MCC950, the selective NLRP3 inflammasome inhibitor protects mice against traumatic brain injury

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Running title: MCC950 confers protection after traumatic brain injury

Number of Pages: 23 (including References); Number of Figures: 08

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Abstract

Nucleotide oligomerization domain (NOD)-like receptor protein-3 (NLRP3) inflammasome may intimately contribute to sustaining damage following traumatic brain injury (TBI). This study aims to examine whether specific modulation of NLRP3 inflammasome by MCC950, a novel selective NLRP3 inhibitor confers protection after experimental TBI. Unilateral cortical impact injury was induced in young adult C57BL/6 mice. MCC950 (50 mg/kg, i.p.) or saline was administration at 1 h and 3 h post TBI. Animals were tested for neurological function, and then sacrificed for edema at 24 or 72 h post TBI. Immunoblotting and histological analysis were performed to identify markers of NLRP3 inflammasome and pro-apoptotic activity in peri-contusional areas of the brains at 24 or 72 h post TBI. MCC950 treatment provided a significant improvement in neurological function and reduced cerebral edema in TBI animals. TBI upregulated NLRP3, apoptosis-associated speck-like adapter protein (ASC), cleaved caspase-1 and interleukin-1β (IL-1β) in perilesional area. MCC950 efficiently repressed caspase-1 and IL-1β with a transient effect on ASC and NLRP3 post TBI. MCC950 treatment also provided protection against pro-apoptotic activation of poly (ADP-ribose) polymerase (PARP) and caspase-3 associated with TBI. A concurrent inhibition of inflammasome priming was also detectable at NFκB-p65 and caspase-1 level. Our findings support the implication of NLRP3 inflammasome in the pathogenesis of TBI, further suggests the therapeutic potential of MCC950.

Keywords: Traumatic brain injury, Edema, neurological deficits, NLRP3-inflammasome inhibitor, MCC950, inflammation
Introduction

Traumatic brain injury (TBI) is a major cause of morbidity and mortality in young population in the United States contributing to around 35%–40% of all injury deaths, with falls and traffic crashes as the most common external causes.\(^1\)\(^2\) TBI therapies are utterly limited, as many promising compounds like progesterone analogues have failed in clinical trials due to intolerable side effects and therapeutic insufficiency.\(^3\)\(^4\) Current available therapies in TBI mainly focus on management of secondary complications as the primary necrotic insult is highly irreversible. As such, there is essential requirement to target the deleterious signals leading to programmed cell death and inflammation to rescue the imperiled CNS cells.\(^5\)\(^6\)\(^7\)

Pile of concrete evidences suggest that inflammatory responses are likely the prominent and early emerging pathological feature of TBI.\(^5\)\(^8\)\(^9\) Besides regional inflammatory responses in microglial and neural cells, the insult triggers an invasion of macrophages and neutrophils into the impact area escalating the tissue damage at least partly through pro-inflammatory cytokines. Over production of interleukin-1 \(\beta\) (IL-1\(\beta\)) is well documented, providing clear evidence for a pivotal role of this cytokine in TBI-associated inflammation.\(^9\)\(^10\)\(^11\)

IL-1\(\beta\) maturation and secretion to systemic circulation is predominantly regulated by a multiprotein complex the receptor nucleotide oligomerization domain (NOD)-like receptor protein (NLRP3) inflammasome, as a recently described mechanism to govern inflammatory responses during tissue injury.\(^12\)\(^13\) Mainly settled in immune cells, the NLRP3 inflammasome is composed of oligomers of NLRP3, apoptosis-associated speck-like (ASC) adapter protein and pro-caspase-1 to serve as a platform for caspase-1 activation and IL-1\(\beta\) maturation. The NLRP3 inflammasome assembly occurs following stimulation by either pathogen-associated molecular pattern (PAMPs) or danger-associated molecular patterns (DAMPs).\(^14\) According to recent reports TBI appears to coincide with ASC and NLRP3-inflammasome oligomerization inducing caspase-1 activation as well as IL-1\(\beta\) and IL-18 maturation.\(^15\)\(^16\) Such implication might come to high significance when NLRP3 has been proposed as a biomarker in cerebrospinal fluid in TBI patients with poor prognosis.\(^17\)
Despite all these emerging background, little has been examined in application of NLRP3 inhibitors in TBI. Applying general neuroprotective like omega-3 fatty acids or telmisartan in experimental TBI, with some earlier studies suggest the association with NLRP3 inhibition account for the therapeutic effects.\textsuperscript{16, 18, 19} Nevertheless generally acting as antioxidants or pleiotropic agents, implication of a wide array of effectors may not be ruled out for such multipotential compounds.\textsuperscript{20, 21}

Based on such background, we used the newly developed NLRP3 inhibitor MCC950, as a highly selective and potent inhibitor, to specify the involvement of NLRP3.\textsuperscript{22} Experimental studies have underlined the beneficial role of MCC950 treatment in several inflammatory disease models.\textsuperscript{22-24} The present study aims to specify whether selective NLRP3-inflammasome inhibition by MCC950 administration confers neuroprotection in experimental TBI. For the existing data addressing NLRP3 activation as early as 24 h following control cortical impact (CCI) model of experimental TBI, our experiments focus on acute phase of TBI injury as a window for high therapeutic potentials. Our findings support the contribution of NLRP3 inflammasome in TBI, provide evidences for specific NLRP3 inhibition as a promising therapeutic tool for TBI.

METHODS

\textbf{Animals and experimental groups}

Wild-type C57Bl/6 mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) were maintained in the animal facility in standard conditions at 12-h light/dark cycle and food and water \textit{ad libitum}. All animal care and manipulations were carried out according to the Institutional Animal Care and Use Committee (IACUC). Young adult animals (9–10 weeks) were quarantined and individually housed in cages for one week before being randomly assigned to experimental groups: sham, TBI and TBI + MCC950. Each group was divided to two sub-groups for acute and sub-acute assessment of neurological damage at 24 and 72 h after TBI. Following the neurological assessments all animals were euthanized at 24 h or 72h post TBI, and the brain samples were harvested according to the prerequisite procedure for immunoblotting or histological evaluations. MCC950 (Adipogen Life Science, Inc., Shanghai, China) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 mM and then diluted with saline to a concentration of 0.3 mg/kg for intraperitoneal (i.p.) injection.
MCC950, the selective NLRP3 inflammasome inhibitor protects mice against traumatic brain injury (DOI: 10.1089/neu.2017.5344)

This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

sciences, USA) was dissolved in sterile saline and dosed in animals (50 mg/kg, i.p.) at 1 h and 3 h post-TBI, based on published reports.22,24

**Induction of traumatic brain injury (TBI)**

C57Bl/6 mice were subjected to CCI or the sham surgery under isoflurane (2-3%) anesthesia. Briefly, mice were placed in a stereotaxic frame (Leica Biosystems Lab, USA) and a 3.5 mm craniotomy was made in the right parietal bone midway between bregma and lambda with the medial edge 12 mm lateral to the midline, leaving the dura intact. Mice were impacted at 4 m/s with a 20-ms dwell time and 2-mm depression using a 3-mm-diameter convex tip, mimicking a moderate TBI. Sham mice underwent the identical surgical procedures except the impaction. Body temperature was maintained at 37°C using a small animal temperature controller throughout all procedures. The incision was closed with VetBond tissue adhesive (1469SB; 3M animal care products, USA) and the animals were allowed to recover.

**Assessment of neurological severity score (NSS)**

Neurobehavioral assessment was performed at 24 and 72 h after TBI in a blinded fashion using a 10-point neurological severity score (NSS).25 One point was given for each failure to account for the cumulative score with a maximum of 10. The assessment consisted of 10 different task: (1) presence of mono- or hemiparesis, (2) Inability to walk on a 3-cm-wide beam, (3) Inability to walk on a 2-cm-wide beam, (4) Inability to walk on a 1-cm-wide beam, (5) Inability to balance on a 1-cm-wide beam, (6) Inability to balance on a round stick (0.5 cm diameter), (7) Failure to exit a 30-cm-diameter circle (for 2 min), (8) Inability to walk a straight line, (9) Loss of startle behavior and (10) Loss of seeking behavior.

**Assessment of cerebral edema**

Brain water content (BWC), a sensitive measure of cerebral edema, was quantified using the wet-dry method, as detailed by our group.26 BWC was estimated in 3 mm coronal sections of the ipsilateral cortex (or corresponding contralateral cortex), centered upon the impact site. Tissue was immediately weighed (wet weight), then dehydrated at 65°C. The sample was reweighed 48 h later to obtain a dry weight. The percentage of tissue
water content was calculated using the following formula: \[ \text{BWC} = \left( \frac{\text{wet weight} - \text{dried weight}}{\text{wet weight}} \right) \times 100\% . \]

**Western Blotting**

The peri-contusional cerebral cortex was microdissected from the brains harvested from euthanized animals and immediately frozen in liquid nitrogen. Tissue samples were homogenized in RIPA buffer containing protease inhibitor cocktail and processed for western blotting as previously described.\(^2\) Thirty-microgram of sample proteins were loaded and separated trough electrophoresis on a 4-20% polyacrylamide gel (Bio-Rad). Proteins were then transferred to PVDF membrane. The membranes were blocked for non-specific binding and probed with primary antibodies against NLRP3, caspase-1, ASC (1:1000; AG-20B-0014; AG-20B-0042; AG-25B-0006 Adipogen life sciences), IL-1β, caspase-3, p-Akt, Akt (1:000; CST-12242; 9664; 9271; 9272; Cell signaling technology), PARP (1:1000; ab32138; Abcam, USA), NFκB p65 (1:1000; Sc-8008; Santa Cruz Biotechnology) following TBS-T washes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000; Sigma). The bands were then visualized by means of a chemiluminescent substrate system (Thermo fisher scientific). The bands’ density were quantified using ImageJ (NIH) and normalized with the expression of beta actin as the housekeeping gene, and expressed as fold change.

**Immunofluorescence Staining**

Mice were anesthetized with ketamine/xylazine and transcardially perfused with PBS followed by 10% formalin (Fischer Scientific). Brains were removed and postfixed in the same fixative overnight at 4 °C and then with 30 % sucrose in PBS for 72 h. The sunk brains were frozen-sectioned in the coronal plane at a thickness of 10 μm. Sections were blocked with Serum-Free Protein Block (X0909, DAKO) and then incubated with primary antibodies against NLRP3, caspase-1 (1:200; 1: 250; Adipogen life sciences) and IL-1β (1:100; Cell signaling technology, USA) overnight at 4 °C in a humid chamber. After washing, slides were incubated with fluorescent anti mouse secondary antibodies (1:200; 072-04-18-03; Dylight-549, KPL) for 1 hour at room temperature and mounted with ProLong™ Diamond Antifade Mountant with DAPI (Invirogen), and viewed using a Zeiss 710 confocal laser.
scanning microscope. Negative controls were prepared by omitting the primary antibodies. Immunofluorescence intensities were quantified using ImageJ software (NIH) in five different fields per section digitized from the perilesional area using a ×40 objective lens.

**Statistical Analysis**

Sample size estimation: The sample size determinations were made at alpha=0.05 and data is reported as mean ±S.E. Based on IL-β expression value of 4.92±10% for saline TBI, and a reduction to 1.87±10% for mice treated with MCC950 + TBI, a sample size of 5/group was provided at least 90% power to detect this difference. One-way analysis of variance was performed to detect any difference in a variable in more than two groups. Tukey’s post-hoc was then used to specify the significant differences. 2-tailed Student t test was applied in cases the variable was compared in two groups like NSS comparison. Statistical significance was accepted at the 95% confidence level (P<0.05).

**RESULTS**

**Effect of MCC950 on neurological severity score (NSS) and brain water content (BWC) after TBI**

NSSs data were compared to track the effect of MCC950 on functional outcome at 24 and 72 h after TBI (Figure 1A). To ensure the normal neurological background, all included animals were tested for the 10-point NSS task before TBI induction and scored with zero. MCC950 treatment showed a trend toward the improvement in the NSS but significant (p < 0.05) improvement was found only at 72 h compared to the saline treated group.

Furthermore, MCC950 affects TBI-induced cerebral edema induced by TBI as assessed by BWC. As shown in Figure 1B, TBI mice treated with MCC950 showed a trend to decrease in BWC (78.04 ±2.03) compared to saline-treated (80.98 ±0.64) counterparts at 24 h after TBI, however the difference did not achieve statistical significance.

**MCC950 attenuates inflammasome activation (NLRP-3, cleaved caspase-1, ASC and IL-1β) after TBI**

According to the illustrated data in figure 2 (A-E), our immunoblot analysis showed the expression the principal constituents of NLRP3 inflammasome including ASC and NLRP3 as
well as their presumptive downstream molecules cleaved caspase-1 and IL-1β demonstrate significant over-expression in samples from peri-contusional area after 24 h and 72 h of TBI. Such augmentations were significantly reversed at 24 h after TBI in MCC950 treated brains.

To confirm the localized NLRP3 inflammasome activation, the TBI affected tissues were probed to detect positive cells for NLRP3, caspase-1 and IL-β. As shown in figure 3 representative sections and the following quantification of the fluorescent signal, the obvious augmentations in cytosolic caspase 1 and IL-1β were remarkably reduced in corresponding samples from MCC950 animals either 24 h or 72 h post injury.

**The inhibitory effect of MCC950 coincides with minute changes in TXNIP and pAkt expression after TBI**

Along our main investigations on MCC950 effects on TBI we tested the probable involvement of thioredoxin interacting protein (TXNIP) as a pivotal regulator of NLRP3 (Figure 4). For the existing evidences for TXNIP modulation by Akt signaling, we also estimated Akt activation through the phosphorylated Akt (pAkt)/Akt ratio (Figure 5). According to our immunoblot analysis TBI was associated with significant (p < 0.05) increase in TXNIP expression and Akt activation as determined by pAkt/Akt ratio. MCC950 did not discernibly alter TXNIP level as well as Akt activity at 24 h after TBI.

**MCC950 treatment represses TNF-α and NFkB-p65 after TBI**

We further elucidated the effect of MCC950 on TNF-α and expression as an immediate pleiotropic response to TBI and NFk-B-p65 level (Figure 6A and 6B), the transcriptional regulator of inflammatory genes. According to our densitometry analysis the very low levels of TNF-α expression in the sham brains turned to pretty high levels in TBI group (p<0.05), and were marginally reversed in MCC950-treated TBI animals. The total NFk-B-p65 also increased in whole cell lysate in TBI brains. MCC950 induced NFk-B-p65 repression was rather tangible than TNF-α.
MCC950 attenuates activation of caspase-3 and PARP after TBI

To examine the effect of MCC950 on pro-apoptotic markers, activation of caspase-3 and PARP was further examined at 24 h after TBI (Figure 7A-C). The expression of cleaved PARP and caspase-3 were significantly increased in TBI mice compared to shams (P<0.01). According to our densitometry analysis in TBI brains, part of this effect is attributed to enhanced levels of the pro molecule caspase 3 but less likely to PARP. MCC950-treated brain samples demonstrated a remarkable reduction in PARP and caspase-3 cleavage (P<0.01). The discernible un-cleaved caspase-3 overexpression in TBI animals was also slightly attenuated by MCC950 treatment.

DISCUSSION

The sufonylurea derivative MCC950 is amongst the recently described small molecule NLRP3 inhibitors developed in effort to produce safe specific leading compound.\textsuperscript{27} It has been considered in several studies for searching potential therapeutic advantages. In different disease models, MCC950 has provided conspicuous protection either in CNS disease models i.e. Alzheimer\textsuperscript{28} or systemic disorders like diabetic vascular dysfunction.\textsuperscript{29-32} Here we specified that MCC950 could also alleviate TBI and the consequent pro-inflammatory and in particular pro-apoptotic signals in perilesional area.

There is pile of solid evidence highlighting the role of NLRP3 inflammasome in pathology of CNS disorders commonly involve neuroinflammation. In this connection, NLRP3 ablation has been shown to ameliorate experimental Alzheimer’s disease (AD), stroke, Huntington’s disease and Pneumococcal meningitis.\textsuperscript{33-35} Our histological evaluations affirm TBI instigated activation of NLRP3 inflammasome in peri-lesional area as evident by the increased protein expression of NLRP3, ASC, cleaved caspase-1 and IL-1β. Noteworthy, number of neuroprotective chemicals (eg. propofol) and natural compounds (eg. mangiferin) have been shown to provide remarkable NLRP3 repression which is claimed to explain their therapeutic advantages in TBI.\textsuperscript{16, 18, 19, 36} To our knowledge MCC950 as the most specifically developed pharmacological tool to accurately address such contribution. The neurological evaluation of MCC950 treated animals showed a promising improvement. This alleviating effects turned to significant values following 72 h, probably when the
effects of molecular events come to affect the CNS function. Edema leading to an increase of brain water content has a crucial impact on morbidity and mortality following TBI as it increases intracranial pressure, and contributes to additional secondary injuries. We found that treatment with MCC950 results in a trend toward decrease in edema in terms of BWC, as expected, but it did not achieve statistical significance. Furthermore, we demonstrated TBI induces NLRP3 inflammasome activation as evident by increased protein expression of NLRP3, ASC, caspase-1, cleaved caspase-1 and mature IL-1β. Inhibition of NLRP3 inflammasome with specific small molecule inhibitor, MCC950, attenuated the corresponding aggravating upregulations in TBI animals. As spotted with either immunoblots or immunostained sections, MCC950 treatment demonstrated a differential impact in acute and sub-acute phases post TBI. That is MCC950 repressed all the mentioned inflammasome markers in 24 h post injury, with exceptional effects on caspase-1 and IL-1β maturation persisting till 72 h following TBI. Conspicuously, this persisting attenuation of caspase-1 activation and subsequent IL-1β repression parallels with the significant NSS improvement in MCC950 treated TBI animals, a noteworthy observation that may underline the central place of IL-1β in TBI pathology and MCC950 neuroprotection. As a proinflammatory cytokine, IL-1β plays a pivotal role in acute neuronal injury and its blockage has been associated with better prognosis in various clinical conditions. Clinical studies supports the existence of correlation between IL-1β level and extent of tissue damage following TBI.

The effect of MCC950 on in TBI-associated inflammation was further evaluated through estimating TNF-α and NFk-B-p65 expression. TBI induced marked expression of TNF-α and NFk-B-p65 subunit in perilesional area. MCC950 treatment was partially efficient to prevent NFk-B-p65 upregulation in TBI animals. Even minimal changes in NFk-B-p65 might be meaningfully valued in two views. First, it has been shown to be involved in the pathogenesis of TBI. Secondly, NFk-B-p65 is a well-established priming signals for NLRP3 inflammasomes. Transcriptional priming of inflammasome constituents like caspase-1 is an essential prerequisite step for further NLRP3 inflammasome activation and subsequent IL-1β maturation. Therefore the associated NFk-B-p65 alterations may provide mechanistic views on MCC950 mode of action in TBI animal. Based on
immunoblots analysis at 24 h, TBI induced remarkable overexpression of caspase-1 (supplementary figure 1) and caspase-3 as a consequent of the priming signal mediated by NFκB-p65. Interestingly, MCC950 associated NFκB-p65 repression was associated with reduced un-cleaved forms of caspase-1 and caspase-3 indicating MCC950 is also efficient to block the priming step besides its well-characterized inhibitory effect on NLRP3 inflammasome activation and caspase-1 cleavage.

Programmed cell death namely pyroptosis and apoptosis are critical targets in TBI as they lead to irreversible ultimate injury in salvageable part of the TBI brain. Pyroptosis could be recognized from apoptosis as it is triggered by caspase 1 and is associated with the release of pro-inflammatory cytokines IL-1β and IL-18. Typically NLRP3 co-localization with caspase-1 is an indicator for pyroptosis. Accordingly the alleviating effects MCC950 in our TBI animals might be somehow linked to attenuated pyroptosis.

Another factor intimately contribute to the pathology of TBI is the apoptosis of neurons and glia. Our TBI brains showed increased protein expression of the pro-apoptotic molecule caspase-3 but not that of PARP at 24 h post TBI. Alternatively, the cleavage activation of both molecules was augmented in TBI brains and were strongly prevented by MCC950 treatment which is highly suggestive of protective effects against apoptotic cell death in TBI perilesional area.

To provide more details about NLRP3 regulation along with MCC950 treatment we were also interested to test the corresponding changes in TXNIP and Akt activation. Extensively studied for contribution to insulin resistance and oxidative stress, TXNIP directly associates with NLRP3 and induce inflammasome oligomerization and activation and modulate the expression of various cytokines and pro-apoptotic signals. TXNIP has been also found be upregulated in response to brain injury coincident with inflammasome activation. Presumptively this takes place downstream to ROS release which in turn promotes TXNIP expression trough transcription factor Forkhead box O3 (FoxO) in TBI brains. Based on our immunoblot analysis TBI induced a significant increase in TXNIP protein, which was slightly modulated by MCC950 treatment.
The serine-threonine kinase, Akt is implicated in neural survival in acute TBI\textsuperscript{50, 51} and may partially demonstrate MCC950 therapeutic effects on TBI animals.\textsuperscript{52, 53} However Akt phosphorylation following TBI strictly vary in different cellular compartments. Akt with the very vast effects, may also independently affect TXNIP transcription and thus subsequent NLRP3 activation. In fact phosphorylation of FoxOs by activated Akt may inhibit the transcriptional functions of FoxOs and thus interrupt ROS-induced TXNIP expression.\textsuperscript{54} However our experiments may not specify such a role for Akt, further investigations are needed to clarify probable involvement of TXNIP and presumptive regulatory effect of Akt signaling.

This “proof of concept study” aims to obtain preclinical information to address whether acute treatment with MCC950, a specific NLRP3 inflammasome inhibitor would improve TBI outcomes. This is the first report indicating acute MCC950 dosing confers beneficial effects in mouse model of TBI. However, longer term studies with multiple days/doses will be needed to assess the actual impact of the manipulation of this promising small molecule NLRP3 inhibitor. One might expect the impact to be even greater at later time points, where inflammation and apoptosis are even more important contributors to the ultimate secondary damage after TBI. Furthermore, although unilateral cortical impact injury is a widely used pre-clinical model, given the prevalence of particular TBI forms like combat blast injury model, may improve translational value of pre-clinical studies on MCC950.

Inflammation is a pivotal degenerating signal persisting in white matter for years in patients with traumatic brain injury.\textsuperscript{55} Despite several evidence supporting detrimental impact neuroinflammation and edema in TBI patients, no anti-inflammatory drug has been yet admitted to clinical practice. Even corticosteroids widely used as unique pluripotent immune-suppressive agents TBI patients \textsuperscript{56}, has failed to prove beneficiary rather than harmful effects in large clinical trials.\textsuperscript{57, 58} The prevalence of infections is another concern in chronic administration of immunosuppressive in TBI patients.\textsuperscript{59} As such, specific targeting of major pro-inflammatory signals may provide efficient and safe medications.
Laboratory studies implicate IL-1β in the pathophysiology of TBI, supporting investigations on NLRP3 inflammasome inhibitors in preclinical studies. The present study provides evidences implicative of particular place of NLRP3 inflammasome in TBI and the therapeutic potential of MCC950 (Figure 8). After further examinations MCC950 as a selective potent NLRP3 inhibitor might represent a promising candidate for clinical trials for its suitable bioavailability and pharmacokinetic properties.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Health [R01-NS097800 (TI)]. The authors are thankful to Dr. Anton J. Reiner, for providing controlled cortical impact (CCI) device (Impact One, Leica Biosystems, Buffalo Grove, IL 60089 US) to induce TBI in mice.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.
References


Legends

Figure 1: Effect of MCC950 on neurological severity score and edema following TBI. (A) C57B1/6 mice were subjected to a ten point neurobehavioral assessment in 24 h and 72 h after TBI. Animals treated with MCC950 remarkably showed ameliorated TBI associated neurological impairment. (B) Edema was assessed at 24 h by measuring brain water content. Administration of MCC950 caused a trend toward the decrease in the edema compared to saline treated group, but it was not statistically significant. Values are expressed as mean ± SEM (n=12), # p <0.05 vs corresponding TBI.
Figure 2: Effect of MCC950 on expression of principal constituents of acute NLRP3 inflammasome activation at 24 h and 72h following TBI. (A) Representative and quantitative analysis of (B) NLRP3, (C) caspase/ cleaved caspase-1, (D) ASC and (E) IL-1β expression demonstrate the corresponding differences in peri-contusional cerebral cortex at 24 h and 72 h after TBI. MCC950 treatment post injury reduced levels of NLRP3 and the subsequent caspase-1 and IL-1β maturation. Values are expressed as mean ± SEM (n=8). * p <0.05 vs corresponding Sham, **p <0.01 vs corresponding Sham # p < 0.05 vs corresponding TBI, ## p < 0.01 vs corresponding TBI.
Figure 3: Effect of MCC950 on immunohistochemical analysis of principal markers of acute NLRP3 inflammasome at 24 and 72 h following TBI. Expression of NLRP3, Caspase 1 and IL-1β were evaluated in peri-contusional cerebral cortex at 24 h (A) and 72 h (C) after TBI as an index for acute NLRP3 activation. Fluorescent intensity was calculated and represented by bar graph (B) and (D). According to the representative probed sections mice treated with MCC950 remarkably reduce TBI-associated NLRP3 inflammasome stimulation. Blue staining: CNS cells nuclei, Red staining: probing of the corresponding primary antibodies (n=8, scale bar = 20 μm). Values are expressed as mean ± SEM (n=8). * p <0.05 vs corresponding Sham, **p <0.01 vs corresponding Sham # p < 0.05 vs corresponding TBI, ## p < 0.01 vs corresponding TBI.
MCC950, the selective NLRP3 inflammasome inhibitor protects mice against traumatic brain injury (DOI: 10.1089/neu.2017.5344)

Figure 4: Effect of MCC950 on TXNIP expression at 24 h following TBI. Representative and quantitative analysis of Western blots showed that TXNIP expression increase significantly at 24 h in saline treated TBI mice compared to shams. MCC950 treatment at post injury induced a minimal decrease in TXNIP levels. Values are expressed as mean ± SEM (n=8). * p < 0.05 vs Sham.
Figure 5: Effect of MCC950 on Akt phosphorylation at 24 h following TBI. Representative and quantitative analysis showed significant elevation in Akt activity as determined by pAkt/Akt ratio in TBI saline treated and MCC950 treated animals compared to shams. Akt activation was not significantly increased with MCC950 treatment compared to saline TBI only. Values are expressed as mean ± SEM (n=8). * p < 0.05 vs Sham.
Figure 6: Effect of MCC950 on inflammation (TNF-α and NFkB-p65) at 24 h following TBI.

Representative and quantitative analysis of Western blots showed significant increase in TNF-α (A) and NFkB-p65 (B) expression at 24 h following TBI compared to Shams. MCC950 treatment showed a trend toward decrease in NFkB-p65 level. Values are expressed as mean ± SEM (n=8). *p < 0.05 vs Sham.
Figure 7: Effect of MCC950 on expression of pro-apoptotic markers at 24 h following TBI.

Representative and quantitative analysis of (A) Caspase-3 and cleaved caspase-3 as well as (B) PARP and cleaved PARP immunoblots illustrate significant activation at 24 h following TBI. MCC950 treatment significantly attenuated Cleavage of both pro-apoptotic markers. Values are expressed as mean ± SEM (n=8). * p < 0.05 vs Sham, ** p < 0.01 vs Sham, ## p < 0.01 vs TBI.
Figure 8. Pictorial description of MCC950 therapeutic effects on experimental TBI. In a simplified look, TBI instigate several deteriorating signals i.e. through ROS or DAMP stimulation interfacing with the protective effectors like Akt phosphorylation. The damage signals are sensed by NLRP3 to recruit ASC to NRPL3 inflammasome assembly. TXNIP interacts with NLRP3 inflammasome to augment its cleavage activity on caspase-1 precursor leading to caspase-1 and the subsequent IL-1β maturation. IL-1β may trigger a
wide range of inflammatory reactions which parallels NF-κB transcriptional activity and TNF-α over production. Apoptotic cell death involving caspase-3 and PARP follows the inflammatory propagation. According to our findings MCC950 mitigates TBI-induced neuroinflammation and associated apoptotic cascades as determined by the red arrows with significant changes detected for the shaded ones. Such ameliorating effect of MCC950 is mainly ascribed to specific inhibition of NLRP3 oligomerization and subsequent IL-1β maturation. Through a likely feedback mechanism, MCC950 may rescue Akt survival activity from deteriorating TBI outcomes. Abbreviations: TBI; traumatic brain injury; ROS: reactive oxygen species; TXNIP: thioredoxin interacting protein; NLRP3: NOD-like receptor protein-3; IL-1β: Interleukin 1 beta; TNF-α: Tumor necrosis factor alpha; p65: NF-κB-p65 subunit.
Figure 1: Effect of MCC950 on expression of Caspase-1 at 24 h and 72 h following TBI. Representative (A) and quantitative analysis of (B) Caspase-1 expression demonstrate the corresponding differences in peri-contusional cerebral cortex at 24 h and 72 h after TBI. MCC950 treatment post injury reduced expression of Caspase-1. Values are expressed as mean ± SEM (n=8). **p < 0.01 vs corresponding Sham # p < 0.05 vs corresponding TBI.
Figure 2: Effect of MCC950 on expression of principal constituents of acute NLRP3 inflammasome activation at 24 h and 72h following TBI (A) NLRP3 (B) and caspase-1

Sham: 1
TBI (24 hour): 2
TBI (24 hour)+MCC950: 3
TBI (72 hour): 4
TBI(72hour)+MCC950: 5

The representative images used in manuscript are highlighted with box.
Figure 3: Effect of MCC950 on expression of principal constituents of acute NLRP3 inflammasome activation at 24 h and 72h following TBI (A) ASC (B) and IL-1β

- Sham 1
- TBI (24 hour): 2
- TBI (24 hour)+MCC950: 3
- TBI (72 hour): 4
- TBI(72 hour)+MCC950: 5

The representative images used in manuscript are highlighted with box.
MCC950, the selective NLRP3 inflammasome inhibitor protects mice against traumatic brain injury (DOI: 10.1089/neu.2017.5344)

This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

[Image of a gel electrophoresis result showing protein bands for different conditions and time points.]

The following figure shows the protein expression levels of the NLRP3 inflammasome components in hippocampal tissue at different time points after traumatic brain injury (TBI). The figure compares control (C) and treatment groups (T) over time. The bands at different molecular weights represent the levels of the proteins of interest. The results suggest a significant reduction in the expression of NLRP3 and inflammasome components in the treatment group compared to the control group, indicating a potential protective effect of MCC950 against TBI-induced inflammation.
Figure 5: Effect of MCC950 on TXNIP expression at 24 h following TBI.
Sham: 1
TBI (24 hour): 2
TBI (24 hour)+MCC950: 3
The representative images used in manuscript are highlighted with box.
Figure 6: Effect of MCC950 on pAkt/Akt expression at 24 h following TBI.
TBI (24 hour): 2
TBI (24 hour)+MCC950 : 3
The representative images used in manuscript are highlighted with box.
Figure 7: Effect of MCC950 on inflammation (A) TNF-α and (B) NFkB-p65 at 24 h following TBI.
Sham: 1
TBI (24 hour): 2
TBI (24 hour)+MCC950 : 3
The representative images used in manuscript are highlighted with box.
Figure 8: Effect of MCC950 on expression of PARP at 24 h following TBL.

Sham: 1
TBI (24 hours): 2
TBI (24 hours)+MCC950: 3

The representative images used in manuscript are highlighted with box.
Figure 9: Effect of MCC950 on expression of Caspase-3 at 24 h following TBI.

Sham: 1
TBI (24 hour): 2
TBI (24 hour)-MCC950 : 3

The representative images used in manuscript are highlighted with box.