TNF-alpha receptor antagonist, R-7050, improves neurological outcomes following intracerebral hemorrhage in mice

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HIGHLIGHTS

- R-7050, a novel TNFR antagonist, reduces neurovascular injury after ICH.
- Pharmacological inhibition of TNFR improves outcomes after ICH.
- TNFR may represent a viable therapeutic target after ICH.

ARTICLE INFO

ABSTRACT

Intracerebral hemorrhage (ICH), the most common form of hemorrhagic stroke, exhibits the highest acute mortality and the worst long-term prognosis of all stroke subtypes. Unfortunately, treatment options for ICH are lacking due in part to a lack of feasible therapeutic targets. Inflammatory activation is associated with neurological deficits in pre-clinical ICH models and with patient deterioration after clinical ICH. In the present study, we tested the hypothesis that R-7050, a novel cell permeable triazoloquinoxaline inhibitor of the tumor necrosis factor receptor (TNFR) complex, attenuates neurovascular injury after ICH in mice. Up to 2 h post-injury administration of R-7050 significantly reduced blood–brain barrier opening and attenuated edema development at 24 h post-ICH. Neurological outcomes were also improved over the first 3 days after injury. In contrast, R-7050 did not reduce hematoma volume, suggesting the beneficial effects of TNFR inhibition were downstream of clot formation/resolution. These data suggest a potential clinical utility for TNFR antagonists as an adjunct therapy to reduce neurological injury and improve patient outcomes after ICH.

1. Introduction

Spontaneous intracerebral hemorrhage (ICH), the most prevalent form of hemorrhagic stroke, induces one-year mortality rates >60% and induces the worst long-term neurological outcomes of all stroke subtypes [29]. ICH is caused by the rupture of small vessels damaged by chronic hypertension or amyloid angiopathy, inducing the formation of an intracranial space-occupying hematoma. Neurosurgical clot evacuation improves outcomes in some ICH patients, although many patients are not amenable to surgical intervention due to an inaccessible location or concurrent intraventricular hemorrhage [27]. As such, conservative management remains a clinical mainstay. The lack of efficacious therapeutic options reinforces the notion that ICH is the least treatable form of stroke and stresses the need for improved medical approaches.

Hemolysis promotes spontaneous hematoma resolution, although this process simultaneously induces the release of pro-inflammatory mediators adjacent to the hematoma. Inflammatory activation is associated with increased neurovascular damage, neurological deterioration, and a poor functional recovery after ICH [15,22,28,37], although the precise mechanisms remain undefined. In particular, elevated plasma concentrations of the pro-inflammatory mediator, tumor necrosis factor-α (TNF-α), clinically correlated with acute hematoma enlargement, edema development, and poor patient outcome following ICH [3,8,9,17,35]. Similarly, increased TNF-α expression was observed in several different species and in multiple experimental models of ICH. Importantly, our laboratory and others reported a functional association between peri-hematomal TNF-α expression and the development brain edema and neurological injury after ICH

[16,19,26,34]. As a whole, these findings support the notion that TNF-α represents a rationale therapeutic target after ICH.

Although emerging pre-clinical and clinical evidence suggests a detrimental role, small molecule TNF-α pathway inhibitors remain largely unexplored in the context of a brain hemorrhage. TNF-α induces biological activity via stimulation of the tumor necrosis factor receptor (TNFR) [1,23]. TNFR interacts with downstream adaptor proteins, including TRADD, TRAF and RIP1, providing specificity of the response toward a pro-inflammatory and/or a cell death response. R-7050 is a novel cell-permeable triazolo-quinoxaline compound that selectively inhibited TNF-α induced cellular signaling using differential screening of a 300,000 compound library [14]. Unlike biologic TNF inhibitors (e.g. infliximab, etanercept and adalimumab) that directly bind TNF-α and function as decoy receptors, R-7050 does not affect binding of TNF-α to TNFR. In contrast, R-7050 selectively inhibits the association of TNFR with intracellular adaptor molecules (e.g. TRADD and RIP), limits receptor internalization, and prevents subsequent cellular responses after TNF-α binding [14]. In the present study, we tested the hypothesis that R-7050 reduces neurovascular injury after ICH.

2. Materials and methods

2.1. ICH model

Animal studies were reviewed and approved by the Committee on Animal Use for Research and Education at Georgia Health Sciences University, in compliance with NIH guidelines. A mouse collagenase model of ICH was utilized for all studies, as detailed by our laboratory [19,32]. Briefly, male CD-1 mice (8–10 weeks old; Charles River, Wilmington, MA, USA) were placed into a stereotactic frame and a 0.5 mm diameter burr hole was drilled over the parietal cortex, 2.2 mm lateral to the bregma. A 26-gauge Hamilton syringe, loaded with 0.04 U of bacterial type IV collagenase in 0.5 μL saline, was lowered 3 mm deep from the skull surface directly into the left striatum. The syringe was depressed at a rate of 450 nL/min and left in place for several minutes after the procedure to prevent solution reflux and excess diffusion. Sham animals underwent the same surgical procedure, except that saline was stereotactically injected rather than collagenase. After the syringe was removed, bone wax was used to close the burr hole, the incision was surgically stapled, and mice were kept warm until recovery of the righting reflex. R-7050 (EMD Biosciences, 6–18 mg/kg) was administered via intraperitoneal route at the time of injury or up to 2 h post-ICH.

2.2. Hematoma volume

Hematoma volume was quantified using by QuantiChrom Hemoglobin Assay Kit (Bioassay Systems, Hayward, CA), per manufacturer's recommendations and as routine to our laboratory [19]. The amount of hemoglobin in each hemisphere was calculated using the following: [(optical density of sample/optical density of calibrator) $\times$ 100.

2.3. Blood–brain barrier (BBB) integrity

BBB integrity was quantified following administration of Evans blue (20 mg/mL in PBS, i.v.) 2 h prior to sacrifice, as detailed by our laboratory and others [19,25]. Absorbance in brain tissue was determined at 620 nm using a Synergy HT plate reader. The amount of Evans blue within brain tissue was calculated using a standard curve and expressed as μg Evans blue/g brain tissue.

2.4. Assessment of cerebral edema

Brain water content, an established measure of cerebral edema, was quantified in a 2 mm coronal tissue sections of the ipsilateral or corresponding contralateral striatum, as routine to our laboratory [18–20]. Tissue was immediately weighed (wet weight), then dehydrated at 65 °C. Samples were reweighed 48 h later to obtain a dry weight. The percentage of water content in each sample was calculated as follows: %Brain water content = [((wet weight − dry weight)/wet weight) $\times$ 100].

2.5. Neurological outcomes

Neurological injury was determined using a modified 24-point scale, per our laboratory and others [5,19,30]. This scale was comprised of six behavioral tests, each of which was graded from 0 (performs with no impairment) to 4 (severe impairment). A composite score was calculated as the sum of the grades on all six tests. All data were scored independently by two investigators blinded to experimental treatment groups.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) followed by Student Newman-Keuls or Two-way ANOVA followed by Tukey's post hoc test were used for multiple group comparisons. Data are expressed as mean $\pm$ SEM. A p-value of <0.05 was considered to be significant.

3. Results

R-7050 attenuates neurovascular injury after ICH. Blood–brain barrier opening contributes to the development of vasogenic edema, an important cause of neurological deterioration after ICH. Evans blue extravasation, a sensitive estimate of blood–brain barrier integrity, increased from 12.2 $\pm$ 1.5 μg Evans blue/g brain tissue in sham-operated mice to 47.2 $\pm$ 5.8 μg Evans blue/g brain tissue at 24 h post-ICH ($p < 0.01$ vs. sham) (Fig. 1). R-7050 (6 mg/kg) reduced Evans blue extravasation to 28.7 $\pm$ 5.9 μg and 30.3 $\pm$ 1.9 μg Evans blue/g brain tissue when administered at 0.5 h or 2 h post-ICH, respectively ($p < 0.05$ and $p < 0.01$ vs. ICH, respectively; not significantly different from sham).

Brain water content, a measure of brain edema, increased from 75.6 $\pm$ 0.3% in sham-operated mice to 81.5 $\pm$ 0.5% at 24 h post-ICH
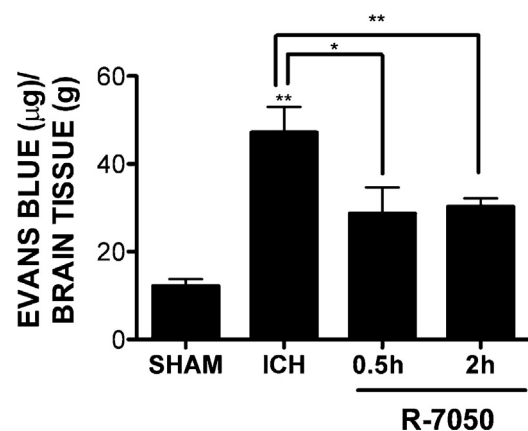

Fig. 1. R-7050 maintains blood–brain barrier integrity after ICH. Mice were administered 6 mg/kg R-7050 at 0.5 h or 2 h after collagenase-induced ICH. Evans blue extravasation, a sensitive measure of BBB disruption was assessed 24 h later. Data are expressed as mean $\pm$ SEM and were analyzed by one-way ANOVA followed by Student Newman Keul's post hoc test (**$p < 0.01$, $n = 8$–9 per group).

Fig. 3. R-7050 does not affect hematoma volume after ICH. R-7050 (6 and 12 mg/kg) administration at the time of ICH failed to reduce hematoma size at 72 h post-ICH. Hematoma volume was quantified by determining the hemoglobin content of each hemisphere at 72 h post-ICH. Data are expressed as mean ± SEM (*$p < 0.05$, ***$p < 0.001$ vs. sham; $n = 8$ per group).
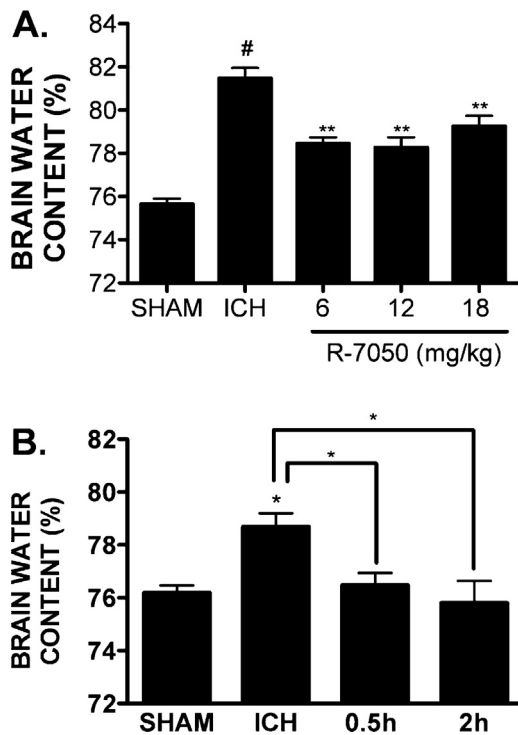

Fig. 2. R-7050 reduces edema development after ICH. (A) Mice were administered R-7050 (6, 12 and 18 mg/kg) just prior to collagenase-induced ICH. Brain water content, a measure of cerebral edema, was assessed in the ipsilateral hemisphere at 24 h post-ICH. (B) Mice were administered 6 mg/kg R-7050 at 0.5 h or 2 h after collagenase-induced ICH. Brain edema was assessed 24 h later. Comparisons within each hemisphere between different treatments groups were done using a one-way ANOVA followed by Student Newman Keul's post hoc test (#$p < 0.05$ vs. sham, *$p < 0.05$, **$p < 0.01$). No significant differences were observed between groups in the contralateral hemispheres. Data are expressed as mean ± SEM from 8 to 9 mice/group.

Fig. 4. R-7050 improves neurological outcome after ICH. Mice were administered R-7050 (6 and 12 mg/kg, i.p.) 0.5 h after collagenase-induced ICH. Neurological outcomes were assessed at 24 h, 48 h, or 72 h following sham or ICH using a 24 point neurological scale score. Data are the mean ± SEM ($n = 9–10$ per group) and were analyzed using repeated measures ANOVA followed by Bonferroni's post hoc test (*$p < 0.05$, **$p < 0.01$).

($p < 0.05$ vs. sham). 6, 12, or 18 mg/kg R-7050 reduced brain water content to 78.5 ± 0.3%, 78.3 ± 0.3%, or 79.3 ± 0.5%, respectively (all treatments $p < 0.05$ vs. ICH; treatments not significantly different from each other) (Fig. 2B). Notably, mice treated with 18 mg/kg exhibited a reduction in general activity/locomotion; thus, follow up studies did not utilized this dose. As was observed with Evans blue extravasation, R-7050 (6 mg/kg) significantly reduced brain water content after ICH. Administration of R-7050 at 0.5 h or 2 h post-ICH attenuated brain water content to levels observed in sham-operated mice ($p < 0.05$ vs. ICH, not significantly different from sham) (Fig. 2B).

R-7050 does not reduce hematoma volume after ICH. Hematoma volume is directly correlated with functional outcomes; thus, the effect of R-7050 on hematoma volume was ascertained. In contrast to the reduction in BBB opening and edema formation, R-7050 (6 and 12 mg/kg) did not significantly reduce hematoma volume over the first 72 h, as assessed by quantification of hemoglobin content within the ipsilateral hemisphere (Fig. 3). Specifically, hemoglobin content was increased within the injured hemisphere from 30.1 ± 2.0 mg/dL in sham-operated mice to 117.9 ± 16.7 mg/dL following ICH ($p < 0.05$ vs. sham). Similarly, neither 6 mg/kg nor 12 mg/kg R-7050 affected hemoglobin content, as compared to placebo-treated ICH mice (101.7 ± 17.0 mg/dL and 111.1 ± 17.3 mg/dL, respectively).

R-7050 improves neurological outcomes after ICH. A protective effect of R-7050 was observed across the first 3 days post-ICH, as compared to placebo treated mice, with a complete reduction in neurological deficits observed by 72 h ($p < 0.05$ vs. ICH, not significantly different from sham) (Fig. 4). Similarly, an intermediate
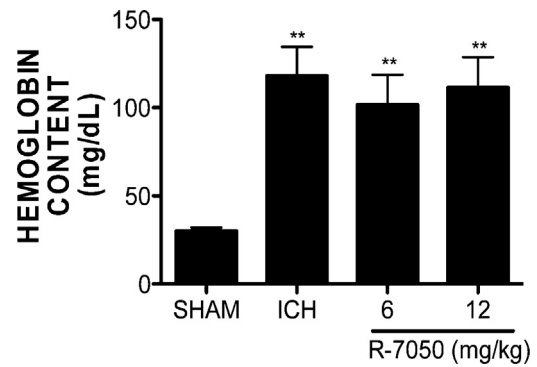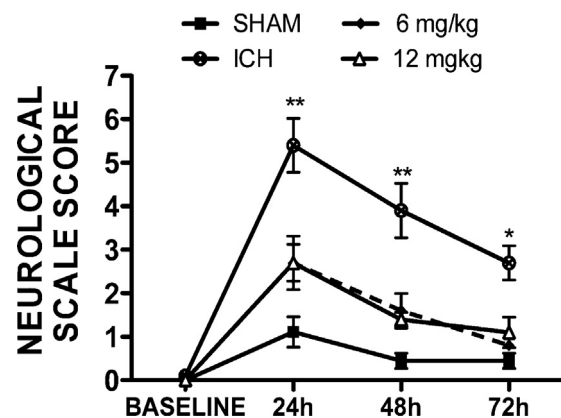
protective effect was observed with both 6 mg/kg and 12 mg/kg R-7050 at 24 h and 48 h post-ICH ($p < 0.01$ vs. ICH, $p < 0.05$ vs. sham). Placebo treatment had no significant effect on neurobehavioral outcomes, as compared to ICH with no treatment.

4. Discussion

ICH induces the highest mortality of all stroke subtypes and <20% ICH survivors recover functional independence after 6 months [13,29]. Hematoma volume directly correlates with neurological deterioration and patient mortality and neurosurgical clot evacuation produces more favorable outcomes in subsets of ICH patients [4,29]; however, many patients remain poor surgical candidates and the efficacy of surgical intervention for spontaneous supratentorial ICH remains controversial [2,27]. These data emphasize the devastating nature of ICH and indicate the dire need for efficacious medical treatment options to improve long-term patient prognoses.

Erythrocyte extravasation induces the formation of a space-occupying hematoma within the brain parenchyma that may result in microvascular compression, herniation, and neurovascular dysfunction [4,36]. Spontaneous hematoma resolution alleviates the mass effect of the clot, although the simultaneous release of toxic hemoglobin metabolites enhances immune activation and exacerbates neurological injury. Along these lines, acute expression of

TNF-α was associated with increased neurovascular injury after ICH and elevated cerebrospinal fluid and plasma TNF-α concentrations correlated with patient mortality after spontaneous ICH [3,9,16]. Consistent with a possible detrimental role in acute brain injury, TNF-α expression temporally preceded glial cell death following exposure to hemin, a major hemoglobin oxidation product within intracranial hematomas [21]. Similarly, heme exposure increased programmed necrosis through autocrine production of TNF-α [12]. Furthermore, unconjugated bilirubin, a heme catabolite that contributed to inflammatory activation and increased brain edema after ICH in mice [24], was associated with increased microglial TNF-α production and enhanced TNFR-dependent gliotic responses [10,11,31]. These findings support the assertion that TNF-α signaling exerts an acute detrimental role after ICH.

In the present study, a single administration of R-7050 reduced neurovascular injury after ICH. In contrast to biologic approaches that directly bind and neutralize TNF-α activity, R-7050 selectively inhibits the association of TNFR with intracellular adaptor molecules, such as TRADD and RIP1, limiting receptor internalization and preventing subsequent cellular responses after TNF-α binding [14]. Consistent with the assertion that TNFR signaling promotes acute brain injury after ICH, we found that necrostatin-1, a selective RIP1 inhibitor [7], limited peri-hematoma cell death, attenuated neuroinflammatory activation, reduced neurovascular injury, and improved neurobehavioral outcomes after ICH (King and Dhandapani, unpublished results). RIP1 functionally mediates TNF-α-induced activation of the pro-inflammatory transcription factor, NFκB [6]; thus, our pre-clinical findings are in line with clinical reports suggesting a direct correlation between inflammatory activation at the time of admission and early neurological deterioration in ICH patients [22]. Furthermore, our laboratory and others reported that NFκB activation reduces blood–brain barrier integrity, increases edema development, and exacerbates neurobehavioral deficits after experimental ICH [15,19,37]. Taken together, these results suggest TNF-α may initiate a detrimental signaling cascade after ICH.

Although most accurately mimicking the spontaneous intracerebral bleeding and evolving hematoma expansion observed in patients, the intrastriatal injection of bacterial collagenase used in this model may induce an exaggerated inflammatory response, as compared to the blood injection model of ICH. Thus, the beneficial effects of R-7050 may be overestimated. Nonetheless, TNF-α was elevated in ICH patients [9] and increased peri-hematoma TNF-α expression contributed to edema development after ICH in rodents [16]. These data suggest the beneficial effects of R-7050 observed herein are unlikely to be model specific and rather, may have more widespread therapeutic implications. Despite a clear damaging role in acute brain injury, TNF-α may also prime cerebral endothelial cells for erythropoietin-induced angiogenesis and enhance functional recovery after a brain hemorrhage [33]. This potential caveat indicates both beneficial and detrimental effects of TNF after brain injury and suggests the need for additional research prior to clinical translation of these data. Future work by our laboratory will further delineate the specific roles of TNF–TNFR signaling after ICH.

In conclusion, we identified a novel role for the triazoloquinoxaline TNF-α inhibitor, R-7050, in the attenuation of neurovascular injury and neurobehavioral improvement in a pre-clinical model of ICH. These data suggest therapeutic targeting of TNF-α represents a feasible means to limit neurological injury after a brain hemorrhage; however, we cannot exclude the possibility that R-7050 may exert neuroprotective effects via off-target effects independent of TNFR. Future work by our laboratory will continue to explore the translational potential of this compound, including further studies of drug specificity, as well as the establishment of a full therapeutic window and dosing paradigm in multiple pre-clinical models of ICH, including both collagenase and blood injection models of ICH.

Conflict of interest

The authors declare no competing interests or conflicts.

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