Selective Sphingosine-1-Phosphate Receptor 1 Modulation Attenuates Experimental Intracerebral Hemorrhage

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Background and Purpose—Preclinical studies and a proof-of-concept clinical study have shown that sphingosine-1-phosphate receptor (S1PR) modulator, fingolimod, improves the clinical outcome of intracerebral hemorrhage (ICH). However, the specific subtype of the S1PRs through which immune modulation provides protection in ICH remains unclear. In addition, fingolimod-induced adverse effects could limit its use in patients with stroke because of interactions with other S1PR subtypes, particularly with S1PR3. RP101075 is a selective S1PR1 agonist with superior cardiovascular safety profile. In this study, we investigated the impact of RP101075 treatment in a mouse model of ICH.

Methods—ICH was induced by injection of autologous blood in 294 male C57BL/6J and Rag2−/− mice. ICH mice randomly received vehicle, RP101075, or RP101075 plus S1PR1 antagonist W146 by daily oral gavage for three consecutive days, starting from 30 minutes after surgery. Neurodeficits, brain edema, brain infiltration of immune cells, blood–brain barrier integrity, and cell death were assessed after ICH.

Results—RP101075 significantly attenuated neurological deficits and reduced brain edema in ICH mice. W146 blocked the effects of RP101075 on neurodeficits and brain edema. RP101075 reduced the counts of brain-infiltrating lymphocytes, neutrophils, and microglia, as well as cytokine expression after ICH. Enhanced blood–brain barrier integrity and alleviated neuronal death were also seen in ICH mice after RP101075 treatment.

Conclusions—S1PR1 modulation via RP101075 provides protection in experimental ICH. Together with the advantageous pharmacological features of RP101075, these results warrant further investigations of its mechanisms of action and translational values in ICH patients. (Stroke. 2016;47:00-00. DOI: 10.1161/STROKEAHA.115.012236.)

Key Words: intracerebral hemorrhage • immune modulation • RP101075 • S1PR1 modulator

Original Contribution

Intracerebral hemorrhage (ICH) is a devastating disease, which accounts for ≈10% to 15% of strokes with high mortality and morbidity.1,2 Effective treatments for ICH remain sparse.3 Accumulating evidence has demonstrated that brain inflammation provoked by microglia and infiltrating lymphocytes exacerbates ICH-induced brain injury.4 After ICH, brain-intrinsic microglia are the first immune responder and followed by inflammatory infiltrates such as neutrophils, monocytes, macrophages, and subsets of lymphocytes.4,5 Activation of these cellular components together with factors they produce and cell death products, further contribute to brain inflammation, which results in the development of perihematomal edema.6–7 Perihematomal edema can aggravate the mass effect and secondary brain injury through subsequent oligemia and inflammatory insults.7,8 Edema, as a surrogate marker for inflammation in ICH,1,9,10 and the persistence of perihematomal edema after ictus makes it amenable for intervention.1 Thus, targeting inflammation could be a viable approach for treating ICH.

Sphingosine-1-phosphate (S1P) is a ligand for 5 G-protein-coupled receptors: S1P receptors 1 to 5 (S1PR1–5) are responsible for numerous cell-intrinsic and extrinsic activities.11,12 Fingolimod (FTY720, Glenya), an oral therapy for multiple sclerosis (MS), is a S1PR modulator that binds to S1PR1, 3, 4, and 5. The beneficial effects of fingolimod in MS may be mediated by S1PR1 expressed on lymphocytes, vascular endothelia, neurons, and glia.13 Recent preclinical and clinical studies have demonstrated that fingolimod can attenuate neurodegeneration and brain edema in ICH.13–18 However, it remains unclear whether immune modulations via S1PR1 are sufficient for fingolimod to provide...
beneficial effects in ICH. In addition, many adverse events, including hypertension, macular edema, pulmonary toxicity, and hepatotoxicity, have been associated with fingolimod, because of its off-target interactions with other S1PR subtypes, particularly with S1PR3.19–22 Considering that disorders such as coronary artery disease are relatively common among ICH patients,23 the clinical application of fingolimod in these patients is limited.

RPC1063 is a potent and selective S1PR1 modulator. RPC1063 has recently emerged as a new option for immune intervention.24 Its efficacy and safety has been confirmed in relapsing remitting MS in phase III trials and inflammatory bowel disease in phase II trials.25 Of interest, distinguished from fingolimod, RPC1063 has a reduced first-dose effect on heart rate with a dose-titration regimen and does not cause QTc prolongation, as demonstrated in a thorough clinical QT study.26–28 The profibrotic activity observed in heart and lung in patients treated with fingolimod was also not observed after RPC1063 treatment, suggesting its superior safety features versus fingolimod.24 RP101075 is an active metabolite of RPC1063 with high potency and S1PR1 selectivity (>100-fold over S1PR5 and >10000-fold over S1PR 2, 3, and 4).27,28 In addition, RP101075 has a high steady-state brain: blood ratio of 52.26,27 Because of these advantageous pharmacological features of RP101075, here we examined the effect of RP101075 on ICH in a mouse model.

Materials and Methods

Animals

Adult male C57BL/6J mice (7–8 weeks old) were obtained from Charles River Laboratories. Rag2−/− mice (8–10 weeks old) were purchased from Taconic Biosciences, Inc. This study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals. Experimental protocols were approved by the Tianjin Medical University General Hospital Animal Care and Use Committee. Adequate measures were taken to minimize the number of experiment animals used and to ensure minimal pain or discomfort in animals.

ICH Model

ICH was induced by a double-injection method described previously,29 with minor modifications. Briefly, 30-μL nonheparinized blood was withdrawn from the angular vein after anesthesia and quickly transferred into a 50-μL syringe with a 26 G needle (Hamilton Company). Then mouse was fixed on a stereotactic frame (RWD Life Science) and anesthesia was induced by a mixture of isoflurane and oxygen. A 1-mm burr hole was drilled, and blood was infused at the caudate nucleus (2.3 mm lateral to midline, 0.5 mm anterior to bregma, and 3.5-mm depth below the surface of the skull). Each ICH model received 30-μL whole blood at a rate of 1 μL/min through the infusion pump (KD Scientific). During the process, needle was paused for 5 minutes before injection, then the first 5 μL was injected to generate a clotting along the needle track, after an additional 5-minute pause, the remaining 25 μL was injected during the following 25 minutes at the same rate of 1 μL/min. After completing the infusion, the needle was held in place for 20 minutes, and then it was slowly withdrawn at a rate of 1 mm/min over the course of 3 movements with 5-minute intervals. Finally, the burr hole was sealed with bone wax (Johnson & Johnson), sterilized and the wound was sutured. After surgery, animals were placed in a cage with free access to food and water. Warming lamps were used to provide thermal support throughout the procedure.

Study Design and Drug Administration

All animal experiments were designed, performed, and reported according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.30,31 Age-matched, male mice (C57BL/6J, 7–8 weeks; Charles River Laboratories, China) and Rag2−/− mice (8–10 weeks old) were used in this study. All animals were housed in the animal care facility at Tianjin Medical University General Hospital (Tianjin, China) or Barrow Neurological Institute (Phoenix, AZ). This study was conducted in accordance with the local authorities, and the use of animals in research and all protocols was approved by the institutional animal care and use committee. All animal experiments to assess the efficacy of RP101075 were performed in both sites. ICH was induced by injection of autologous blood in 294 mice. Wild-type mice were randomly assigned into 3 groups: vehicle group (n=124), RP101075 group (n=124), and RP101075 plus W146, a S1PR1 antagonist, group (n=30). All animals received magnetic resonance imaging (MRI) tests for lesion location detection, and mice with lesions in the ipsilateral basal ganglia were included, whereas those with no lesions or with lesions only in the ventricles or deceased within 1 day were excluded. RP101075 was a gift from Receptos Inc., RP101075 was dissolved in 5% DMSO+5% Tween 20+90% 0.1 N HCl as the vehicle and stored at 4°C. The S1PR1 antagonist W146 was purchased from Tocris Bioscience and dissolved in 10% DMSO+25% Tween 20 in sterile water and stored at −20°C. To determine the therapeutic dosage and time window, mice were treated with either 0.3 or 0.6 mg/kg RP101075 at 30 minutes, 24 hours, or 72 hours after ICH induction, respectively, and 0.6 mg/kg RP101075 at 30 minutes after ICH was finally adopted. The 0.6 mg/kg RP101075, vehicle, or 0.6 mg/kg RP101075+10 mg/kg W146 were administered by oral gavage for 3 consecutive days starting from 30 minutes after ICH. For the S1PR1 targeting study, Rag2−/− mice (n=16) that lack of T, B, and NKT cells (Barrow Neurological Institute) were used, with RP101075 administered using the same regimen and receiving the same measurements as wild-type mice. Data from groups of mice were collected and analyzed by at least 2 investigators who were blind to group assignment.

Neurological Function and Survival Evaluation

Neurological tests were performed at baseline, day 1, and day 3 after ICH induction. To comprehensively evaluate the motor, sensory, reflex, and balance functions, a battery of tests consisting of the modified Neurological Severity Score, Corner Turning test, and forelimb placing test were performed as previously described.32,33 The 14-day survival index were evaluated between groups.

Magnetic Resonance Imaging

MRI detection for hematoma, lesion volume, and hemispheric edema was performed by serial MRI on a 7.0 T Siemens ClinicScan scanner at day 1 and day 3 after ICH. Susceptibility-weighted images (repetition time, 21.0 ms; echo time, 8.0 ms; 17 slices; 0.3-mm thickness) were acquired to determine hematoma volume and T2-weighted images (turbo RARE pulse sequence, echo time 41.0 ms, repetition time, 3380.0 ms; 16 slices; 0.5-mm thickness) were acquired to detect lesion volume and hemispheric volume. The volumes were manually outlined and calculated by multiplying the sum of the volume by the distance between sections (0.3 mm in susceptibility-weighted images and 0.5 mm in T2-weighted images) using MIPAV software. Brain edema (%) was expressed as ((ipsilateral hemisphere volume–contralateral hemisphere volume)/contralateral hemisphere volume)×100%.34

Brain Water Content Assessment

At days 1 and 3 after surgery, brains were removed for brain water content measurement as described in the online-only Data Supplement.

Hemoglobin Quantification

At days 1 and 3 after surgery, brains tissues were homogenized for hemoglobin measurement as described in the online-only Data Supplement.

Isolation of Cellular Components From Central Nervous System and Peripheral Blood for Flow Cytometry Analysis

Brain tissues and venous blood at day 1 and day 3 after surgery were collected for flow cytometry analysis to detect microgli
(CD45intCD11b+), neutrophils (CD45Gr-1+), and lymphocyte subpopulations (CD45CD4+T, CD45CD8+T, CD45CD19+B, CD45NK1.1+NK cells) in the brain and peripheral as described in the online-only Data Supplement.

ELISA
Ipsilateral hemisphere tissues and serum were collected at day 1 and day 3 for ELISA detection for interleukin-1β and tumor necrosis factor-α as described in the online-only Data Supplement.

Assessment of Blood–Brain Barrier Permeability
At day 3 after surgery, Evans Blue extravasation was quantified for detecting blood–brain barrier permeability as described in the online-only Data Supplement.

Western Blot Analysis for Tight Junctions
At day 3 after ICH, ipsilateral hemispheres were homogenized for detecting expression of tight junctions using Western blot as described in the online-only Data Supplement.

Analysis of Neuronal Apoptosis
Three days after ICH, the extent of cell death was assessed and quantified by TdT-mediated Biotin-dUTP Nick End labeling (TUNEL) stain using a TUNEL kit (Roche) as described in the online-only Data Supplement.

Statistical Analysis
All data were expressed as mean±SEM and analyzed by SPSS 18.0 software. Statistical differences were measured by unpaired two-tailed Student t test for comparison of 2 groups or ANOVA followed by Bonferroni post hoc test for multiple comparisons. The survival index was analyzed by Gehan–Breslow Wilcoxon test, and P<0.05 was considered statistically significant.

Results
RP101075 Attenuated Neurodeficits and Increased Survival After ICH
To comprehensively determine the impact of RP101075 on ICH, modified Neurological Severity Score, corner turning, and forelimb placing tests were performed to evaluate motor, sensory, reflex, and balance functions, respectively. RP101075 significantly attenuated clinical severity in all three tests (Figure 1A–1C) and improved survival after ICH (Figure 1D). A selective S1PR1 antagonist, W146, blocks the effects of RP101075 on the neurological outcomes and survival index after ICH (Figure 1A–1D). These data indicate that modulation of S1PR1 using RP101075 is sufficient to improve neurological outcomes and survival after ICH.

RP101075 Ameliorated Brain Edema After ICH
MRI showed significant midline shift and brain edema at day 1 and 3 after ICH (Figure 2A). When compared with the vehicle-treated group, RP101075 reduced lesion volume, brain edema, and midline shift at day 1 and 3 in MRI (Figure 2A

Figure 1. RP101075 attenuated neurological function and increased survival index. A battery of neurological tests were performed at baseline, day 1 and 3 after intracerebral hemorrhage (ICH) to comprehensively detect the motor, sensory, reflex, and balance functions in groups of ICH mice receiving vehicle, RP101075 and RP101075+W146. Survival of ICH mice were observed for 14 d. A–C, RP101075 improved clinical scores in modified Neurological Severity Score (mNSS), corner turning test and forelimb placement (FP) test. W146 blocked the impact of RP101075 on neurological outcomes. D, RP101075 also significantly increased survival after ICH. Data were presented as mean±SEM, *P<0.05, **P<0.01, compared with vehicle group, #P<0.05, ##P<0.01, compared with RP101075+W146 group, n=20 in vehicle or RP101075 group, n=10 in RP101075+W146 group.
and 2B). Furthermore, measurements of brain water content showed a significant decrease in the ipsilateral hemisphere at day 1 and 3, as well as in the contralateral hemisphere at day 3, in the RP101075 group when compared with the vehicle- or W146+RP101075–treated groups (Figure 2C; Figure I in the online-only Data Supplement). In addition, hematoma volume shown in susceptibility-weighted images and hemoglobin level were not significantly different across these groups (Figure 2B and 2C). These data suggest that RP101075 mainly has an impact on brain edema but not on hematomas.

**Impact of RP101075 on Inflammatory Response After ICH**

Next, we sought to understand how RP101075 affects brain edema after ICH. After examination of cellular components in the brain, we found that RP101075 treatment significantly reduced counts of microglia (CD45<sup>+</sup>CD11b<sup>+</sup>), neutrophils (CD45<sup>+</sup>Gr-1<sup>+</sup>), and lymphocyte subpopulations (CD45<sup>+</sup>CD4<sup>+</sup>T, CD45<sup>+</sup>CD8<sup>+</sup>T, CD45<sup>+</sup>CD19<sup>+</sup>B, CD45<sup>+</sup>NK1.1<sup>+</sup>NK cells) in the brain at day 3 after ICH (Figure 3). In addition, RP101075 reduced levels of interleukin-1β and tumor necrosis factor-α.
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in brain homogenates from the ipsilateral hemisphere at
days 1 and 3 after ICH (Figure 3). Similar alterations were
also seen in the peripheral blood of ICH mice treated with
RP101075 versus vehicle (Figure II in the online-only Data
Supplement).

Impact of RP101075 on Blood–
Brain Barrier Permeability
To determine the impact of RP101075 on neurovascular func-
tion in ICH, we examined blood–brain barrier (BBB) perme-
ability and the integrity of tight junctions after RP101075
treatment. RP101075 significantly reduced Evans Blue
e extravasation at day 3 after ICH (Figure 4A and 4B), sug-
gestig a decrease in BBB permeability after RP101075
treatment. Significantly increased expression of occludin
and zonula occludens-1 was seen in ICH mice after receiv-
ing RP101075 when compared with vehicle-treated controls
(Figure 4C and 4D). An increase of claudin-5 expression was
also noticed but was not statistically significant (Figure 4C
and 4D).

Impact of RP101075 on Neuronal Death After ICH
Three days after ICH, brain sections from ICH mice receiv-
ing RP101075 or vehicle controls were stained with TUNEL
to determine cell death. Quantitation of TUNEL+ cells in the
perihematomal area suggests reduced cell apoptosis after
RP101075 treatment (Figure 5).

Discussion
Here, we demonstrate that RP101075 treatment attenuated
neurological deficits, and brain edema in a mouse model of
ICH. RP101075 can reduce brain-infiltrating immune cells,
enhance BBB integrity, and attenuate cell death after ICH.
A S1PR1 antagonist W146 diminished the neuroprotective
effects of RP101075. Our results suggest that selective S1PR1
modulation using a novel compound RP101075 is sufficient to
attenuate brain inflammation and provide protection after ICH.
Neurodeficits and brain edema are 2 surrogate markers
for clinical evaluation of ICH. In this study, we chose to use
an autologous blood infusion model because of its ability to
mimic the rapidly developing bleeding without actually ruptur-
ing vessels or using exogenous factors.5,7 Thirty microliters of
blood infused in mice reflects a 60-mL intracerebral hematoma
in humans29 and provides a reproducible brain edema size dur-
ing hematoma evolution. These advantages suggest autologous
blood infusion model as a suitable model for this study.
Recently, fingolimod as a S1PR modulator was shown
to be effective at reducing brain injury after ICH in rodents

Figure 3. RP101075 reduced cellular components and cytokine production in the brain after intracerebral hemorrhage (ICH). At days 1 and 3 after ICH, brain tissues were obtained to harvest isolated cells for flow cytometry analysis or brain homogenates for ELISA assays. A, Flow cytometry plots show the gating strategy of CD4+ T cells (CD45+CD4+), CD8+ T cells (CD45+CD8+), CD19+ B cells (CD45+CD19+), natural killer (NK) cells (CD45+NK1.1+), neutrophils (CD45+Gr-1+), and microglia (CD45+CD11b+). B, At day 3 after ICH, flow cytometry analysis showed a significant decline in microglia, neutrophils, and lymphocyte subpopulations in RP101075 group compared with vehicle. Interleukin (IL)-1β and tumor necrosis factor (TNF)-α levels were also decreased at day 1 and 3 after RP101075 treatment. Data were presented as mean±SEM, *P<0.05, **P<0.01, n=12 per group. APC indicates allophycocyanin; FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; and SSC, side scatter.
and in patients.13–18 Thereafter, increasing attention has been focused on S1PR modulation as a potential immune intervention approach for ICH.11,35–37 However, several limitations of fingolimod may preclude its clinical applications. First, the diverse subtypes of S1PR expressed on peripheral immune cells and brain-intrinsic cells preclude a better understanding of the cell types and compartments responsible for the mechanisms of action of fingolimod. Of note, despite the pivotal role of S1PR1 in the mediation of fingolimod’s effects, it remains unclear whether S1PR1 modulation is sufficient and necessary for fingolimod to provide beneficial effects. In addition, the optimal timing for immune interventions using S1PR modulators is still undefined in ICH. Last but not the least, the activation of S1PR3 by fingolimod was at least partially responsible for its side effects on the cardiovascular system and organ fibrosis, which may cause prominent safety issues.19–22

RP101075 is S1PR1 selective and has extremely low affinity to other subtypes of S1PRs such as S1PR3.27 The clinical pharmacokinetic properties of RP101075 are characterized by a Tmax of ≈7 hours and a half-life of 19 hours.27 The toxicology data also suggest that the dose of 0.6 mg/kg is safe in preclinical studies.24–27 The efficacy of RP101075 has been tested in experimental allergic encephalomyelitis mice and compared with FTY720.27 Dose of 0.6 mg/kg RP101075 can cause ≈60% reduction in the counts of circulating lymphocytes, which resembles 1-mg/kg FTY720.27 In addition, we show that 0.6 mg/kg of RP101075 can produce sufficient efficacy of protection, in comparison with 0.3-mg/kg RP101075 or 1-mg/kg FTY720 (Figure I in the online-only Data Supplement). In terms of the potency, selectivity, and dose–effect relation of RP101075, we therefore chose the dose of 0.6 mg/kg in the current study.

We showed that RP101075, a selective S1PR1 modulator, improves neurological outcomes and attenuates brain edema after ICH. Of note, the beneficial effect of RP101075 on neurological outcomes and brain edema was blocked by W146, a selective RP101075 antagonist.38–40 Together with the reduced cellular infiltration and inflammatory cytokine expression after RP101075 treatment, these results support the notion that modulation of S1PR1 is sufficient to suppress brain inflammation.

Figure 4. RP101075 attenuated Evans Blue leakage and loss of tight junction. A and B, RP101075 reduced Evans Blue leakage at day 3 after ICH. C and D, Results from Western blot analysis showed that RP101075 prevented the loss of occludin and zonula occludens-1 (ZO-1). Scale bar, 2.5 mm. Data were presented as mean±SEM, *P<0.05, **P<0.01, compared with vehicle group, n=5 per group for Evans Blue leakage and n=4 per group for tight junction Western blot.

Figure 5. RP101075 reduced cell death in the brain after intracerebral hemorrhage (ICH). Brain sections from ICH mice receiving RP101075 or vehicle controls were stained with TdT-mediated Biotin-dUTP Nick End labeling (TUNEL) to measure cell death. A, Representative images showed reduced TUNEL+ cells at day 3 after ICH in group mice receiving RP101075 compared with vehicle. B, The scheme of coronal brain section illustrated the location for cell death measurements. C, A decrease of perihematomal apoptotic cells was seen at day 3 in ICH mice receiving RP101075. Scale bar, 400 μm. Data were presented as mean±SEM, *P<0.05, **P<0.01, compared with vehicle group, n=4 per group per time point. DAPI indicates 4’,6-diamidino-2-phenylindole.
and provide protection in ICH. The superior pharmacological features along with its efficacy may qualify RP101075 as a promising candidate for future ICH investigations.

Our findings cannot conclude that modulation of S1PR1 on immune cells is the sole mechanism responsible for the protection offered by RP101075. Considering the wide distribution of S1PR1 on a variety of cells involved in the process of ICH and the preferential distribution of RP101075 in the brain, other potential cellular targets beyond immune cells and their relevant anatomic locations for immune interventions await further investigations. It is noteworthy that our results are consistent with previous findings that early immune interventions are beneficial in acute ICH. However, the current study does not provide sufficient information on the optimal time window for S1PR1 modulation to achieve maximal beneficial effects after ICH. Because of its importance for the design of immunomodulatory therapies, this information is critical and should be examined in future studies. The decreased TUNEL+ cells after RP101075 treatment also indicates that the protection of RP101075 may not entirely depend on peripheral lymphocytes including T, B, and NKT cells. Nevertheless, given the widespread expression of S1PR1 on multiple types of cells, the operating mechanisms of RP101075 in ICH require future investigation.

In conclusion, we demonstrate that selective S1PR1 modulation using RP101075 and FTY720 suggest that the beneficial effect of FTY720 in ICH may be largely mediated by its impact on S1PR1 (Figure I in the online-only Data Supplement). This finding is consistent with previous reports that the benefit of FTY720 requires S1PR1 in animal models of ischemic stroke and MS. Of interest, one recent report suggests the benefit of FTY720 in experimental ischemic stroke depends on lymphocytes because of the prothrombotic impact of lymphocytes on microperfusion. Another report shows that the benefit of FTY720 in a mouse model of MS depends on its action on astrocytes. In addition to these findings, we found that although to a lesser extent, RP101075 can still reduce neurodeficits and brain edema in Rag2−/− mice (Figure IV in the online-only Data Supplement). These results suggest that the protection of RP101075 may not entirely depend on peripheral lymphocytes including T, B, and NK cells. Nevertheless, given the widespread expression of S1PR1 on multiple types of cells, the operating mechanisms of RP101075 in ICH require future investigation.

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Disclosures
None.

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### Supplemental Methods

#### Brain water content assessment
At days 1 and 3 after surgery, brains were removed and divided into three parts: left hemisphere, right hemisphere and cerebellum. Tissues were quickly weighed on an electronic analytical balance to obtain wet weight. Then the tissues were dried for 24 hrs at 100 °C to obtain dry weight. The formula for calculation was as follows: \[
\text{[(Wet Weight - Dry Weight) / Wet Weight] x 100%}.
\]

#### Hemoglobin quantification
We also detected the amount of extravasated blood in the brain tissue by performing hemoglobin ELISA using a commercial kit (Kamiya Biomedical Company, USA) and a microplate photometer (Thermo Scientific, Varioskan Flash, USA) capable of reading at 450 nm and 570 nm were used to calculate quantity of hemoglobin.

#### Isolation of cellular components from CNS and peripheral blood for flow cytometry analysis
Brain tissues were mechanically homogenized through a 40 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) in PBS on ice, then cell suspension was collected and centrifuged at 400 x g for 10 min; the pellet was resuspended in 5 ml of 60 % Percoll (GE Healthcare Bio Science AB, Uppsala, Sweden) and 5 ml 30 % Percoll was overlaid on the top of the 60 % solution. The gradient was then centrifuged at 500 x g for 20 min and cells were collected on the interface between 30 % and 60 % Percoll. Isolated cells were washed once (1500 x rpm, 8 min) in 15 ml PBS and were resuspended in 1 % bovine serum albumin (BSA) for staining. Venous blood (500 μl per mouse) was simultaneously collected and lymphocytes were isolated according to the manufacturer’s instructions for the Ficoll gradient media (GE Healthcare Bio Science AB, Uppsala, Sweden) and RBC lysis buffer (GE Healthcare Bio Science AB, Uppsala, Sweden). Flow cytometry staining buffer (100 μl) was added to resuspend the isolated cells for flow cytometry analysis. Cells isolated from the either the CNS or venous blood were diluted to 1 x 10^6/ml and stained for anti-CD45 FITC (Cat#103107, Biolegend), anti-CD4 PE (Cat#100407, Biolegend), anti-CD8 APC (Cat#100711, Biolegend), anti-NK1.1 PE-Cy5 (Cat#108715, Biolegend), anti-CD19 PerCP (Cat#115531, Biolegend), anti-Gr-1 PE-Cy7 (Cat#108415, Biolegend) and anti-CD11b APC-Cy7 (Cat#101225, Biolegend), and isotype control respectively. Flow cytometry was performed using a FACS Calibur (Aria III, BD Bioscience) and data were analyzed by Flow Jo software (Tree Star, US).

#### Enzyme-linked immunosorbent assay (ELISA)
Ipsilateral hemisphere tissues (~100 mg) were homogenized and supernatants were collected after centrifugation. Supernatants from brain homogenates and blood serum samples were used to analyze the expression of IL-1β and TNF-α using a commercial ELISA kit (Biolegend, USA) and a microplate photometer (Thermo Scientific, Varioskan Flash, USA) capable of reading at 450 nm and 570 nm.

#### Assessment of blood-brain barrier permeability
Evans Blue dye (Sigma, USA) was used as a tracer to measure the BBB permeability at day 3 as previously described. 2 % Evans blue (2 ml/kg) was injected intravenously 2 h prior to sacrifice. The ipsilateral hemisphere was weighed on an electronic balance (with an accuracy of 0.1 mg) and homogenized into a test tube with 5 ml of formamide (Sigma, USA), then incubated in a 60 °C water bath for 72 h. After centrifugation at 1000 x rpm for 5 min, the supernatants were collected for testing. A microplate reader (Thermo Scientific, Varioskan Flash, USA) was used (λ = 450, 570 nm) to measure the OD of the supernatant and the standards (80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 μg/ml). The concentration of EB was calculated by the formula as follows: EB content in brain tissue (μg/g wet brain) = EB concentration x formamide (ml)/wet weight (g).

#### Western blot analysis for tight junctions
At day 3 after ICH, ipsilateral hemispheres were homogenized in RIPA lysis buffer (Sigma, USA) and 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) (Sigma, USA). After centrifugation, the supernatant was used for analysis. Proteins were loaded and transferred to a PVDF membrane (Millipore, USA). After being blocked, membranes were incubated overnight at 4 °C with anti-occludin (1:1000, Millipore), anti-zonula occluden-1 (ZO-1) (1:1000, Millipore), anti-claudin-5 (1:1000, Millipore) rabbit polyclonal antibodies. Membranes were then incubated for 1 h at room temperature with horseradish peroxidase (HRP) labeled goat anti-rabbit secondary antibody (1:4000, Vector). The membranes were placed into a gel imaging system (Bio-Rad), and then exposed. The intensity of blots was quantified using the Quantity One software (Bio-Rad). β-actin was used as an internal control.

Analysis of neuronal apoptosis
Three days after ICH, the extent of cell death was assessed by TUNEL stain using a TdT-mediated Biotin-dUTP Nick End labeling (TUNEL) kit (Roche, USA), and nuclei were stained with DAPI (Sigma-Aldrich, F6057, USA). Once stained, the specimens were analyzed under a fluorescence microscope (Nikon C-HGFI, Japan). The total number of TUNEL positive cells was counted in four random fields at the edge of the hematoma in 20 x views, and the ratio of apoptotic cells to nuclei was calculated as apoptotic cells (%).

Primary neuronal culture
Cortical tissue was dissected from embryonic day 18 (E18) Sprague-Dawley rat embryos under the 10 × microscope. Cortical tissue was then minced with scissors in ice-cold Neurobasal medium (Invitrogen, USA). Thereafter, minced tissue was digested with Papain (20 U/mg; Worthington) at 30 °C for 20 min in tubes shaken at 120 rpm in a water bath. After digestion, the reaction was stopped by adding inactivated fetal bovine serum. After trituration and centrifugation, cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum and then seeded in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum and then seeded in polylysine (Sigma, USA) coated 12-well plates at 5 × 10⁵ cells per well. Then, the medium was replaced by serum-free Neurobasal® medium containing 2 % B27 medium-supplement (GIBCO, USA), 0.5 mM glutamine and antibiotics 4 hours later. The cultures were essentially free of astrocytes and microglia and maintained at 37 °C in a humidified incubator with 5 % CO₂. Half of the culture media was changed every 3 days. The neurons were used for experiments after 7 days.

After 7 days in vitro, cortical neurons were treated with either PBS or RP101075 (100 nM; Receptos Inc, USA) for 24 hours and then exposed to hemin (100 μM; Sigma, USA) for an additional 24 hours. Neuronal apoptosis was observed using TUNEL staining.

Immunostaining
Immunostaining of anti-microtubule-associated protein 2 (MAP2) was used to identify neurons. A primary MAP2 antibody (Abcam, ab5392, USA) and a secondary antibody (Alexa Fluor 488 donkey anti-mouse Ab) (Abcam, ab150105, USA) were used.

Reference
Supplemental Figure I. The dose-effect-relation of RP101075 in ICH mice.
ICH mice were treated with vehicle, 0.3 or 0.6 mg/kg RP101075, or 1 mg/kg FTY720 at 30 min, 24h or 72h after injection of autologous blood. RP101075 0.6 mg/kg at 30 min after ICH reduced neurodeficits and brain edema at day 1 and 3 after ICH which resembled the FTY720 1 mg/kg at 30min. Data are expressed as mean ± SEM. **p < 0.01, versus vehicle group. n = 20 per group for the evaluation of mNSS, n = 40 per group for the assessment of brain edema.
Supplemental Figure II. Alteration of immune cells and cytokines in peripheral blood between groups.

At days 1 and 3 after ICH, venous blood was collected to harvest isolated cells for flow cytometry analysis or serum for ELISA assays. After RP101075 administration, flow cytometry analysis showed a significant decline in lymphocyte subpopulations, neutrophils and leukocytes compared with vehicle. IL-1β and TNF-α levels also decreased after RP101075 treatment. Data are presented as mean ± SEM, *p < 0.05, **p < 0.01, n=12/group.
Supplemental Figure III. RP101075 attenuates neuronal apoptosis in vitro.

At day 7 in vitro, primary cortical neurons were treated with vehicle PBS or 100 nM RP101075 for 24 h. Thereafter, cortical cultures were exposed to hemin (100 μM; Sigma, USA) for an additional 24 h. Neuronal apoptosis was directly assessed by counting cells that are TUNEL⁺ (A) or MAP2⁺ (C, yellow arrow: normal; red arrow: diffusion injury; gray arrow: mild injury). Summarized results are shown in B (percentage of total number of cells) and D (percentage of untreated controls). ***p < 0.001 versus control; #p < 0.05, ##p < 0.01 versus vehicle. n = 6. Data were from three independent experiments. Scale bars = 200 μm in A; 40 μm in B. Con = Control, Ve = Vehicle, RP = RP101075.
**Supplemental Figure IV.** RP101075 reduces neurodeficits and brain edema in Rag2−/− mice.

(A) Neurological function was evaluated using mNSS at indicated time points after ICH. RP101075 improved mNSS.

(B) At day 3 after ICH, 7T MRI was performed to assess hematoma size (SWI), lesion volume (T2WI) and hemispheric volume (T2WI). Brain edema (%) was expressed as [(ipsilateral hemisphere volume − contralateral hemisphere volume)/contralateral hemisphere volume] × 100%. Data are presented as mean ± SEM, *p < 0.05 versus vehicle, n = 8 mice per group.