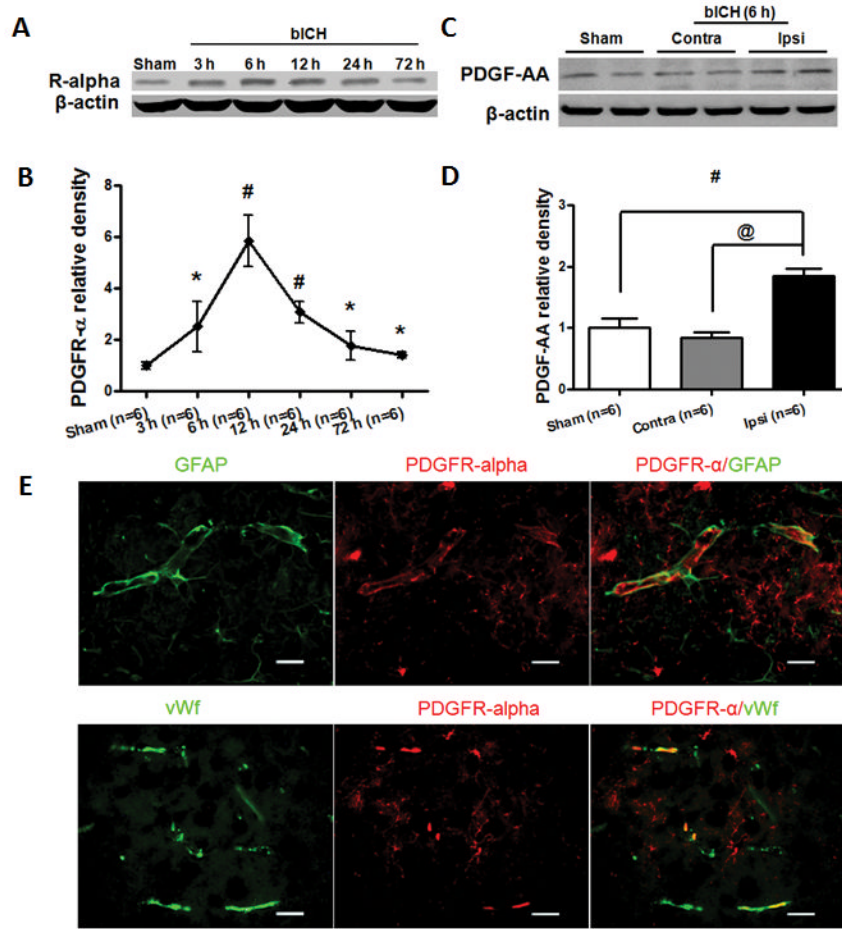
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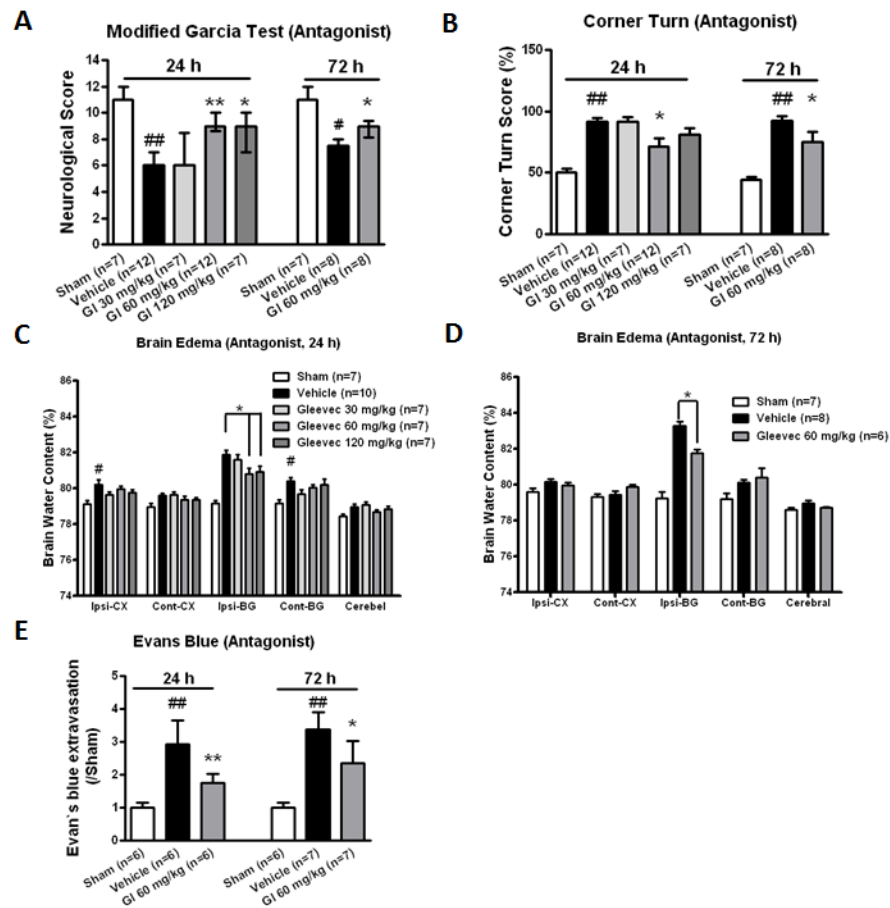
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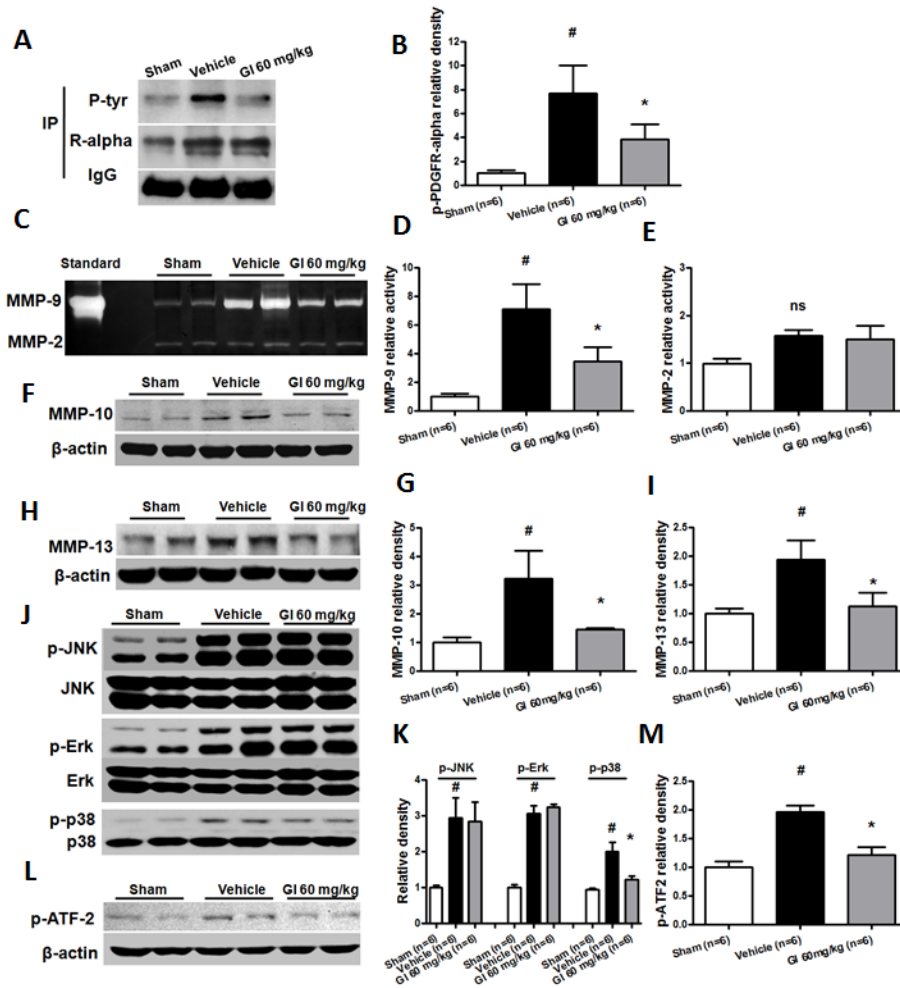
**Figure 1.** Experimental design and animal groups classification. bICH = autologous arterial blood-induced intracerebral hemorrhage; Zymo = zymography assay; WB = western blotting; EB = Evans blue assay; Anti-PDGF-AA Ab = Anti-PDGF-AA antibody.



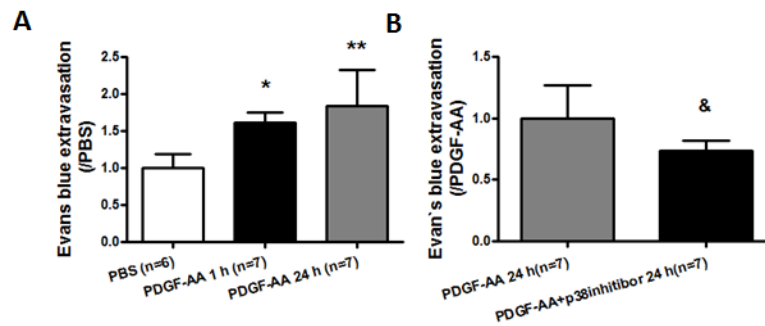
**Figure 2.** Expression of PDGFR- $\alpha$  and PDGF-AA after autologous arterial blood induced intracerebral hemorrhage (bICH). (A) Western blot assay for the profiles of PDGFR- $\alpha$  expression in the ipsilateral hemisphere in sham and bICH mice 3, 6, 12, 24 and 72 hours following operation. (C) Western blot assay for PDGF-AA expression in sham, ipsilateral (Ipsi) and contralateral (Contra) hemisphere in bICH mice 6 hours following operation; (E) Representative photographs of immunofluorescence staining for PDGFR- $\alpha$  (red) expression in astrocytes (GFAP, green) and endothelial cells (vWf, green) in the perihematoma area 6 hours following bICH. Scale bar: 50  $\mu$ m. Quantification of A and C is shown in B and D, respectively. n = 6 mice per group and per time point. Error bars represent mean  $\pm$  standard error of the mean. #  $p < 0.05$  vs Sham; \*  $p < 0.05$  vs bICH (6 h); #  $p < 0.05$  vs Sham; @  $p < 0.05$  vs Contra.



**Figure 3.** PDGFR- $\alpha$  suppression improved neurological functions, reduced brain edema and Evans blue extravasation at 24 and 72 hours following bICH. PDGFR- $\alpha$  antagonist, Gleevec was administered 1 hour following bICH. Modified Garcia test (A) and corner turn (B) at 24 and 72 hours following operation in sham, vehicle and GI treatment groups (24 hours: 30, 60 and 120 mg/kg; 72 hours: 60 mg/kg). Brain edema at 24 hours (C) and 72 hours (D) following operation in sham, vehicle and GI treatment groups (24 hours: 30, 60 and 120 mg/kg; 72 hours: 60 mg/kg). Brain sections (4 mm) were divided into four parts: ipsilateral basal ganglia (Ipsi-BG), ipsilateral cortex (Ipsi-CX), contralateral basal ganglia (Cont-BG), contralateral cortex (Cont-CX). Cerebellum (Cerebel) is the internal control. (E) Evans blue extravasation at 24 and 72 hours in the ipsilateral hemisphere following operations in sham, vehicle and GI treatment groups (60 mg/kg).  $n = 6-12$  mice per group. Error bars represent median  $\pm$  25th–75th percentiles (A) or mean  $\pm$  standard error of the mean (B, C, D and E). #  $p < 0.05$  vs Sham; ##  $p < 0.01$  vs Sham; \*  $p < 0.05$  vs Vehicle; \*\*  $p < 0.01$  vs Vehicle.



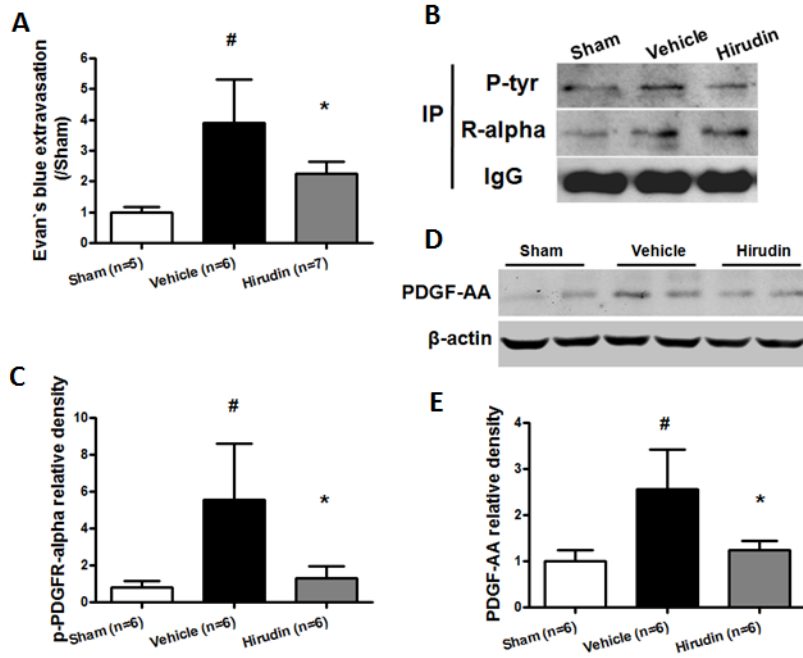
**Figure 4.** Characterization of PDGFR- $\alpha$  pathway at 6 hours following bICH in mice. PDGFR- $\alpha$  antagonist, Gleevec (60 mg/kg) was administered 1 hour following bICH. (A) Immunoprecipitation assay (IP) for phosphor-PDGFR- $\alpha$  level with phosphotyrosine-specific antibody (P-tyr) in the ipsilateral hemisphere in sham, vehicle and G1 treatment (60 mg/kg) mice. The precipitated protein was also visualized with PDGFR- $\alpha$ -specific antibodies (R-alpha). IgG was visualized as a loading control. (C) Gelatin zymography assay for MMP-9 and MMP-2 activity in the ipsilateral hemisphere in sham, vehicle and G1 treatment (60 mg/kg) mice; Western blot assay for MMP-10 (F), MMP-13 (H), JNK/p-JNK, Erk/p-Erk and p38/p-p38 (J), p-ATF-2 (L) in the ipsilateral hemisphere in sham, vehicle and G1 treatment (60 mg/kg) mice. Quantification of A, C, F, H, J, and L is shown in B, D, E, G, I, K, and M, respectively, n = 6 mice per group. Error bars represent mean  $\pm$  standard error of the mean. #  $p < 0.05$  vs Sham; \*  $p < 0.05$  vs Vehicle; ns indicates not significant.



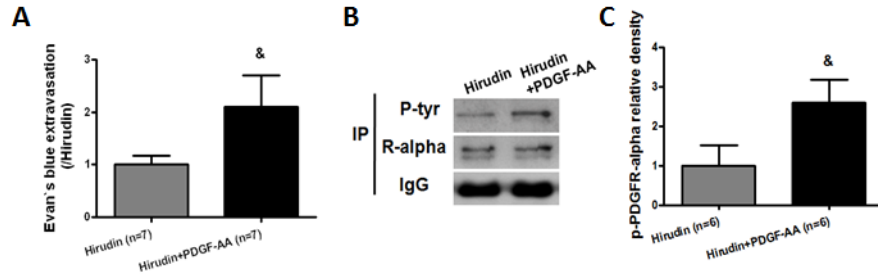
**Figure 5.**

PDGFR- $\alpha$  activation by exogenous PDGF-AA increased Evans blue extravasation in naïve mice. (A) Evans blue extravasation in the ipsilateral hemisphere at 1 and 24 hours following PDGF-AA injection or 24 hours following PBS injection in naïve mice; (B) Evans blue extravasation in the ipsilateral hemisphere at 24 hours in PDGF-AA or PDGF-AA+p38 inhibitor co-injection naïve mice.  $n = 6-7$  mice per group. Error bars represent mean  $\pm$  standard error of the mean. \*  $p < 0.05$  vs PBS; \*\*  $p < 0.01$  vs PBS; &  $p < 0.05$  vs PDGF-AA (24 hours).

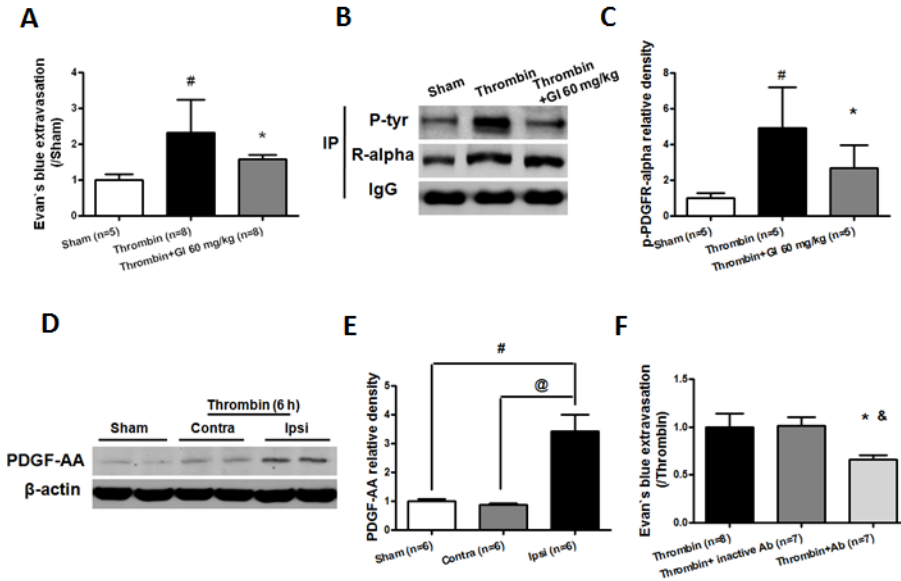




**Figure 6.** Thrombin inhibition reduced Evans blue extravasation, phosphor-PDGFR- $\alpha$  and PDGF-AA levels following bICH injury. Thrombin inhibitor, hirudin (5 U) was co-injected with autologous arterial blood. (A) Evans blue extravasation in the ipsilateral hemisphere 24 hours following operation in sham, vehicle and hirudin treatment (5 U) mice; (B) Immunoprecipitation assay (IP) for phosphor-PDGFR- $\alpha$  level with phosphotyrosine-specific antibody (P-tyr) in the ipsilateral hemisphere 6 hours following operation in sham, vehicle and hirudin treatment (5 U) mice. The precipitated protein was also visualized with PDGFR- $\alpha$ -specific antibodies (R-alpha). IgG was visualized as a loading control. (D) Western blot assay for PDGF-AA level in the ipsilateral hemisphere 6 hours following operation in sham, vehicle and hirudin treatment (5 U) mice. Quantification of B and D is shown in C and E, respectively, n = 5–7 mice per group. Error bars represent mean  $\pm$  standard error of the mean. #  $p < 0.05$  vs Sham; \*  $p < 0.05$  vs Vehicle.



**Figure 7.** Activation of PDGFR- $\alpha$  by PDGF-AA reversed thrombin inhibition by hirudin following bICH. Thrombin inhibitor, hirudin (5 U) with or without PDGF-AA (200 ng) was co-injected with autologous arterial blood. (A) Evans blue extravasation in the ipsilateral hemisphere 24 hours following bICH in hirudin (5 U) and hirudin (5 U) + PDGF-AA (200 ng) mice; (B) Immunoprecipitation assay (IP) for phosphor-PDGFR- $\alpha$  level with phosphotyrosine-specific antibody (P-tyr) in the ipsilateral hemisphere 6 hours after bICH in hirudin (5 U) and hirudin (5 U) + PDGF-AA (200 ng) mice. The precipitated protein was also visualized with PDGFR- $\alpha$ -specific antibodies (R-alpha). IgG was visualized as a loading control. Quantification of B is shown in C. n = 6–7 mice per group. Error bars represent mean  $\pm$  standard error of the mean. &  $p < 0.05$  vs Hirudin.



**Figure 8.** Gleevec and PDGF-AA neutralizing antibody reduced Evans blue extravasation 24 hours following thrombin injection in mice. PDGFR-α antagonist, Gleevec (60 mg/kg) was administered 1 hour following thrombin (5 U) injection. Inactive PDGF-AA antibody (PDGF-AA Ab) or PDGF-AA antibody (PDGF-AA Ab, 1.2 μg) was co-injected with thrombin (5 U) into right basal ganglia. (A) Evans blue extravasation in the ipsilateral hemisphere 24 hours following operation in sham, thrombin (5 U) and GI treatment (60 mg/kg) groups; (B) Immunoprecipitation assay (IP) for phosphor-PDGFR-α level with phosphotyrosine-specific antibody (P-tyr) in the ipsilateral hemisphere 6 hours following thrombin injection in sham, thrombin (5 U) and GI treatment (60 mg/kg) mice. The precipitated protein was also visualized with PDGFR-α-specific antibodies (R-alpha). IgG was visualized as a loading control. (D) Western blot assay for PDGF-AA in Sham, ipsilateral (Ipsi) and contralateral (Contra) hemisphere in thrombin injection mice 6 hours following operation; (F) Evans blue extravasation in the ipsilateral hemisphere 24 hours following operation in thrombin (5 U), thrombin (5 U)+inactive PDGF-AA antibody (PDGF-AA Ab), and PDGF-AA antibody (PDGF-AA Ab, 1.2 μg) mice. Quantification of B and D is shown in C and E, respectively. n = 5–8 mice per group. Error bars represent mean ± standard error of the mean. # *p* < 0.05 vs Sham; \* *p* < 0.05 vs Thrombin; @ *p* < 0.05 vs Contra; & *p* < 0.05 vs Thrombin+inactive Ab.