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Heat Shock Protein 70 Upregulation by Geldanamycin Reduces Brain Injury in a Mouse Model of Intracerebral Hemorrhage

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Abstract

This study investigated the effect of geldanamycin post-treatment on the development of secondary brain injury and neurological deficits in a mouse model of intracerebral hemorrhage. CD-1 mice received stereotactic injection of collagenase type VII into the right basal ganglia. Treatment groups were administered 1mg/kg (low dose) or 10mg/kg (high dose) of geldanamycin. Mice were euthanized at two time-points: 24 hrs or 72 hrs. Blood-brain-barrier permeability, brain edema, and neurobehavioral deficits were assessed. Additionally, the effects of geldanamycin on heat shock protein 27 and 72; tumor necrosis factor-alpha and interleukin 1 beta expressions were evaluated.

High dose geldanamycin significantly attenuated blood brain barrier disruption and brain edema after intracerebral hemorrhage. Neurobehavioral outcomes were significantly improved in some parameters by high dose treatment. Molecular results showed a marked increase in heat shock protein 72 expression in ipsilateral brain of geldanamycin treated groups with a reduction in the pro-inflammatory tumor necrosis factor-alpha.

CONCLUSION—Geldanamycin post-treatment is neuroprotective in the mouse model of intracerebral hemorrhage. Geldanamycin administration results in reduction of inflammation, preservation of blood-brain-barrier and amelioration of neurobehavioral deficits after an insult possibly by upregulation of heat shock protein 72.

Keywords

geldanamycin; intracerebral hemorrhage; edema; neurobehavior; neuroprotection

INTRODUCTION

Intracerebral hemorrhage (ICH) is a devastating clinical event with greater than 40% initial mortality, leaving many of the survivors permanently disabled (Woo et al. 2002). Elevated

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intracranial pressure, increased blood-brain-barrier (BBB) permeability, and resultant edema and hemorrhage are all contributors to the poor prognosis after ICH (Qureshi et al. 2001). Of the dynamic and complex processes that encompass this devastating disease, inflammation appears to play a key role (Hua et al. 2006). The identification of inflammatory mediators in the pathogenesis of ICH is a key element in determining novel targets for therapeutic intervention.

One such target has been heat shock proteins (HSPs). There are four major families of mammalian HSPs according to their molecular size: HSP90, HSP70, HSP60 and the small HSPs. HSP90 and HSP60 are constitutively expressed in mammalian cells, and HSP27 and HSP72 which are synthesized in response to stress and are mediators of inflammatory and apoptotic pathways (Sinn et al. 2007). Studies have demonstrated that induction of HSPs, such as HSP 27 or HSP 72, is related to protection against ischemic damage (Aoki et al. 1993, Kato et al. 1994), motor neuron disease (Katsuno et al. 2005), and severe heat stress (Uney et al. 1993). These findings provide a basis for investigating the role of HSP induction in an ICH setting. Geldanamycin, an inducer of HSPs, belongs to the family of ansamycin antibiotics. Biochemical and structural studies have demonstrated that geldanamycin binds specifically to HSP 90, allowing for the activation of HSF 1 (Whitesell et al. 1994) and subsequent production of other HSPs, in particular HSP 72 (Voellmy et al. 2007). Geldanamycin-induced HSP 72 upregulation has been shown in a variety of cell types including neurons, astrocytes, and visual epithelial cells (Xu et al. 2003, Ouyang et al. 2005, Shen et al. 2005, Kaarniranta et al. 2005), and has been found to be neuroprotective in experimental models of middle cerebral artery occlusion (Whitesell et al. 1994, Lu et al. 2002, Kwon et al. 2008) and Parkinson's disease (Shen et al. 2005). Moreover, geldanamycin has been demonstrated to decrease the protein of mixed-lineage kinase (MLK) 3 and activate Akt. Wen et al. (2008) showed enhancement of the association of HSP 90 and MLK3, association with Src and activation of c-Jun N-terminal kinase (JNK) 3. They demonstrated geldanamycin as a pre-treatment neuroprotectant in vitro (using hippocampal cells); and in vivo (twenty-minutes after cerebral ventricular injection of geldanamycin, their group performed a 4-vessel occlusion model of global ischemia followed by reperfusion.)

To our knowledge, no study to date has assessed the effects of geldanamycin on the outcomes of intracerebral hemorrhage. Accordingly, the objective of this study was to determine whether geldanamycin may improve the consequences of ICH in a mouse model. To this end, edema, BBB permeability, neurobehavioral deficits, HSP expression, and markers of inflammation were assessed at 24 or 72 hours after the onset of ICH.

MATERIALS AND METHODS

Animal Groups and Treatment Method

This study was conducted in accordance with the National Institutes of Health guidelines for the treatment of animals and was approved by the Animal Care and Use Committee at Loma Linda University. Male CD-1 mice (34–43 grams, Charles River, MA, USA) were housed with a 12-hour light/dark cycle with access to water and food *ad libitum*. A total of 109 mice were randomly divided into five groups: sham (needle-trauma) group, naïve with 10 mg/kg geldanamycin, ICH (collagenase-injection) with vehicle, ICH with 1 mg/kg geldanamycin, and ICH with 10 mg/kg geldanamycin. Geldanamycin was dissolved in 2.5% dimethylsulfoxide (DMSO) then administered intraperitoneally one hour post-ICH. Vehicle mice received 2.5% DMSO in saline. Mice were euthanized at 24 hours or 72 hours post-ICH. The drug concentration given in the 72 hour group was based on the results of the 24 hour study. Only that optimal dosage was used in the 72 hour study.

Surgical Procedure

The collagenase-induced intracerebral hemorrhage model (Rosenberg et al. 1990) was used as previously described in mice (Tang et al. 2004). Briefly, under general anesthesia [ketamine (100 mg/kg), xylazine (10 mg/kg)] the mice were positioned prone in a stereotaxic frame (Stoelting, Wood Dale, IL, USA), the calvarium was exposed by a midline scalp incision from the nasion to the superior nuchal line, and the skin was retracted laterally. Using a variable speed drill (Fine Scientific Tools, Foster City, CA, USA) a 1.0 mm burr hole was made 0.9 mm posterior to the bregma and 1.45 mm to the right of midline. A 26-gauge needle on a Hamilton syringe was inserted 4.0 mm into the right deep cortex/basal ganglia at a rate 1 mm/min. Collagenase (0.075 units in 0.5 µl saline, VII-S; Sigma, St Louis, MO, USA) was infused into the brain at a rate of 0.25 µl/min over 2 minutes using an infusion pump (Stoelting, Wood Dale, IL, USA). Afterwards, the needle was left in place for an additional 10 minutes following injection to prevent the possible leakage of collagenase solution. Upon removal of the needle, the incision was closed and the mice were allowed to recover. Sham operation was performed with needle insertion only.

Evaluation of Brain Edema

Edema was measured as described previously (Manaenko et al. 2009, Zhang et al. 2004). Whole brain specimens were removed immediately and divided into five parts for evaluation of brain edema: ipsilateral and contralateral basal ganglia, ipsilateral and contralateral cerebral cortex, and cerebellum. The cerebellum was used as an internal control for brain water content. Tissue samples were weighed on a high-precision balance (model AE 100; Mettler Instrument Co., Columbus, OH, USA) to obtain the wet weight. The tissue was then dried at 100°C for 24 hours to determine the dry weight. Brain water content (%) was calculated as [(wet weight - dry weight)/wet weight]/100.

Evaluation of BBB Permeability

2 % Evans Blue in normal saline (4 mL/kg BW) was injected into the jugular vein and allowed to circulate for 1 hr. The mice were then transcardially perfused until colorless fluid was obtained from the right atrium. Afterwards, the brain tissue was removed and divided into right and left hemispheres, frozen in LN₂, and stored at -80°C. Brain samples were homogenized in 600µL of PBS and centrifuged (30 min, 15000 rcf, 4°C). The supernatant was collected and an equal amount of 50% trichloroacetic acid was added and then centrifuged (30 min, 15000 rcf, 4°C). Evans Blue dye was measured by spectrophotometer (ThermoSpectronic; excitation 600 nm, emission 650 nm) and quantified according to a standard curve.

Neurobehavioral Evaluation

Mice were tested by an evaluator blinded to animal groups. Three tests were implemented for evaluation of neurological deficits: 1) Modified Garcia test, in which mice were given a score of 0 to 21 (normal) (Garcia et al.1995). The scoring system consists of 7 tests, (spontaneous activity, axial sensation, vibrissae proprioception, limb outstretching, lateral turning, forelimb walking and climbing) with a possible score range of 0 to 3 (0=worst; 3=best). The minimum total score is 0 and the maximum is 21; 2) Wire hanging test and 3) Beam balance test (Manaenko et al. 2009). The latter two tests utilized bridges (550cm wire or 590cm beam) between two platforms on which the mice were placed in the center. Mice were evaluated according to six criteria based on the subject's ability to reach the platform and/or use its limbs in a symmetrical manner, for which they were assigned a score of 0 to 5 (normal). The average of three trials per test for each animal was calculated.

Western Blot Analysis

Mice were perfused transcardially with 40 ml of cold PBS. Hemispheres were isolated and stored at -80°C until analysis. Protein extraction from whole-cell lysates were obtained by gently homogenizing in RIPA lysis buffer (Santa Cruz Biotechnology, Inc, sc-24948) and centrifuging (14,000g at 4°C for 30 minutes). The supernatant of the extract was collected and the protein concentration was determined using a detergent compatible assay (Bio-Rad, Dc protein assay). Equal amounts of protein (50 μg) were loaded and subjected to electrophoresis on an SDS-PAGE gel. Primary antibodies were: rabbit polyclonal anti-HSP 27 (R&D System), rabbit polyclonal anti-HSP 72 (Assay Designs, Inc.), rabbit polyclonal anti-TNF- α (Millipore Inc), and rabbit polyclonal anti-IL-1 β (Abcam). Incubation with secondary antibodies (Santa Cruz Biotechnology) was done for 1 hour at room temperature. Immunoblots were then probed with an ECL Plus chemiluminescence reagent kit (Amersham Biosciences, Arlington Heights, IL) and visualized with an imaging system (Bio-Rad, Versa Doc, model 4000). Data was analyzed using Quantity One 4.6.1 software (Bio-Rad).

Statistical Analysis

Quantitative data were expressed as mean $\pm$ SEM. Statistical significance was verified by analysis of variance/ANOVA. Significance was accepted at $p < 0.05$.

RESULTS

High-dose Geldanamycin Attenuates Brain Edema

At 24 hrs post-ICH, edema was significantly increased in the ipsilateral basal ganglia (Fig. 1A). Treatment resulted in a dose-dependent effect where low-dose geldanamycin had no effect on improving edema, while high-dose geldanamycin resulted in a marked decrease in brain edema as compared to vehicle. At 72 hrs post-ICH, edema had progressed to bilateral basal ganglia as well as the ipsilateral cortex (Fig. 1B). High-dose geldanamycin reduced water content significantly in all these regions (Fig. 1B). Since there was no statistical difference between the brain water content of naïve (non-operated animals) and sham-operated animals at the 24 hour point, we did not expect the development of brain edema at a latter time-point and therefore used sham-operated animals in both studies.

High-dose Geldanamycin Preserves BBB Integrity

Although no differences were observed between groups at 24 hours post-ICH in any brain region (Fig. 2A), at 72 hours post-ICH mice treated with high-dose geldanamycin showed a significant decrease in BBB permeability in the ipsilateral hemisphere as compared to their vehicle-administered counterparts (Fig. 2B). Because there was no observed difference in Evans Blue dye extravasation between the right and left hemispheres and cerebellum of sham operated animals at 24 hours, we used these animals in both the 24 hour and 72 hour segments of our study.

High-dose Geldanamycin Improves Neurological Function

Neurological deficits 24 hrs post-ICH were evident as tested by the modified Garcia test, and neither low- or high-dose geldanamycin was able to attenuate these ICH-induced effects (Fig. 3A). However, 72 hours post-ICH, high-dose treatment significantly improved neurological parameters assessed by the modified Garcia test (Fig. 3B). Again neither treatment dosages were able to ameliorate the ICH-induced neurological deficits assessed by the wire hanging and beam balance tests at 24 hrs post-ICH (Fig. 4A); while at 72 hours post-ICH high-dose geldanamycin improved scoring on the wire hanging test (Fig. 4B).

Effect of Geldanamycin on HSP Expression

Although neither low nor high doses of geldanamycin enhanced expression of HSP 27 (Fig. 5A), high-dose treatment resulted in a significant increase in HSP 72 expression in the ipsilateral hemisphere at 24 (Fig. 5B) and ipsilateral and contralateral hemisphere 72 hours (Fig. 6A) post-ICH. There was a strong tendency of increased HSP 72 in the contralateral hemisphere at 24 hours, however it did not reach significance. There was no noted increase of HSPs in naïve (non-operated animals) at 24 hours, so this was not tested. Based on this, we did not test naïve animals at the 72 hour time point.

Effect of Geldanamycin on Inflammation

High-dose geldanamycin significantly attenuated the ICH-induced increase of TNF- α in the ipsilateral hemisphere (Fig. 6B). No differences were observed between groups in relation to IL-1b expression (Fig. 6C).

DISCUSSION

In the present study, we tested the dose-dependent effect of geldanamycin on reducing the detrimental consequences of ICH. We showed geldanamycin-limited BBB disruption, edema formation, and neurological deficits after an ICH-induced injury in mice. Our results suggest that these protective effects are mediated by geldanamycin-induced upregulation of HSP 72 and subsequent reduction of the pro-inflammatory cytokine, TNF- α .

The neuroprotective effects of geldanamycin have been studied in a variety of experimental animal models (Lu et al. 2002, Kwon et al. 2008, Kiang et al. 2006). In our model, we demonstrated that geldanamycin at high concentrations decreased the ICH-induced brain damage as assessed by edema and BBB permeability measurements. The reduction in edema and BBB permeability noted in the 72 hour post-ICH group correlated with improvements in functional outcome. Results in which no neuroprotection was demonstrated we regarded as indicative of sub-therapeutic dosing of geldanamycin. While the high-dosage groups in our study used notably higher concentration than in earlier studies (Lu et al. 2002, Kwon et al. 2008, Kiang et al. 2006, Kiang 2007), they were still significantly below the dosages demonstrated in previous literature to be toxic or lethal (Supko 1995). However, a major established side effect of geldanamycin is hepatotoxicity which is associated with fatigue. In the 24 hour study, the low performance scores of the treated group may have resulted from generalized discomfort from the geldanamycin itself, rather than from a lack of the desired beneficial effect. This is well in agreement with other reports, which demonstrated that HSP72 up-regulation can ameliorate the neurological deficits in the delayed but not acute phase (Sinn et al. 2007).

The neuroprotective effects afforded by the geldanamycin, an inducer of heat-shock proteins, appear to be mediated by HSP 72. HSP 72 is a major inducible stress protein long thought to contribute to cell survival following potentially lethal cellular stresses. In the central nervous system HSP72 can be expressed in neurons, glia and endothelial cells (van Noort 2008; Popp et al. 2009) in response to heat or a variety of other stresses (Yao et al. 2007). States et al. demonstrated that following middle artery occlusion, DNA fragmentation occurs less frequently in HSP 72 positive cells (States et al. 1996). Transgenic animals overexpressing HSP 72 showed fewer apoptotic cells in comparison with wild-type animals after undergoing permanent focal ischemia (Tsuchiya et al. 2003). In a model of neonatal hypoxia/ischemia, Matsumori et al. studied the activation of mitochondrial apoptotic pathways in mice overexpressing HSP 72. They showed that elevated constitutive expression of HSP 72 appears to protect the brain from hypoxia and ischemia in the neonatal period.

The protective effect of pharmacological HSP 72 upregulation in the ICH rat model has also been demonstrated. The authors demonstrated that a combination of pre- and post-treatments with geranylgeranylacetone (another inducer of HSP 72) significantly reduced changes in brain water content and neurologic deficits in animals following ICH (Sinn et al. 2007). However, they failed to demonstrate beneficial, clinically relevant effects of post-treatment on ICH-induced increases in brain water content. Sinn et al did not observe statistically significant up-regulation of HSP 72 or decreased of brain edema in their post-treatment groups. The effects on neurological deficits were not evaluated in post-treatment groups.

In our study, geldanamycin provided neuroprotection as a result of a decrease in the ICH-induced production of pro-inflammatory cytokines and ameliorated BBB-disruption. The effect of geldanamycin is linked to its ability to inhibit HSP 90 and to activate HSF 1 (Whitesell et al. 1994). Several publications demonstrated that HSF 1 activity is essential for production of stress-inducible HSPs in particular HSP 72 and HSP 27 which increase resistance against the pathophysiological challenges (Voellmy et al. 2007). Yan et al demonstrated that HSF 1 deficiency reduces expression of HSP 27 and HSP72, but not HSP60 and HSP90. Down regulation of HSPs alters cardiac redox homeostasis and increases mitochondrial oxidative damage (Yan et al. 2002). However, the arsenite-induced inhibition of HSF 1 drastically suppressed production of HSP 27 and 72 (Liu et al. 2010). The HSP 90 inhibitor, geldanamycin, or 17-Allylamino-demethoxygeldanamycin can increase production of inducible HSP 72 and HSP 27 and increase the cells stress resistance. (Ryhänen et al 2008; McCollum et al 2006). Moreover Kiang et al demonstrated that geldanamycin has no effect on the production of other HSPs such as HSP90, HSP60 and HSP 40 (Kiang et al 2006).

HSP 72 is known to affect proteins and genes involved in inflammation, which is involved with NFkB and its associated pathways. NFkB normally localizes to the cytosol bound to its associated inhibitory protein. Under stress, NFkB translocates to the nucleus. Many genes involved in inflammation, including iNOS, TNF- α and IL-1 undergo activation via these events. It has been demonstrated by Guzhova, I. V. et al. 1997 and others that increased levels of HSP 72 are associated with decreased nuclear NFkB translocation. Interaction of HSP 72 with NFkB decreases inflammatory cell generation via one or more cytokine mediators. Nitric oxide (Feinstein et al. 1996) and other inflammatory cytokines (Ding et al. 2001) have been found to be reduced in the presence of HSP 72. Inhibition of HSP 90 itself has been demonstrated to attenuate inflammation in a similar fashion in other models such as endotoxin-induced uveitis (Poulaki et al. 2007).

Our findings demonstrated that geldanamycin successfully increased expression of HSP 72 but had no effect on HSP 27 expression after ICH. This suggests that the neuroprotective effects observed in our study were related to HSP 72 expression which is consistent with the results of other groups studying HSP 72 (Whitesell 1994, Lu et al. 2002, Kwon et al. 2008).

Activation of the inflammatory cascade is a common finding in the ICH-induced brain. This inflammatory response is characterized by the infiltration of neutrophils and activation of microglia (Wasserman et al. 2008) – thus exacerbating BBB permeability and enhancing edema formation. Up-regulation of cytokines (TNF- α and/or IL-1b) after ICH (Wasserman et al. 2008, Wu et al. 2006, Zhang et al. 2006) can further propagate the inflammatory response and have been shown to correlate with severity of neurofunctional disability in stroke patients (Zaremba et al. 2001). In our study, administration of geldanamycin decreased ICH-induced production of TNF- α . Thus, the attenuation of BBB disruption, edema formation, and neurological dysfunction afforded by geldanamycin treatment may be mediated by HSP 72-induced reduction in TNF- α expression. However, the exact mechanism by which HSP 72 decreases TNF- α level in the hemorrhagic brain remains to be determined.

These results suggest that induction of HSP 72 by geldanamycin may provide major benefits for BBB integrity, edema formation, and neurobehavioral deficits after ICH. All together, these findings provide a basis for further investigation of the therapeutic effects of geldanamycin for ICH.

To test the importance of HSP72 induction for the protection observed here with geldanamycin the use of HSP72 knockouts would be informative (Wacker et al., 2009), while the use of TNFalpha knockout mice (described by Liu et al (Liu et al., 1998)) and TLR4 knockout mice (Chowdhury et al., 2006) would indicate whether these molecules are essential in the downstream pathway for protection by geldanamycin

High lights

Intracerebral hemorrhage is a deadly disease and without a cure.

Geldanamycin upregulated heat shock proteins 27 and 70 and provided neuroprotection in ischemic animal models.

This study investigated the effect of geldanamycin treatment on the development of secondary brain injury and neurological deficits in a mouse model of intracerebral hemorrhage.

geldanamycin significantly attenuated blood brain barrier disruption and brain edema after intracerebral hemorrhage and improved neurobehavioral functions.

Geldanamycin increased heat shock protein 72 expression in ipsilateral brain and reduced pro-inflammatory tumor necrosis factor-alpha.

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Figure 1. Geldanamycin reduced ICH-induced brain-edema in both the 24 hour (A) and 72 hour (B) study groups

A) 24 hour study: Brain edema in the ipsilateral basal ganglia was significantly higher among ICH groups as compared to sham-operated animals (* $p < 0.001$ vs sham). Geldanamycin (10 mg/kg) significantly reduced edema in the ipsilateral basal ganglia (# $p < 0.001$ vs vehicle). [sham = 7; ICH+vehicle = 6; ICH+Geldanamycin 1 mg/kg = 6; ICH+Geldanamycin 10mg/kg = 7]. B) 72 hour study: Brain edema was observed in vehicle mice compared to sham (* $p < 0.001$ vs sham). Geldanamycin (10 mg/kg) significantly reduced brain water content (# $p < 0.001$ vs vehicle). [sham = 7; ICH+vehicle = 6; ICH+Geldanamycin 10mg/kg = 6]

Figure 2. Geldanamycin failed to preserve the blood-brain barrier in the 24 hour study (A), but significantly reduced blood-brain-barrier disruption in the 72 hour study (B)
A) 24 hour study: No differences were found between groups. B) 72 hour study: Geldanamycin (10 mg/kg) significantly reduced blood-brain-barrier permeability in the ipsilateral hemisphere as compared to vehicle (* $p < 0.001$ vs vehicle). [ICH+vehicle = 6; ICH+Geldanamycin 10mg/kg = 6]

Figure 3. Geldanamycin at low and high concentrations failed to improve neurobehavioral deficits in the 24 hour study (A). Significant improvement was observed in the 72 hour study (B)

A) 24 hour study: Significant neurologic deficits were observed in all animals with collagenase-induced ICH. [sham = 7; Naïve+Geldanamycin 10mg/kg = 6; ICH+vehicle = 12; ICH +Geldanamycin 1mg/kg = 6; ICH+Geldanamycin 10mg/kg = 12]. B) 72 hour study: Geldanamycin (10 mg/kg) significantly improved neurological deficits as compared to vehicle (*p < 0.001 vs vehicle). [sham = 7; ICH+vehicle = 25; ICH+Geldanamycin 10mg/kg = 20]

Figure 4. In the 24 hour study, no improvement in neurological deficits were noted (A). High concentrations of geldanamycin significantly improved performance on the wire hanging test (B)
A) 24 hour study: The ICH+vehicle group performed significantly worse on the wire hanging and beam balance tests as compared to sham. Neither treatment dosages were able to attenuate these ICH-induced effects. [sham = 7; Naïve+Geldanamycin 10mg/kg = 6; ICH+vehicle = 12; ICH+Geldanamycin 1mg/kg = 6; ICH+Geldanamycin 10mg/kg = 12]. B) 72 hour study: The ICH+vehicle group performed significantly worse on the wire hanging and beam balance tests as compared to sham. Geldanamycin (10 mg/kg) significantly improved deficits associated with the wire hanging test (*p < 0.001 vs vehicle). [sham = 7; ICH+vehicle = 25; ICH +Geldanamycin 10mg/kg = 20]

Figure 5. High concentrations of geldanamycin increased production of HSP 72 (A) and decreased production of TNF-α (B) in the 72 hour study

A) 24 hour study: No differences in heat shock protein (HSP) 27 were found between groups.
 B) 24 hour study: Geldanamycin (10 mg/kg) significantly enhanced HSP 72 expression in the ipsilateral hemisphere after ICH (* $p < 0.001$ vs vehicle). [sham = 6; Naive+Geldanamycin 10mg/kg = 6; ICH+vehicle = 6; ICH+Geldanamycin 10mg/kg = 6]

Figure 6. Western Blot Protein Expression at 72 Hours

A) 72 hour study: Geldanamycin (10 mg/kg) significantly enhanced HSP 72 expression in the ipsilateral hemisphere after ICH (* $p < 0.001$ vs vehicle). B) 72 hour study: Geldanamycin (10 mg/kg) significantly attenuated the ICH-induced rise of TNF- α expression in the ipsilateral hemisphere. C) No differences in IL-1 β were found between groups (* $p < 0.001$ vs sham; # $p < 0.001$ vs vehicle). [sham = 6; ICH+vehicle = 6; ICH+Geldanamycin 10mg/kg = 6]