Published in final edited form as:

Brain Res. 2011 June 13; 1395: 62–73. doi:10.1016/j.brainres.2011.04.022.

Post-injury Administration of NAAG Peptidase Inhibitor Prodrug, PGI-02776, in Experimental TBI

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Abstract

Traumatic brain injury (TBI) leads to a rapid and excessive increase in glutamate concentration in the extracellular milieu, which is strongly associated with excitotoxicity and neuronal degeneration. *N*-acetylaspartylglutamate (NAAG), a prevalent peptide neurotransmitter in the vertebrate nervous system, is released along with glutamate and suppresses glutamate release by actions at pre-synaptic metabotropic glutamate autoreceptors. Extracellular NAAG is hydrolyzed to *N*-acetylaspartate and glutamate by peptidase activity. In the present study PGI-02776, a newly designed di-ester prodrug of the urea-based NAAG peptidase inhibitor ZJ-43, was tested for neuroprotective potential when administered intraperitoneal 30 min after lateral fluid percussion TBI in the rat. Stereological quantification of hippocampal CA2-3 degenerating neurons at 24 hrs post injury revealed that 10 mg/kg PGI-02776 significantly decreased the number of degenerating neurons ($p < 0.05$). Both average latency analysis of Morris water maze performance as well as assessment of 24-hour memory retention revealed significant differences between sham-TBI and TBI-saline. In contrast, no significant difference was found between sham-TBI and PGI-02776 treated groups in either analysis indicating an improvement in cognitive performance with PGI-02776 treatment. Histological analysis on day 16 post-injury revealed significant cell death in injured animals regardless of treatment. In vitro NAAG peptidase inhibition studies demonstrated that the parent compound (ZJ-43) exhibited potent inhibitory activity while the mono-ester (PGI-02749) and di-ester (PGI-02776) prodrug compounds exhibited moderate and weak levels of inhibitory activity, respectively. Pharmacokinetic assays in uninjured animals found that the di-ester (PGI-02776) crossed the blood-brain barrier. PGI-02776 was also readily hydrolyzed to both the mono-ester (PGI-02749) and the parent compound (ZJ-43) in both blood and brain. Overall,

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these findings suggest that post-injury treatment with the ZJ-43 prodrug PGI-02776 reduces both acute neuronal pathology and longer term cognitive deficits associated with TBI.

Keywords

Traumatic brain injury (TBI); Glutamate; *N*-acetylaspartylglutamate (NAAG); Hippocampus; Morris water maze

1. Introduction

Traumatic brain injury (TBI) remains one of the leading causes of death and disability globally. In the United States, an estimated 1.7 million persons sustain TBI resulting in 275,000 hospitalizations and 52,000 deaths each year (Faul et al., 2010). However, no truly efficacious and approved therapies are currently available for the treatment of TBI. Glutamate, the principal excitatory neurotransmitter in the central nervous system (CNS), is one of the most common targets for drug therapy following TBI as excessive glutamate release leads to neurotoxicity (Meldrum, 2000). Extracellular glutamate is elevated immediately after TBI, causing excitotoxic damage to neurons through excessive activation of AMPA- and NMDA-type glutamate receptors (Faden et al., 1989; Globus et al., 1995; Katayama et al., 1990). Pharmacological blockade of these receptors has led to reductions in neurotoxicity and improvements in behavioral outcome (Faden et al., 1989; Hayes et al., 1988; Kawamata et al., 1992), but the translation of this strategy into clinical application remains overwhelmingly disappointing (Bullock et al., 1999; Narayan et al., 2002).

An emerging alternative strategy for reducing glutamate excitotoxicity is through pharmacological inhibition of glutamate carboxypeptidase II (GCP II) which hydrolyzes *N*-acetylaspartylglutamate (NAAG). NAAG is an abundant peptide neurotransmitter found in millimolar concentrations in the mammalian brain and is co-distributed with small amine transmitters including glutamate and GABA (Coyle, 1997; Neale et al., 2000) and selectively activates the group II metabotropic glutamate receptor subtype 3 (mGluR3) (Neale et al., 2000; Schweitzer et al., 2000; Wroblewska et al., 1997; Wroblewska et al., 1998; Wroblewska et al., 2006). During intense neuronal stimulation the peptide neurotransmitter NAAG is released into the synapse where it activates presynaptic mGluR3 and thereby modulates (reduces) further synaptic release of glutamate (Sanabria et al., 2004; Xi et al., 2002; Zhao et al., 2001; Zhong et al., 2006) therefore creating a negative feedback loop. Synaptically released NAAG is hydrolyzed to NAA and glutamate by the NAAG peptidase catalytic enzymes, GCP II and GCP III (Bzdega et al., 2004; Luthi-Carter et al., 1998). Thus, any neuroprotective potential of NAAG to reduce excitotoxicity following TBI is likely to be short-lived as NAAG is rapidly inactivated in the synapse by this peptidase activity.

A series of in vitro and in vivo studies demonstrated that inhibition of GCP II and GCP III increases the extracellular levels of NAAG neuropeptide, reduces glutamate release, and moderates glutamate related pathologies in animal models of several human disorders (Neale et al., 2005; Tsukamoto et al., 2007). For example, NAAG peptidase inhibitors provide neuroprotection in models of excitotoxicity including ischemic-hypoxic damage, diabetic neuropathy and glutamate-induced motor neuron death (Ghadge et al., 2003; Slusher et al., 1999; Zhang et al., 2006). These data support the hypothesis that NAAG peptidase inhibitors will augment an endogenous protective mechanism to reduce excitotoxicity and thus, may represent a potentially important therapeutic approach to attenuate TBI-induced glutamate-mediated excitotoxicity.

In the present study, PGI-02776, a newly designed di-ester prodrug of the urea-based NAAG peptidase inhibitor ZJ-43, was tested for neuroprotective potential when administered systemically 30 min after fluid percussion TBI in the rat. PGI-02776 was also tested for brain penetration in uninjured animals following systemic administration and for inhibition of NAAG peptidase activity with an in vitro assay. Acute hippocampal neuronal loss, long-term hippocampal neuronal survival, and cognitive deficits were assessed following fluid percussion TBI in rats to determine the efficacy of PGI-02776 as a potential treatment for TBI.

2. Results

2.1 NAAG Peptidase (GCP II) Inhibition in vitro Assay

This experiment compared the efficacy of PGI-02776 (di-ester), PGI02749 (mono-ester), and ZJ-43 (parent compound) as inhibitors of the NAAG peptidase, GCP II, using an in vitro Chinese hamster ovary (CHO) cell membrane assay. The di-ester PGI-02776 had weak inhibitory actions on GCP II activity with an inconsistent dose response (1 nM, $p < 0.05$; 10 nM–10 μ M, not significant; 100 μ M, $p < 0.01$). The mono-ester PGI-02749 inhibited the peptidase at 1 nM ($p < 0.05$), 10 μ M ($p < 0.01$), and 100 μ M ($p < 0.001$). In contrast, the parent compound, ZJ-43, inhibited the enzyme activity at concentrations of 10 nM through 100 μ M ($p < 0.001$) (Fig. 1).

2.2 Pharmacokinetics

These experiments assessed brain penetration of ZJ-43 and PGI-02776 following intraperitoneal (i.p.) administration as well as the conversion of the di-ester (PGI-02776) to the mono-ester (PGI-02749) and to the parent compound (ZJ-43). Measurements were made in blood and brain homogenates from uninjured mice.

Following injection of the parent compound (ZJ-43, 100 mg/kg, i.p.), blood and brain levels of ZJ-43 were rapidly elevated reaching maximal values in blood at 15 min and in brain at 30 min (Fig. 2A,B).

Measurements of the di-ester (PGI-02776) and the parent compound (ZJ-43) were made in the blood and brain following injection of the di-ester, PGI-02776 (100 mg/kg, i.p.), in uninjured mice. Blood and brain levels of the di-ester, PGI-02776 were maximal at 30 and 60 min, respectively (Fig. 2C,D). The maximum elevation in blood of the mono-ester, PGI-02749 occurred at 4 hrs (Fig. 2E). Brain levels of the mono-ester, PGI-02749, were detected from 2–6 hrs post administration (Fig. 2F). The maximum elevation in blood of the parent compound, ZJ-43, also occurred at 4 hrs (Fig. 2G). Brain levels of the parent compound were detected from 4–8 hrs post administration (Fig. 2H).

2.3 Therapeutic Evaluation Experiments

2.3.1 Acute histology—This experiment was designed to evaluate a dose-response effect of PGI-02776 administered 30 min post-injury on TBI-induced neuronal degeneration measured at 24 hrs post-injury. There were no significant differences between groups in pre-TBI body weight, injury magnitude, righting time, temporalis muscle temperature, or rectal temperature values (Table 1).

Neuronal degeneration was detected with Fluoro Jade-B (FJ-B) at 24 hrs after TBI and quantified in the stratum pyramidale of the ipsilateral CA2-3 region. Pyramidal cells staining positive for FJ-B fluoresced brightly with prominent somas and extensive dendritic arborization into the stratum radiatum (Figs. 3A,B). The number of degenerating neurons in the ipsilateral hippocampus was significantly different between groups [$F(3,41) = 3.098$,

$p < 0.05$]. Post hoc Dunnett test revealed that compared to the TBI+saline control group there was a near significant effect of the 1 mg/kg dose ($p = 0.053$), a significant reduction of degeneration neurons in the 10 mg/kg dose of PGI-02776 treated group ($p = 0.035$), and no change in the 50 mg/kg dose PGI-02776 group ($P > 0.40$). Compared to the TBI+saline control, the middle dose (10 mg/kg) reduced acute neuronal degeneration by 32% (Fig. 3C). Visual inspection at 20X of all groups did not detect obvious FJ-B positive neurons in the hippocampus contralateral to the fluid percussion injury.

2.3.2 Chronic Behavior and Histology—This experiment evaluated the most effective dose of PGI-02776 determined from the acute histology experiment (10 mg/kg, i.p., 30 min post-injury) on Morris water maze (MWM) performance. Acquisition of spatial learning and memory retention was assessed on days 11–15 after injury by measuring time to find the hidden platform. At the conclusion of the MWM probe test on day 16 post-injury, cumulative cell survival in the CA2-3 in the dorsal hippocampus was quantified using stereological techniques. The mean righting time of sham-TBI rats was significantly less than TBI groups, as expected. Pre-TBI body weight, injury magnitude, righting time, temporalis muscle temperature, and rectal temperature values were not significantly different between groups (Table 1).

A repeated measure analysis of variance (ANOVA) revealed a significant main effect of group on latency to find the platform (Fig. 4A) [$F(2,22) = 8.719$, $p < 0.005$]. There was also a significant main effect of time [$F(4,88) = 96.92$, $p < 0.001$] and a significant time by group interaction [$F(8,88) = 4.06$, $p < 0.001$]. Post hoc Dunnett tests indicated that the TBI+saline group took significantly more time to locate the hidden platform ($p < 0.001$) compared to the sham TBI group on days 11–15 post-injury. Latency to locate the hidden platform was not significantly different between the TBI+PGI-02776 group and sham TBI group. A one-way ANOVA performed on the day 15 data revealed that there was a significant difference between groups on the final test day [$F(2,22) = 7.105$, $p = 0.004$]. Bonferroni post hoc tests revealed that the TBI+saline group had significantly longer latency to find the hidden platform compared to the sham TBI group ($p = 0.007$). There was no difference in latency to find the hidden platform between the TBI+ PGI-02776 and the sham TBI groups ($p = 0.733$). The TBI+PGI-02776 group had significantly shorter latency to find the hidden platform compared to the TBI+saline group ($p = 0.033$).

In addition to measuring the mean latency to platform, more detailed trial-by-trial analysis was performed to assess both short-term and long-term components of memory (Fig 4B). We defined short-term memory as a reduced latency to find the hidden platform over the four trials within each day (4 min inter-trial interval). All three groups demonstrated a similar degree of intact short-term memory within each day with no significant differences between groups ($p > 0.05$).

Long-term memory was defined as a reduced latency to platform between trial 4 of one day and trial 1 of the subsequent day (24-hour inter-trial interval) and was termed “saved latency to platform”. ANOVA revealed a significant effect of group on saved latency to platform [$F(2,22) = 3.970$, $p < 0.05$]. The sham TBI group had an 11.2 sec mean saved latency to platform (Fig. 4C) on trial 1 compared to trial 4 of the prior day, indicative of long-term memory. The TBI+saline group had a significantly longer mean saved latency to platform (–19.5 sec, Fig. 4C) compared to the prior day. Furthermore, there was a significant difference in saved latency to platform between the sham TBI and TBI+saline groups (Dunnett test $p < 0.05$). The mean saved latency to platform for the TBI+PGI-02776 group was close to zero (–1.6 sec), intermediate between the sham TBI and TBI+saline groups, and was not significantly different from the sham-TBI group (Dunnett $p > 0.40$). On day 16 post-injury, probe trials revealed no significant differences between groups in time spent in

the platform quadrant (sham TBI, 20.0 sec; TBI+saline, 18.1 sec; TBI+PGI-02776, 21.5 sec) [$F(2,22)=0.637$, $p > 0.50$].

Measures of swim speed were used to differentiate motor deficits from cognitive deficits. The visible platform test was used to evaluate deficits in visual acuity which could confound interpretation of cognitive performance. Swim speeds (sham TBI, 25.6 cm/sec; TBI+saline, 28.9 cm/sec; TBI+PGI-02776, 29.7 cm/sec) were significantly different between groups [$F(2,22)=10.329$, $P<0.01$]. Post hoc Dunnett tests indicated that the TBI+saline and the TBI+PGI-02776 groups had significantly ($p<0.05$) faster swim speeds than the sham TBI group. The visual acuity test revealed no differences between groups (sham TBI, 7.90 sec; TBI+saline, 7.58 sec; TBI+PGI-02776, 7.50 sec) [$F(2,22)=0.036$, $p > 0.90$], which indicated no confounding visual deficits.

Following the MWM probe trials on day 16 post-injury, animals were euthanized and brain sections were stained with cresyl violet. Pyramidal neurons in the ipsilateral CA2-3 region were quantified using stereological techniques. The CA2-3 stratum pyramidale in TBI animals (Fig. 5A,B) consistently appeared narrower than in sham TBI animals (Fig. 5C,D). Analysis of cell counts identified significant differences in neuronal number in the CA2-3 region [$F(2,22)=4.842$, $p<0.02$] and post hoc Dunnett test indicated both TBI groups, regardless of saline or PGI-02776 treatment, had significantly fewer surviving neurons compared to the sham-TBI group ($p<0.05$) (Fig. 5E).

3. Discussion

These data demonstrate the therapeutic potential of post-injury administration of PGI-02776, a novel di-ester prodrug modified from the urea-based NAAG peptidase inhibitor ZJ-43. A dose-response analysis of PGI-02776 administered 30 min after lateral fluid percussion TBI in rats determined that PGI-02776 (10 mg/kg, i.p.) significantly reduced acute neuronal degeneration in the CA2-3 hippocampus assessed at 24 hrs post-injury. In chronic behavioral experiments, a single 10 mg/kg dose of PGI-02776 administered 30 min post-injury improved cognitive performance on the MWM, specifically long-term memory, as assessed on days 11–15 post injury. Histological analysis on day 16 post-injury revealed that TBI produced a significant reduction in surviving neurons in the dorsal CA2-3 hippocampus regardless of treatment.

PGI-02776 is a newly designed di-ester prodrug based upon the urea-based NAAG peptidase inhibitor ZJ-43 (Olszewski et al., 2004; Yamamoto et al., 2004; Zhong et al., 2005; Zhong et al., 2006) modified with a lipophilic carrier to enhance BBB penetration. Pharmacokinetic data indicated that PGI-02776 crossed the BBB in uninjured rodents. However, greater drug delivery to the brain would likely occur following TBI when BBB integrity is compromised (Jiang et al., 1992). Inhibition studies demonstrated that the parent compound (ZJ-43) exhibited potent GCPII inhibitory activity while the mono-ester (PGI-02749) and di-ester (PGI-02776) compounds exhibited moderate and weak levels of inhibitory activity, respectively. Pharmacokinetic assays in uninjured animals revealed that the di-ester (PGI-02776) was hydrolyzed to the mono-ester (PGI-02749) and to the parent compound (ZJ-43) in both blood and brain over several hours. In summary, the di-ester prodrug, PGI-02776, crossed the BBB but had weak GCPII activity. Furthermore, since it took several hours for PGI-02776 to be hydrolyzed to ZJ-43 (which more potently inhibited GCPII), it is possible that additional mechanism(s) of therapeutic action other than GCPII inhibition may play a role in the protection afforded by PGI-02776.

Previous experimental TBI studies have shown that NAAG peptidase inhibitors produce effects consistent with reduction in glutamate excitotoxicity when administered prior to or

immediately after TBI (Zhong et al., 2005; Zhong et al., 2006). For example, administration of the NAAG peptidase inhibitor, ZJ-43, 15 min prior to lateral fluid percussion TBI in rats increased and prolonged the brain levels of NAAG and reduced the brain levels of glutamate (both measured by microdialysis) compared to vehicle-treated TBI rats (Zhong et al., 2006). Immediate post injury injection of ZJ-43 followed by two additional doses at 8 and 16 hrs significantly reduced acute neuronal degeneration and acute loss of GFAP immunoreactivity at 24 hrs post-injury (Zhong et al., 2005). Co-administration of a selective Group II mGluR antagonist abolished those effects, indicating that the actions of ZJ-43 were mediated through Group II mGluRs activated by the elevated levels of NAAG (Zhong et al., 2005). The present results demonstrate the efficacy of delayed post injury administration of a novel prodrug formulation of ZJ-43 in a model of TBI.

The present findings demonstrated that PGI-02776 produced a U-shaped dose response with the 10 mg/kg dose providing significant reduction in the numbers of degenerating neurons in the CA2-3 at 24 hrs post-injury. The 24 hr evaluation was chosen as a time of maximal CA2-3 degeneration based on previous TBI studies examining FJ staining from 30 min to 7 days post-TBI (Hallam et al., 2004; Zhao et al., 2003; Zhong et al., 2005). Quantification of surviving neurons at 16 days after TBI revealed significant cumulative neuronal loss in the CA2-3 regardless of treatment. Therefore, the significant decrease in FJ positive neurons with PGI-02776 treatment did not translate to an increase in long-term cell survival possibly because the drug delayed, but did not prevent neuronal degeneration. The cognitive protection afforded by the PGI-02776 may be due to the drug positively affecting cell function rather than simply reducing the number of dying neurons. Other experimental therapeutics have shown behavioral protection without concomitant effects on cell death (Bramlett et al., 1995; Bramlett et al., 1997). Additionally, previous studies have demonstrated significant decrement in cognitive performance in the absence of cell death following experimental TBI (Gurkoff et al., 2006; Lyeth et al., 1990). Regardless of the effect on long-term neuronal cell loss, a significant improvement in MWM performance was observed suggesting that PGI-02776 applied 30 minutes following injury improved outcome in TBI rats.

Detailed analysis of the MWM data revealed an important finding with respect to short- and long-term memory performance. The typical MWM data analysis evaluates the average performance per day (average of 4 trials per day) over 4 to 5 days of consecutive testing (e.g., Figure 4A). In the present analysis, short-term and long-term memory were isolated by evaluating performance over consecutive trials within a day (short-term memory; inter-trial interval of 4 min) versus evaluating the difference in latency between the last trial of a day and the first trial of the subsequent day (long-term memory; inter-trial interval of 24 hrs) (Hunsaker et al., 2008). Examination of Figure 4B indicates that regardless of injury or treatment, animals maintained intact short-term memory. In contrast, saline-treated TBI rats had an increase in latency between trial 4 on one day and trial 1 on the subsequent day (e.g., Figure 4B; between D11 and D12) and therefore a decrement in long-term memory. The present study demonstrated that the PGI-02776 treated group had a performance intermediate between the sham and TBI saline groups indicating that PGI-02776 improved long-term memory in TBI rats.

The present results provide further evidence for the continued therapeutic development of NAAG peptidase inhibitors for TBI. This data contributes to the proof of concept that prodrugs of a well-studied NAAG peptidase inhibitor, ZJ-43, are efficacious in reducing acute neuronal degeneration and cognitive deficits in this model of TBI and suggest their potential usefulness in animal models of other NAAG responsive disorders.

4. Experimental Procedures

4.1 NAAG peptidase Inhibition Assay

PGI-02776 and ZJ-43 were synthesized in the laboratory of Dr. Zhou (PsychoGenics, Tarrytown NY). PGI-02776 is a di-ester prodrug of the urea-based NAAG peptidase inhibitor ZJ-43 (Olszewski et al., 2004).

The inhibition assay used membranes harvested from CHO cells that had been stably transfected with rat GCPII and was based on GCPII hydrolyzing NAAG (radiolabeled in the glutamate moiety) to [^3H]-glutamate and unlabeled NAA (Bzdega et al., 1997). Following the reaction, tritiated NAAG and glutamate were separated via cation exchange. Test compounds were dissolved and subsequently diluted for dose-response assays in molecular grade water. Each dose was tested four times ($n=4$). Each reaction sample contained 5 μL of the test compound, 15 μg of membranes from CHO/GCPII cells, 4 μM NAAG (not labeled), trace amounts of tritium-labeled “hot” NAAG (~100,000 dpm), CoCl_2 (1 mM), and 50 mM Tris pH 7.5 to a final volume of 50 μL . Control samples included: blank (no inhibitors, no membranes) and positive (no inhibitor). The signal from the blank represented [^3H]-glutamate (or other tritiated amines) that were present in the radiolabeled NAAG stock. Samples were incubated 3 hrs at 37°C and the reaction was stopped with 0.2 M HCl. Samples were applied to a 0.5 ml cation exchange AG 50W-X8 column previously equilibrated with 0.1 M HCl, then washed with 3 ml 0.1 M HCl to collect ^3H -NAAG, and with 3 ml of 1 M HCl to collect [^3H]-glutamate. Results were calculated as a percentage of total dpm in the glutamate fraction/dpm in glutamate + NAAG fractions and normalized to the no inhibitor control value which was set as 100% enzyme activity.

4.2 Pharmacokinetic Experiments

4.2.1 Subjects—Forty-two C57B/6 mice (Vital River Laboratory Animal technology Co. Ltd., Beijing, China) weighing 18–22 grams were used for pharmacokinetic data. Animals were housed 10/cage in polypropylene cages in a temperature and humidity-controlled animal facility with 12-hour light/dark cycle and with free access to food and water.

4.2.2 Bioanalysis of Drugs in Blood and Brain after i.p. Administration—Mice were dosed with PGI-02776 and ZJ-43 at the level of 100 mg/kg i.p. At 0.25, 0.5, 1, 2, 4, 6, and 8 hrs after drug injection ($n=3$ /time point), 0.30 ml of whole blood was withdrawn from the femoral artery into sodium heparin-coated tubes. Mice brains were then rapidly harvested after exsanguination from femoral vein and artery and homogenized with 2-fold distilled water. Whole blood and homogenized brain samples were added to 3-fold volume of methanol immediately to stabilize PGI-02776 in the biosamples after collection. After centrifugation at 4 °C under 20,000 g for 15 min, 200 μL of the supernatant was collected and injected onto a Liquid Chromatography-Electrospray Ionization Mass Spectrometry (LC/MS/MS) (AB Sciex, model API 4000 Qtrap) for quantitative analysis. The calibration standard and quality control samples were used to calculate the concentrations of PGI-02776 and ZJ-43 in the samples. Average blood and brain concentration-time data were analyzed using a standard non-compartment method (WinNonlin Enterprise, version 5.2.1) to obtain pharmacokinetic parameters.

4.3 Therapeutic Evaluation Experiments

4.3.1 Subjects—Seventy male Sprague-Dawley rats (Harlan) weighing 300–330 grams were used in this study. Animals were housed in individual cages in a temperature (22°C) and humidity-controlled (50% relative) animal facility with a 12-hour light/dark cycle. Animals had free access to food and water during the duration of the experiments. Animals were held in the animal facility for at least 7 days prior to surgery. The Institutional Animal

Care and Use Committee at the University of California at Davis approved all animal procedures in these experiments.

4.3.2 Experimental Design—Investigators were blind to the injury and treatment for all experiments included in this study. PGI-02776 was dissolved in sterile 0.9 percent saline and injected i.p. at a volume of 1 ml/kg. Drugs were administered 30 min after TBI in all experiments.

The dose-response effects of PGI-02776 were evaluated on TBI-induced neuronal degeneration measured at 24 hrs post-injury. Four groups of animals were subjected to lateral fluid percussion followed 30 min later by administration of one of three dose of PGI-02776 (1.0, 10, or 50 mg/kg) or an equal volume of saline vehicle (n=10, n=9, n=10, n=16, respectively). Animals were euthanized at 24 hrs after TBI and brain tissue processed for analysis of acute neuronal degeneration.

The effects of PGI-02776 were evaluated on cognitive performance in the MWM on days 11–16 post-injury and neuronal survival on day 16 post-injury. Two groups of animals were subjected to TBI followed 30 min later by administration either the most effective dose of PGI-02776 (10 mg/kg) as determined from the acute neuronal degeneration experiment or an equal volume of saline (n=10 per group). A third group of sham-TBI animals (n=5) was administered an equal volume of saline 30 min after sham-TBI. Animals were euthanized following the MWM probe trial (day 16) and brain tissue was processed for analysis of neuronal survival.

4.3.3 Surgical Procedure—Rats were anesthetized with 4% isoflurane in a 2:1 nitrous oxide/oxygen mixture, intubated, and mechanically ventilated with a rodent volume ventilator (Harvard Apparatus model 683, Holliston, MA). A surgical level of anesthesia was maintained with 2% isoflurane. Rats were mounted in a stereotaxic frame, a scalp incision made along the midline, and a 4.8 mm diameter craniectomy was performed on the right parietal bone (centered at –4.5 mm Bregma and right lateral 3.0 mm). A rigid plastic injury tube (modified Leur-loc needle hub, 2.6 mm inside diameter) was secured over the exposed, intact dura with cyanoacrylate adhesive. Two skull screws (2.1 mm diameter, 6.0 mm length) were placed into burr holes, 1 mm rostral to Bregma and 1 mm caudal to Lambda. The injury tube was secured to the skull and screws with cranioplastic cement (Plastics One, Roanoke, VA). Rectal temperature was continuously monitored and maintained within normal ranges during surgical preparation by a feedback temperature controller pad (CWE model TC-1000, Ardmore, PA). Temporalis muscle temperature was measured by insertion of a 29-gauge needle temperature probe (Physitemp unit TH-5, probe MT-29/2, Clifton, NJ) between the skull and temporalis muscle.

4.3.4 Traumatic Brain Injury—Experimental TBI was produced using a fluid percussion device (VCU Biomedical Engineering, Richmond, VA) (Dixon et al., 1987) using the lateral orientation (McIntosh et al., 1989). The device consisted of a Plexiglas cylindrical reservoir filled with isotonic saline. One end of the reservoir had a Plexiglas piston mounted on O-rings and the opposite end had a transducer housing with a 2.6 mm inside diameter male Leur-Loc opening. Injury was induced by the descent of a pendulum striking the piston, which injected a small volume of saline epidurally into the closed cranial cavity, producing a brief displacement and deformation of neural tissue. The resulting pressure pulse was measured in atmospheres (ATM) by an extracranial transducer (model SPTmV0100PG5W02; Sensym ICT) and recorded on a digital storage oscilloscope (model TDS 1002; Tektronix Inc., Beaverton, OR).

Rats were disconnected from the ventilator, the injury tube connected to the fluid percussion cylinder, and a moderate fluid percussion pulse (~ 2.15 ATM) was delivered within 10 sec. Immediately after TBI, rats were ventilated with a 2:1 nitrous oxide/oxygen mixture in the absence of isoflurane. The plastic injury tube and skull screws were removed and the scalp incision was closed with 4-0 braided silk sutures. As soon as spontaneous breathing was observed the rats were extubated and assessment of the righting reflex began by placing the rat in a supine position at regular intervals (~20 sec) to test the rat's ability to spontaneously recover to a prone position. The duration of suppression of the righting reflex was used as an additional indicator of injury severity. Sham TBI rats were subjected to all anesthetic and surgical procedures but were not delivered a fluid pulse to the brain.

4.3.5 Tissue Collection and Sectioning—Rats were euthanized 24 hrs (acute histology experiment) or on day 16 (chronic histology experiment) after TBI, by deep sodium pentobarbital anesthesia (100 mg/kg, ip) followed by transcardial perfusion with 100 ml of 0.1 M sodium phosphate buffer (PB) (pH = 7.4) followed by 350 ml of 4% paraformaldehyde (pH 7.4). Brains were removed and post-fixed for 1 hr in 4% paraformaldehyde at 4°C. Brains were next cryoprotected in 10% sucrose solution for 1 day followed by 2 days in a 30% sucrose solution, and then frozen on powdered dry ice. Using a sliding microtome (American Optical, Model 860), 45 µm coronal sections were cut. Every serial section starting at -2.12 mm Bregma and ending at -4.80 mm Bregma was saved in 24-well cell culture plates. Systematic random sampling techniques were used for selecting tissue sections for staining and stereological analysis.

4.3.6 Acute Histology: Neuronal Degeneration—Neuronal degeneration quantified using stereological techniques at 24 hrs after TBI using the histofluorescent stain, FJ-B (Schmued et al., 1997; Schmued and Hopkins, 2000). Tissue sections were mounted on gelatin-coated slides in 0.1 M PB and distilled H₂O (1:1 ratio) and air-dried overnight. The slide-mounted tissue sections were subsequently immersed in 100% alcohol (3 min), 70% alcohol (1 min), dH₂O (1 min), and 0.006% potassium permanganate (15 min). Sections were rinsed in dH₂O (1 min), incubated in 0.001% FJ-B (Histo-Chem Inc., Pine Bluff, AK) staining solution in 0.1% acetic acid for 30 min, rinsed again in dH₂O (3 min), and air-dried. Finally, the sections were immersed in xylene and coverslipped with DePeX mounting medium (Electron Microscopy Sciences, Fort Washington, PA).

4.3.7 Chronic Behavioral Evaluation—Acquisition of spatial learning and memory was assessed in the MWM on days 11–15 after TBI (Morris, 1984). The test apparatus consisted of a large white circular tank (220 cm diameter by 60 cm high) filled with water to a depth of 22 cm. Water temperature was maintained at 24–28°C. A transparent circular escape platform (12.8 cm diameter, 20 cm high) was placed in a fixed position in the tank 2 cm below the water surface. Four consistent visual cues were located in the test room outside of the maze. Rats were released from one of four starting points (selected randomly on each day for each rat) and allowed 120 sec to find and mount the escape platform. If a rat did not find the platform within 120 sec, the experimenter placed the rat on the platform. Subjects remained on the platform for 30 sec before being removed from the maze. Subjects received a 4-min inter-trial interval in a warmed holding cage before being returned to the maze for subsequent trials. Rats received a total of 4 trials per day, one from each starting point, over 5 consecutive days. Mean latency to find the platform was calculated for each day to assess learning.

We additionally analyzed short-term and long-term memory. Short-term memory was defined as a reduced latency to platform from trial 1 to trial 4 within a day (4 min inter-trial interval). Long-term memory was defined as a reduced latency to platform between trial 4 of one day and trial 1 of the subsequent day (24 hr inter-trial interval) and was termed “saved

latency to platform”. Thus, a positive value for “saved latency to platform” indicated that the MWM performance improved over the 24 hrs between the last trial of a day and the first trial of the subsequent day. Conversely, a negative value for “saved latency to platform” indicated a worsening of MWM performance over 24 hrs and was defined as a deficit in long-term memory.

On day 16, a 60-sec memory probe trial was conducted without the escape platform to access time spent in each of four pool quadrants. One hour later, a visual acuity test was performed using a black-circular platform 1 cm above the water surface, located in a different quadrant from the escape platform. Data from all trials were recorded using a video tracking system (Poly-Track Video Tracking System version 2.1, San Diego Instruments Inc. San Diego, CA).

4.3.8 Chronic Histology: Neuronal Survival—On day 16 post-injury, surviving neurons were stained with cresyl violet and quantified using stereological techniques. Tissue sections were mounted on gelatin-coated slides and dried overnight before staining. Sections were dehydrated at room temperature by a series of ethanol immersions: 70% (2 min × 1), 95% (2 min × 2) and 100% (2 min × 2) followed by immersion in xylene (16 min). Sections were then rehydrated in a series of ethanol immersions: 100% (2 min × 2), 95% (2 min × 2) and 75% (2 min × 1), then rinsed with distilled water (30 sec × 2). Sections were next stained with cresyl violet acetate (0.1%) for 6 min followed by rinsing in distilled water (15 sec × 2), differentiation by immersion in 95% ethanol with 0.15% acetic acid (8 min), and dehydrated in a series of ethanol immersions: 95% (30 sec × 2), 100% (30 sec × 2) and cleared by immersion in xylene (5 min × 2). Sections were coverslipped with Permount (Fisher Scientific, Hampton, NH).

4.3.9 Stereological Cell Counts—The number of FJ-B positive degenerating neurons and cresyl violet stained neurons in the region of interest was quantified using stereological methods. Tissue sections were selected by taking every fifth section starting at Bregma −3.1 mm and ending at Bregma −4.7 mm for a total of 7 sample sections per brain. The FJ-B cell counts were made on an epifluorescent microscope (Nikon E600, Nikon, Tokyo) under a mercury arc lamp with a FITC fluorescence filter cube (Nikon B-2A, Tokyo) using a motorized stage (MS-2000, Applied Scientific Instruments, Eugene OR) and computer software (Stereologer™ version 1.3, Systems Planning & Analysis, Inc., Alexandria, VA). The cresyl violet cell counts were made on a microscope (Nikon E600, Nikon, Tokyo) with a motorized stage (Bioprecision2, Ludl Electronic Products, Inc., Hawthorne, NY) using computer software (Stereo Investigator™ 8.0, Microbrightfield, Inc., Williston, VT). Degenerating neuronal cell counts (24 hrs) and surviving neuronal cell counts (16 days) were made in a region of interest encompassing the stratum pyramidale of the dorsal hippocampal CA2-3. The border of the stratum pyramidale was defined by the pyramidal layer entry into dentate gyrus at the lateral tips of the dorsal and ventral blades of the dentate granule cells on one end and at the other end by the narrowing of the stratum pyramidale at the intersection of the CA1 to CA2. Criterion for counting degenerating neurons (FJ-B positive) included green fluorescing, morphologically distinct cell bodies with at least one clearly identifiable dendrite. The criterion for selection and quantification of surviving neurons (cresyl violet) was a morphologically distinct cell body. The region of interest was outlined using 2X (FJ-B) or 10X (cresyl violet) objectives. Neuronal identification and cell counting were performed with 20X (FJ-B) or 100X oil (cresyl violet) objectives.

4.4 Statistical Analysis

Data analysis was performed using SPSS software (Version 17, Chicago IL) which adheres to a general linear model. Alpha level for Type I error was set at 0.05 for rejecting null hypotheses.

The normalized NAAG peptidase inhibition data were expressed as mean $\pm$ standard deviation (SD) and analyzed using separate one-way ANOVA for each compound with a post hoc Dunnett test comparing each concentration to the no-inhibitor control group. Data for body weights, fluid percussion injury magnitudes, righting times, and temperature measurements were expressed as mean $\pm$ SD and analyzed using separate one-way ANOVA between groups. All other animal data were expressed as mean $\pm$ standard error of the mean (SEM). Degenerating neuronal counts from FJ-B staining and surviving neuronal counts from cresyl violet staining were each analyzed using a one-way ANOVA and a Dunnett post hoc analysis comparing each group to sham.

Behavioral data for MWM latency was analyzed using repeated measures ANOVA with assessment days as the repeated variable within subjects followed by Dunnett post hoc analysis. Additionally, the difference in latency to platform on the four trials within a day as well as the difference in latency to platform between the 1st trial of a daily session as compared the last trial of the previous day's session were used as measures of short-term and long-term memory (Hunsaker et al., 2008) and were analyzed with one-way ANOVAs followed by Dunnett post hoc analysis.

Acknowledgments

This research was supported by NIH NS61352 (JZ), NIH NS38080 (JHN) and NIH NS29995 (BGL).

Abbreviations used

ATM	atmosphere
BBB	blood-brain barrier
CNS	central nervous system
FJ-B	Fluoro-Jade B
GCP II	glutamate carboxypeptidase II
i.p	intraperitoneal
mGluR3	metabotropic glutamate receptor (subtype 3)
MWM	Morris water maze
NAA	<i>N</i> -acetylaspartate
NAAG	<i>N</i> -acetylaspartylglutamate
PBS	phosphate-buffered saline
TBI	traumatic brain injury

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Figure 1. NAAG peptidase (GCP II) inhibition assay

NAAG radiolabeled in the glutamate moiety was hydrolyzed by membranes of CHO cells that had been stably transfected with GCP II. The release of [3H]-glutamate from [3H]-NAAG in the absence of any inhibitor is taken as 100% enzyme activity. The inhibitory efficacy of ZJ-43, PGI-02749 and PGI-02776 were determined over a range concentrations from 1 nM to 100 μ M. The data demonstrate that the parent compound (ZJ-43) strongly inhibits GCP II while the mono-ester (PGI-02749) and the di-ester (PGI-02776) weakly inhibit GCP II. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to the no inhibitor control.

Figure 2. Blood and brain time-concentration curves of compounds

Individual time-concentration curves of compounds in blood and brain were made following i.p. administration at 100 mg/kg in male C57B/6 mice (n=3 per time point). Following injection of ZJ-43, blood (A) and brain levels (B) were maximal after 15 and 30 min, respectively. Following injection of PGI-02776, blood (C) and brain levels (D) were maximal after 30 min and 1 hr, respectively. Following injection of PGI-02776, blood levels (E) of the mono-ester PGI-02749 were maximal at 4 hrs and brain levels (F) were detectable from 2 to 6 hrs. Following injection of PGI-02776, blood levels (G) of the parent compound ZJ-43 were maximal at 4 hrs and brain levels (H) were detectable from 4 to 8 hrs. BLOQ = below level of quantification

Figure 3. Fluoro-Jade B histofluorescence of degenerating neurons 24 hrs after injury

The photomicrographs are representative coronal sections (−3.6 mm Bregma) of the dorsal hippocampal CA2-3 region. **A**: Moderate lateral fluid percussion injury produced fluorescing cells, indicative of degenerating neurons, especially in the ipsilateral parietal cortex and hippocampal CA2-3 region. **B**: Higher magnification in ipsilateral CA3 showed individual, brightly fluorescing degenerating neurons. **C**: Degenerating neurons were quantified using stereological techniques. Administration of PGI-02776 significantly reduced the number of ipsilateral degenerating neurons in the CA2-3. Calibration bars: A = 200 μ m; B = 100 μ m. SR = stratum radiatum, SP = stratum pyramidale, SO = stratum oriens. * $p < 0.05$ compared to 0 mg/kg PGI-02776 (saline vehicle) group

Figure 4. Morris water maze tests for cognitive performance on days 11–15 after injury
A: Latency to platform of daily performance is plotted over days 11–15 after TBI. Each data point represents the mean of 4 daily trials. The sham TBI group had the shortest latency to find the hidden platform. The TBI+saline group had the longest latency to find the hidden platform. There was a significant difference in latency between the sham TBI group and TBI +saline group ($p < 0.01$). There was no significant difference between the sham TBI group and the TBI+PGI-02776 group, indicating that the drug treatment improved cognitive performance. **B:** Latency to platform on individual trials per day is plotted over days 11–15 after TBI. A detailed analysis of trial-by-trial latencies to platform was used to separate short-term and long-term memory performance. Short-term memory was defined as a change in latency to platform from trial 1 to trial 4 within a day. There were no significant differences in short-term memory between groups. **C:** Long-term memory was defined as a reduced latency (“saved latency to platform”) between trial 4 of one day and trial 1 of the subsequent day (24-hour inter-trial interval). The positive value in saved latency to platform for the sham TBI group implied intact long-term memory. The significant difference between the sham TBI group (11 sec) and the TBI+saline group (–19 sec) indicate that TBI

resulted in long-term memory deficits. The TBI+PGI-02776 group had an intermediate phenotype with a saved latency to platform value close to zero. There was no difference between the sham TBI and the TBI + PGI-02776 groups. * $p < 0.05$ compared to sham TBI group

Figure 5. Cresyl violet staining of surviving neurons 16 days after TBI

Photomicrographs of representative coronal sections (−3.6 mm Bregma) show the CA2-3 in a panel **A** for a TBI+saline animal and in panel **B** for a sham TBI animal. Panels **B** and **D** are high magnification views of the outlined boxes in panels **A** and **C**. Note the thinning of the pyramidal cell layer in the TBI+saline animal in panel **B** compared to a sham TBI animal in panel **D**. **E**: Stereological cell counts show that TBI+ saline and TBI+PGI-02776 had significant reductions in surviving CA2-3 neurons compared to sham TBI at day 16 post-injury. Calibration bars: A, C = 200 μ m; B, D = 20 μ m; SR = stratum radiatum, SP = stratum pyramidale, SO = stratum oriens; * $p < 0.05$ compared to the sham + saline group.

Table 1

Groups and Parameters in Experiments (Values are Mean $\pm$ SD)

Group	Sample	Body Weight (g)		TBI (ATM)	Righting Time (min)	Rectal Temp. (°C)		Temporalis Temp. (°C)	
		Pre-TBI	Pre-MWM			Pre-TBI	Post-TBI	Pre-TBI	Post-TBI
Dose-response study: 24-hour histology									
TBI + Saline	16	315 ± 16	-	2.15 ± 0.01	14.7 ± 3.4	37.1 ± 0.3	37.1 ± 0.3	35.7 ± 0.3	35.6 ± 0.3
	10	317 ± 13	-	2.14 ± 0.01	12.1 ± 3.6	37.1 ± 0.4	37.0 ± 0.3	35.8 ± 0.4	35.8 ± 0.4
	9	313 ± 7	-	2.14 ± 0.02	13.6 ± 4.3	37.2 ± 0.3	37.1 ± 0.2	36.0 ± 0.4	36.0 ± 0.3
	10	315 ± 13	-	2.15 ± 0.01	12.9 ± 3.6	37.3 ± 0.2	37.2 ± 0.2	35.9 ± 0.3	35.9 ± 0.3
Optimal dose: Behavior and 16-day histology									
Sham TBI + Saline	5	309 ± 7	322 ± 13	-	3.1 ± 0.3	37.1 ± 0.1	-	36.0 ± 0.2	-
	10	316 ± 17	313 ± 14	2.15 ± 0.01	12.8 ± 2.6	37.0 ± 0.3	36.8 ± 0.3	35.9 ± 0.2	35.8 ± 0.2
	10	314 ± 15	316 ± 18	2.15 ± 0.02	12.7 ± 3.8	36.9 ± 0.3	36.8 ± 0.3	35.9 ± 0.2	35.7 ± 0.3