Evaluating the neurotherapeutic potential of a water-soluble progesterone analog after traumatic brain injury in rats

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A B S T R A C T

The poor aqueous solubility of progesterone (PROG) limits its potential use as a therapeutic agent. We designed and tested EIDD-1723, a novel water-soluble analog of PROG with >100-fold higher solubility than that of native PROG, as candidate for development as a field-ready treatment for traumatic brain injury (TBI). The pharmacokinetic effects of EIDD-1723 on morphological and functional outcomes in rats with bilateral cortical impact injury were evaluated. Following TBI, 10-mg/kg doses of EIDD-1723 or PROG were given intramuscularly (i.m.) at 1, 6 and 24 h post-injury, then daily for the next 6 days, with tapering of the last 2 treatments. Rats were tested pre-injury to establish baseline performance on grip strength and sensory neglect, and then retested at 4, 9 and 21 days post-TBI. Spatial learning was evaluated from days 11–17 post-TBI. At 22 days post-injury, rats were perfused and brains extracted and processed for lesion size. For the edema assay the animals were killed and brains removed at 24 h post-injury. EIDD-1723 significantly reduced cerebral edema and improved recovery from motor, sensory and spatial learning deficits as well as, or better than, native PROG. Pharmacokinetic investigation after a single i.m. injection in rats revealed that EIDD-1723 was rapidly converted to the active metabolite EIDD-036, demonstrating first-order elimination kinetics and ability to cross the blood-brain barrier. Our results suggest that EIDD-1723 represents a substantial advantage over current PROG formulations because it overcomes storage, formulation and delivery limitations of PROG and can thereby reduce the time between injury and treatment.

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1. Introduction

Numerous drugs have shown promise as neuroprotective agents in preclinical animal models of traumatic brain injury (TBI), but none have worked in clinical trials. Despite substantial pre-clinical evidence (Schumacher et al., 2015) supporting the neuroprotective effects of progesterone (PROG), two recently completed phase III clinical trials reported that the neurosteroid did not provide a significant benefit to patients recovering from moderate to severe TBI (Skolnick et al., 2014; Wright et al., 2014). One factor in the failure of the trials may have been treatment delays, which ranged from 4 to 9 h post-injury and could have compromised the neuroprotective benefits of the test drug. A key tenet of brain injury treatment is that the sooner the intervention takes place, the better the functional outcome will be (Stein, 2011), but there must also be good penetration of the agent and its metabolites into the damaged tissue to permit neuroprotective activity. Thus another factor in the negative trial results could have been the poor solubility of PROG, and the intrinsic biological activity of the carrier chosen for the delivery of PROG (Howard et al., 2015; Stein, 2015). Current clinical
protocols for PROG administration require that patients be transported to a hospital setting before treatment can begin, losing valuable time. Time to treatment is an important consideration because many agents which show positive effects in the laboratory, where they can be given minutes to several hours after injury, do not show efficacy when treatment is delayed to later in the injury cascade.

PROG thus has certain limitations as a therapeutic intervention, notably its insolubility in aqueous-based formulations, and its short half-life, which restrict its capability for rapid delivery in emergency conditions. What is needed is a stable, water-soluble and easy-to-administer form of PROG that works immediately to rescue damaged tissue in preparation for a longer course of therapy that can be delivered in the hospital.

As noted, the disappointing outcomes of TBI randomized controlled trials may have been impacted by the choice of vehicle with which PROG was administered intravenously (i.v.). In both phase III trials (Skolnick et al., 2014; Wright et al., 2014) a lipid carrier was used, but these agents can have important effects on physiology and are not completely “neutral” as required for an appropriate control vehicle. Lipid emulsions have been shown to reduce inflammatory reactions in endothelial cells, an effect which may implication on modulating vascular repair in the damaged brain (Harvey et al., 2015). Some lipid carrier effects are beneficial, some are not. Significant elevation in total HDL and LDL cholesterol following 96 h of continuous 10% Intralipid infusion has been reported (Wasan et al., 1994). A meta-analysis of case reports found that Intralipid administration was effective in reversing multiple drug toxicities (Muller et al., 2015). Studies have shown that i.v. lipid emulsions can be effective in the acute stages of drug intoxication, improving Glasgow Outcome Scale (GOS) scores and reducing blood glucose levels up to 6 h after administration (Taufichi et al., 2012). Intralipid administration has also been shown to increase insulin resistance in heart tissue in an experimental model of type II diabetes in rats, indicating that the agent can interfere with glucose metabolism under certain conditions that might also affect the extent of brain injuries (Lou et al., 2015). Thus, as a vehicle in a clinical trial, Intralipid or other lipid compounds may confound and/or mask the effects of PROG given to patients in the acute stage of the injury cascade. In contrast, virtually all pre-clinical experiments preceding the clinical trials used more soluble 2-hydroxypropyl-β-cyclodextrin (HBC) as a vehicle rather than a lipid formulation, another possible reason the pre-clinical reports following dose and prior to catheter removal. All animals were observed at dosing and each scheduled collection. Serial samples were collected from the JVC according to the schedule in Table 1. The animals were not fasted, but the p.o. group was dosed >2 h after “lights on” to ensure that stomachs were empty when dosed. All oral doses were administered via gavage tube and all i.v. doses were administered via tail vein catheter. Catheters were flushed with ~0.5 ml of saline immediately following dose and prior to catheter removal. All animals were observed at dosing and each scheduled collection. Serial samples were collected from the JVC according to the schedule in Table 1. At the 10-h post-dosing, animals were euthanized and brain samples were collected from all the rats. Brains were rinsed with saline, patted dry, weighed, and placed on dry ice prior to storage at −80 °C until transferred to analytical chemistry for analysis. Blood samples were collected into K2EDTA tubes and stored on ice until processed to plasma by centrifugation (3500 rpm at 5 °C) within 1 h of collection. Plasma samples were transferred into matrix tubes and stored at nominal −80 °C until transferred to analytical chemistry for analysis.

2.2. Pharmacokinetics study

A pharmacokinetics (PK) experiment was carried out in rats using per oral (p.o.), i.m. or i.v. administration to investigate the metabolic conversion of EIDD-1723 to its active metabolite EIDD-036. This study was outsourced to Agilux, Inc. (Worcester, MA, USA) under Protocol #CE-0006-DA-RI. Male Sprague Dawley (SD) rats fitted with indwelling jugular vein cannulas (JVC) were allowed to acclimate to the test facility for 2 days prior to the start of the study and then randomly assigned to 5 groups (n = 4/group) as described in Table 1. The animals were not fasted, but the p.o. group was dosed >2 h after “lights on” to ensure that stomachs were empty when dosed. All oral doses were administered via gavage tube and all i.v. doses were administered via tail vein catheter. Catheters were flushed with ~0.5 ml of saline immediately following dose and prior to catheter removal. All animals were observed at dosing and each scheduled collection. Serial samples were collected from the JVC according to the schedule in Table 1. At the 10-h post-dosing, animals were euthanized and brain samples were collected from all the rats. Brains were rinsed with saline, patted dry, weighed, and placed on dry ice prior to storage at −80 °C until transferred to analytical chemistry for analysis. Blood samples were collected into K2EDTA tubes and stored on ice until processed to plasma by centrifugation (3500 rpm at 5 °C) within 1 h of collection. Plasma samples were transferred into matrix tubes and stored at nominal −80 °C until transferred to analytical chemistry for analysis.

2.3. Tissue distribution of EIDD-1723 in rat brain

A study of the distribution of the compound in brain tissue was performed to determine the time-concentration profile of EIDD-1723 in the CNS after an i.m. injection. This study was conducted by Agilux, Inc. under Protocol #CE-0006-DA-RI. Male SD rats (n = 24) were allowed to acclimate to the test facility for 2 days prior to the start of the study. The animals were provided food and water ad libitum. All animals were dosed i.m. with 10 mg/kg of EIDD-1723 and observed at dosing and at each scheduled collection. No abnormalities were recorded. Four animals were euthanized at 2.5 h after the 0.5, 1, 2, 4, 6 and 8 h time-points, and brain samples were collected. Tissue samples were rinsed with saline, patted dry, weighed, and placed on dry ice prior to storage at −80 °C until transferred to analytical chemistry for analysis. Plasma
and tissue samples were analyzed on a Sciex MS API-6500 instrument (SCIEX, Framingham, MA, USA) in positive ionization mode fitted with an ACE-3 C18 column using a gradient (2.5 h) mobile phase with mobile phase A = H2O:ACN:FA (95:5:0.1) and mobile phase B = ACN:MeOH:FA (50:50:0.1). Chrysin was used as an internal standard.

2.4. Efficacy study

2.4.1. Subjects

Male SD rats weighing 200–350 g at the time of injury were used for the efficacy study, which was conducted in our facility at Emory University. The rats were maintained on a reverse 12:12 light-dark cycle at 22°C ± 1°C with appropriate humidity levels. After one week of quarantine the rats were handled at least 3 times prior to surgery. This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) in accordance with all National Institutes of Health guidelines. All experimental animal procedures were approved by the Emory University Institutional Animal Care and Use Committee (Protocol # 2002865).

2.4.2. Contusion injury

Rats were anesthetized using isoflurane gas (5% induction, 1.5% maintenance, 700 mmHg N2O, 500 mmHg O2) and then mounted in a Kopf stereotaxic device (David Kopf Instruments, Tujunga, CA, USA). The scalp incision area was shaved and sterilized with Betadine® antiseptic and 70% isopropanol. Physiological parameters were monitored with pulse oximetry (SurgiVet™ model V3304); heart rate was maintained above ~300 beats per minute and SpO2 kept above 90%. Core body temperature (~37°C) was maintained with a homeothermic heating blanket system (Harvard Apparatus, Holliston, MA, USA). Under aseptic conditions, a midline incision was made into the skin and fascia covering the skull. Cotton swabs were used to staunch and clean any fascial bleeding. Bregma was located and a trephine drill was used to perform a 5-mm diameter mid-sagittal bilateral craniotomy 3 mm anterior to bregma. Controlled cortical impact (CCI) injury to the medial frontal cortex (MFC) was induced with a magnetic cortical pinpoint contusion impactor (4-mm diameter; PC1300; Hatteras Instruments, Cary, NC, USA) to a depth of 2.5 mm at a pressure of 1.7 psi, impact time of 100 ms, and velocity of 2.26 m/s. Sutures were used to close the incision after bleeding stopped. Animals were then placed into heated recovery boxes and allowed to recover from the anesthesia before being returned to their home cages. The sham group received no impact and the incisions were sutured closed after comparable time under anesthesia.

2.4.3. Treatment

Animals were randomly assigned to one of four treatment groups. For the edema assay: sham (n = 3), vehicle (22.5% HBC (n = 4)), PROG (10 mg/kg; n = 4) and EIDD-1723 (10 mg/kg; n = 4). HBC was used because it is water-soluble and because it has limited, if any, toxicity even when given over long periods of time (Gould and Scott, 2005). Treatment was administered i.m. at 1 and 6 h post-TBI. Animals were euthanized at 24 h post-injury and their brains removed and sampled for edema assay. Percent change in brain tissue water content was measured.

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<th>Table 1</th>
<th>Experimental design of pharmacokinetics study.</th>
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<td>Group</td>
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Fig. 1. Schematic of EIDD-1723 synthesis.
For the behavioral study, rats were randomly assigned (n = 8/group) to sham-vehicle, lesion-vehicle, lesion-PROG and lesion-EIDD-1723 groups and the identity of the groups was coded to avoid experimenter bias. Rats received i.m. injections of PROG (10 mg/kg), EIDD-1723 (10 mg/kg), or HBC alternately into the right or left hind limb, at 1, 6, and 24 h and 2, 3, 4, 5, 6, and 7 days post-injury. The last 2 injections were tapered (reduced doses) to reduce PROG withdrawal syndrome (Cutler et al., 2005). After completion of the behavioral tests the rats were perfused and their brains extracted at 22 days post-injury for histological assays.

2.4.4. Edema assay
Edema was measured at 24 h after surgery. Briefly, the rats were decapitated under deep anesthesia and the brain extracted and dissected into anterior and posterior sections. The anterior section contained the entire lesion area. Each section was placed in a pre-labeled and pre-weighed tube that was immediately capped. Each tube was reweighed, and then opened and placed in a 60 °C oven with 15 mmHg vacuum pressure for 48 h. Samples were reweighed after drying. All weighing was done on the same balance. The percent water content was calculated by [(wet wt - dry wt)/wet wt] × 100. The percent difference in water content between the anterior peri-contusional and the posterior distal section was calculated for each sample by: [(anterior H2O% – posterior H2O%)/(anterior H2O%)] × 100.

2.4.5. Behavioral/functional assays
2.4.5.1. Grip strength. The rats were tested pre-injury to establish a baseline performance on grip strength, then retested at 4, 9, and 21 days post-TBI. To check forelimb grip strength, we used an electronic digital force gauge grip-strength meter (Columbus Instruments, Columbus, OH, USA) that measures the peak force exerted by an animal while gripping the sensor bar. A digital reading (in Newtons) of three successive trials was obtained for each rat, and the best (highest) score was used for data analysis.

2.4.5.2. Sensory neglect. The animals were tested pre-injury to establish a baseline performance for sensory neglect, then retested at 4, 9 and 21 days post-TBI. An adhesive removal test was used to evaluate somatosensory function. Adhesive (half-inch round) labels were placed on the ventral surface of the forepaw. The latency to remove the adhesive label for each rat was recorded manually. If a rat was unable to remove the sticker after 120 s passed, the sticker was removed by the experimenter and the animal was given a maximum latency of 2 min. Two trials per animal were given and the best latency was used for final analysis.

2.4.5.3. Morris water maze (MWM) spatial learning performance. All the rats were tested for cognitive and spatial navigational performance through MWM starting at day 11 and ending at day 17 (7 consecutive days) post-TBI. An overhead camera and computer-assisted tracking system (San Diego Instruments Inc., San Diego, CA, USA) recorded the rat's position in the maze, swim distance, heading, and time taken to find the platform (latency). Each rat received 2 trials per day. The trials were separated by a 5-min interval and used a different quadrant location. The rats were allowed to swim in the pool until they reached the platform or until 90 s had passed. If rats were unable to reach the platform within the allotted time, the experimenter guided them to the platform. Rats were allowed to remain on the platform for 20 s and then removed from the pool. After 5 min, subjects were again released into the tank from a new position and allowed to swim to the platform. The platform remained in the same position for both trials on all days of testing. Between and after trials, animals were placed in holding cages in front of a warm air blower to dry.

2.4.6. Tissue preparation
For histological analysis brains were extracted after transcardial perfusion with 4% formalin. After 24 h of post-fixation in 4% paraformaldehyde solution, brains were immersed in 10%, 20% and 30% sucrose solution, then mounted and frozen using dry ice. The brains were cut into 20-μm sections on a cryostat and stored at -80 °C on 1% gelatin-coated slides. Slides were stained in 0.1% cresyl violet solution (0.1 g cresyl violet acetate and 0.3 ml of glacial acetic acid dissolved in 100 ml distilled water) for 10 min at 45 °C, and then rinsed in distilled water. Then slides were washed in 95% alcohol for 5–10 min and 100% alcohol (2 × 5 min each), and then in xylene (3 × 5 min each). Slides were mounted with xylene-based cytoseal.

2.4.7. Necrotic cavity measurements
Six sections from each rat brain (4, 7, 3, 7, 2, 7, 0.7 anterior and 0.3 mm posterior to bregma), were chosen for lesion cavity measurements. Stained slides were scanned using a Silverfast Pathscan Enabler IV scanner. Scanned images were analyzed with Image-J software, which permits precise tracing of an image and calculation of its surface area. The percentage of remaining tissue from a single section was calculated by tracing the perimeter of the remaining brain tissue, determining its surface area, dividing this by an estimate of the total surface area of the section (taken by tracing both the remaining tissue and the estimated perimeter of the necrotic cavity), and multiplying by 100. The percentage of remaining tissue for each rat was determined by averaging the percentage of remaining tissue across the six selected sections.

2.4.8. Immunohistochemistry
Histologic cryosections (20 μm in thickness) were selected for immunohistochemical analysis and immunolabeled for glial fibrillary acidic protein (GFAP) and microglia-specific protein Iba1. Sections were air-dried for 1 h and washed in 1× phosphate buffered saline (PBS) 5 times, then incubated in with protein blocking serum at room temperature (RT) for 1 h. Sections were then incubated with primary antibody GFAP (1:200, Millipore, Billarica, MA, USA) and Iba1 (1:1000, Wako, Richmond, VA, USA) for 1 h at RT and then washed with 1× PBS 5 times followed by incubation with secondary goat anti mouse (1:100, Alexa Fluor 488 IgG (H+L)), A11001, Invitrogen, Carlsbad, CA, USA) and goat anti rabbit (1:100, life technologies IgG (H + L), F2765, for 30 min at RT. Slides were washed with 1× PBS 5 times and covered with mounting medium (Vectashield). The slides were examined under a fluorescence microscope and pictures were taken from the peri-contusion area using a digital camera mounted on a microscope (Olympus BX41, Olympus America, Inc., Center Valley, PA, USA).

2.4.9. Statistics
All results were expressed as mean ± standard error of the mean and calculations were obtained using SPSS 11.0 software. All behavioral data were analyzed by repeated-measures analysis of variance (ANOVA) followed by a Tukey's post-hoc test for individual comparisons among the groups. Other results were analyzed with one-way ANOVA followed by LSD post-tests for multiple comparisons. The criterion for statistical significance was set at p < 0.05.

3. Results
3.1. PK study of EIDD-1723 in rats
EIDD-1723 was dosed i.m. at 3, 10 and 30 mg/kg and the resulting concentration versus time plots (Fig. 2A) show that the parent compound is rapidly cleaved after an i.m. injection and converted to the active metabolite EIDD-036, which was observed in plasma in a dose-dependent manner and demonstrated first-
plasma, thus present in concentrations that were slightly lower than those in the effective dose in our proof-of-concept study, we did not show in the dose-response study of PROG (3, 10, and 30 mg/kg) efficacy at the same dose of PROG, 10 mg/kg, was used to compare the efficacy to PROG in attenuating BWC/edema.

3.3. Functional outcome study

3.3.1. Grip strength

I.m. administration of EIDD-1723 improved grip strength and showed equivalent efficacy to PROG following CCI. Peak force exerted to grip the bar assembly showed significant group effects \((F(3,28) = 13.26, p < 0.001)\) effects. Grip strength decreased significantly \((p < 0.05)\) in rats subjected to CCI \((8.13 \pm 0.64\ N, 10.06 \pm 0.29\ N, 11.38 \pm 0.21\ N\) at 4, 9 and 21 d post-injury, respectively) at all time points compared to sham-operated rats \((12.05 \pm 0.51\ N, 13.26 \pm 0.58\ N, 15.09 \pm 0.72\ N\) at 4, 9, and 21 d post-surgery, respectively). Post-hoc analyses showed that repeated treatments with 10 mg/kg PROG and EIDD-1723 after CCI significantly \((p < 0.05)\) improved grip strength at 4, 9 and 21 d (Fig. 4).

3.3.2. Sensory neglect

I.m. administration of EIDD-1723 improved sensory neglect and showed equivalent efficacy to PROG following CCI. Latency to remove adhesive tape from the forepaw showed significant group effects \([F(3,28) = 15.11, p = 0.001]\) effects. There were significant \((p < 0.05)\) deficits in latency to remove the sticky tape in CCI- + vehicle-treated rats \((108.13 \pm 11.87, 66.13 \pm 10.99, 41.25 \pm 13.75, 14.9, 9, 21\) d post-CCI, respectively) at all time points compared to shams \((10.25 \pm 150, 12.13 \pm 2.31, 6.63 \pm 2.53, 14.9, 9, 21\) d post-surgery, respectively). Repeated i.m. treatments with PROG and EIDD-1723 after CCI significantly \((p < 0.05)\) decreased the latency to remove the sticker at 4 and 9 d but not at 21 d post-CCI when all animals had recovered on this task (Fig. 5).

3.3.3. Spatial navigation performance in the MWM

This task allows measurement of working (short-term, trial-to-trial) and reference (longer-term, day-to-day) memory. During the 7 days consecutive days of testing the latency to escape the water maze and find the platform was measured, and the data were recorded for two trials 5 min apart. The first trial of the day was taken to be a measure of reference or long-term memory (there was a 24-h delay between the last trial of the day and the first trial of the next day). After the CCI, we found significant cognitive impairments in the vehicle group in MWM learning. Repeated measures one-way ANOVA on duration showed significant group effects on Trial

3.2. Brain water content (edema)

Brain water content (BWC) was measured to evaluate the potential efficacy of the EIDD-1723 analog relative to PROG in reducing cerebral edema following injury. In order to use the most effective dose in our proof-of-concept study, we first conducted a dose-response study of PROG (3, 10, and 30 mg/kg) efficacy (data not shown in figures) to reduce edema at 24 h post-CCI. We found decreases in edema of 30.88, 58.17 and 49.80% with 3, 10, and 30 mg/kg doses of PROG, respectively. The most effective dose of PROG, 10 mg/kg, was used to compare the efficacy at the same dose of EIDD-1723.

Significantly \((p < 0.05)\) greater BWC was found in the lesion + vehicle group around the peri-contusional site compared to the sham-vehicle group (Fig. 3). All surgical groups showed higher BWC than the sham group. Compared with the vehicle group, both PROG and EIDD-1723 treatments reduced BWC to significantly \((p < 0.05)\) lower levels. EIDD-1723 showed equivalent efficacy to PROG in attenuating BWC/edema.
Post-hoc tests revealed a significant difference between CCI + vehicle and sham + vehicle rats. Progesterone- (PROG) and EIDD-1723-treated rats showed significantly ($p < 0.05$) improved grip compared to the vehicle group.

Intramuscular treatment with PROG and EIDD-1723 showed progressive reduction in time taken to reach the hidden platform that became significant ($p < 0.05$) at day 7 compared with vehicle groups on both Trial 1 and Trial 2 (Fig. 6). EIDD-1723 showed equivalent efficacy to PROG in latency to escape the water maze and find the platform.

### 3.4. Necrotic cavity

Briefly, the 6 sections from each rat brain were photographed with an Epson scanner and ImagePro™ software. Group means of percent of damaged tissue averaged across 6 anterior-posterior sections were used to determine overall lesion size. There was a significant main effect of lesion/treatment on lesion size ($F (2,21) = 3.53, p < 0.05$). Post-hoc comparisons further revealed a significant difference between the CCI + vehicle and PROG-treated groups ($p < 0.05$) and between the EIDD-1723 and vehicle-treated groups ($p < 0.05$) (Fig. 7).
Gliosis

The immunohistochemical assay showed a significant increase in astrogliosis (GFAP immunoreactivity) and microgliosis (Iba1 immunoreactivity) in the brain tissues of the vehicle group. Both PROG and EIDD-1723 treatments markedly decreased the intensity of GFAP (Fig. 8) and Iba1 (Fig. 9) at 3 weeks post-injury compared to vehicle ($p < 0.05$). EIDD-1723 showed equivalent efficacy to PROG in attenuating gliosis.

Discussion

We compared the neuroprotective actions of a novel PROG analog, EIDD-1723, to those of PROG. In this proof-of-concept study, compared to vehicle-treated animals, EIDD-1723 showed significantly better edema reduction, gait improvement, cognitive recovery, and smaller lesion volumes that were closely comparable to those following PROG treatment.

Multiple studies have reported PROG’s edema-attenuating effects in a number of animal models of brain injury (Guo et al., 2006; Maghool et al., 2013). An inverse correlation between serum PROG level and degree of edema has been reported (Wright et al., 2001). More recently it was demonstrated that compared to dexamethasone, a corticosteroid drug commonly used to reduce brain swelling during neurosurgical procedures, PROG had no side effects and was equally effective in reducing brain edema and more effective in attenuating acute cellular inflammatory responses (Xu et al., 2014).

In previous studies evaluating edema by measuring BWC, we screened multiple PROG analogs for efficacy in a rat CCI model (MacNevin et al., 2009; Guthrie et al., 2012). For the current study we chose the i.m. route of administration because any field-ready therapy for TBI will likely need to be delivered i.m. in an aqueous formulation to allow for rapid release into circulation. Three small, single-center clinical studies have now shown positive outcomes with low-dose i.m. PROG (Xiao et al., 2008; Mofid et al., 2016; Raheja et al., 2016), but large phase III clinical trials using i.v.

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**Fig. 8.** Immunostaining of glial fibrillary acidic protein (GFAP, a marker of astrocytes) in the peri-contusional area at 22 days post-traumatic brain injury. A, D, G and J at low magnification ($10 \times$) and B, E, H and K at higher magnification ($40 \times$). Immunostaining pictures showed higher intensity of GFAP in the vehicle group (B, F, J) compared to the control group (A, E, I). Treatment with PROG (C, G, K) and EIDD-1723 (D, H, L) significantly reduced the expression of astrocytes. The scale bar represents 10 μm. Graph (M) showing quantification of GFAP intensity. Data expressed as mean ± SE. *CCI + Veh vs. Sham + Veh; # CCI + Veh vs. CCI + PROG or EIDD-1723.
administration at much higher doses of the hormone had negative results (Wright et al., 2007, 2014; Skolnick et al., 2014).

We compared the efficacy of the 10-mg dose of EIDD-1723 to that of PROG at the same dose. EIDD-1723 showed equivalent efficacy to PROG in attenuating BWC/edema. We found significant differences in necrotic cavity size in the injury-alone compared to the PROG- and EIDD-1723-treated groups. The reduction of the injury-induced necrotic cavity in the present study is likely the result of the ability of EIDD-1723 to attenuate edema during the acute phase of injury. The current results are in agreement with our earlier work showing that, when given after bilateral brain injury, PROG analogs have functional and edema-attenuating activity and support the potential utility of these compounds for the treatment of brain injury (MacNevin et al., 2009; Guthrie et al., 2012).

Although measurement of edema is useful in the screening and evaluation of efficacy, it alone cannot provide a prognosis concerning the extent of, and recovery from, cognitive, sensory, and motor deficits. Behavioral outcome measures are essential to this purpose. PROG has been shown to promote functional recovery in different brain injury models including stroke and TBI (Djebaili et al., 2004; Gibson and Murphy, 2004; Grossman et al., 2004; Gibson et al., 2005; Chen et al., 2007; Wali et al., 2011; Tang et al., 2013; Geddes et al., 2014; Robertson and Saraswati, 2014; Wali et al., 2014; Yousuf et al., 2014a,b; Peterson et al., 2015). Our results in the present study show that besides reducing the water content in the penumbral region at one day after TBI, EIDD-1723 significantly improved functional recovery and reduced accumulation of astrocytes and microglia, cells associated with post-injury inflammatory processes that can impair recovery of function.

In TBI patients, sensorimotor and cognitive deficits are well recognized (Writer and Schillerstrom, 2009; Sun and Feng, 2014). The sensory neglect test is used in rats to evaluate the sensory-motor deficits caused by brain injury (Bouet et al., 2009) and it can be used to assess long-lasting impairments and recovery in animal models of TBI (Teasdale et al., 1998). In the present study we found that PROG and EIDD-1723 treatment ameliorated sensory

**Fig. 9.** Immunofluorescence of Iba1 (a marker of gliosis) in the peri-contusional area at 22 days post-traumatic brain injury. A, B, C and D at low magnification (10×) and E, F, G and H at higher magnification (40×). Immunostaining pictures showed higher intensity of Iba1 in the vehicle group (B, F, J) compared to the control group (A, E, I). Treatment with progesterone (C, G, K) and EIDD-1723 (D, H, L) significantly reduced the expression of Iba1. The scale bar represents 10 μm. Graph (M) showing quantification of Iba1 intensity at 22 days post-injury. Data expressed as mean ± SE. *CCI + Veh vs. Sham + Veh; # CCI + Veh vs. CCI + PROG or EIDD-1723.
neglect at 4 and 9 days after CCI. The rats' grip strength, impaired by the injury, was also significantly improved in the PROG- and EIDD-1723-treated groups.

Cognitive impairments, such as the partial inability to form or store new memories, and visuo spatial deficits are among the most common characteristics of mild to severe TBI (Dikmen et al., 2009; Sigurdardottir et al., 2009; Bagiella et al., 2010; Meffre et al., 2005). The MWM test is widely used to assess these cognitive functional deficits. The hidden platform test employed in the present study is considered a reliable measure of spatial working memory (Janus, 2004). We note that EIDD-1723 treatment improved spatial learning performance in the MWM task as comparable to the PROG effects observed, and in agreement with previous reports demonstrating PROG-mediated recovery and improved behavioral performance in rats (Roof et al., 1994). In the present study, the brain-injured groups were impaired on the acquisition phase of MWM testing compared to the control group. However, the PROG- and EIDD-1723 treated groups showed improved performance in finding the hidden platform over the Vehicle animals.

After brain injury, including TBI, GFAP expression increases in astrocytes, and activated astrocytes release inflammatory cytokines and disrupt functional recovery (Hoane et al., 2003a,b). A large body of literature now demonstrates PROG is a potent anti-inflammatory agent (Stein, 2008; Schumacher et al., 2015) that can mediate microglial activity after brain injury (Drew and Chavis, 2000). The analysis of reactive gliosis around the lesion showed that PROG and EIDD-1723 markedly decreased gliosis (GFAP and Iba1 immunostaining). Previous studies have shown PROG-induced reduction in GFAP expression following TBI (Liu et al., 2014). These results suggest that EIDD-1723 administration following CCI significantly reduces tissue damage at the site of injury. The frontal cortex is especially vulnerable to working memory deficits and deficits on the adhesive removal test (Hoane et al., 2003a,b), so the reduction in lesion size is likely responsible for the protection from behavioral impairments in the sensory neglect and spatial navigation tasks.

We also found that after i.m. injection, EIDD-1723 is rapidly cleaved, generates high levels of the active steroid EIDD-036, and passes through the blood-brain barrier at therapeutic levels, a critical property for a TBI treatment. EIDD-036 levels in brain were approximately the same as those in plasma at any given time point, indicating that the material readily penetrates the blood-brain barrier. After i.m. injection, only EIDD-036 was observed in plasma, while the parent conjugate, EIDD-1723, was never observed in levels above the limit of detection. Our previous studies have shown the intermediate oximes to be water-insoluble (<15 μg/ml) but intrinsically active metabolites of C-20 PROG analogs (Guthrie et al., 2012).

PROG is a pleiotropic hormone that uses multiple signaling pathways including the regulation of gene expression after binding to intracellular progesterone receptors (PR). In addition to the classical PR, PROG also interacts with other signal transduction mechanisms such as the α1 receptor, for which it is a competitive inhibitor and through which it may reduce N-methyl-D-aspartate (NMDA) glutamate signaling (Hanner et al., 1996; Bergeron et al., 1999). PROG also signals at the nicotinic acetylcholine receptor (nAChR) (Valera et al., 1992) and affects gamma-aminobutyric acid (GABA) through its 5α-reduced metabolite allopregnanolone and positive modulation of the GABA_A receptor (Belletti et al., 2002; Losel et al., 2002; Mani, 2006; Schumacher et al., 2007). PROG is also known to activate the pregnane X receptor (PXR) and the membrane surface receptor 25-Dx (Meffre et al., 2005; Guennoun et al., 2008). Our previous findings have shown that progesterin-mediated pro-survival response following TBI is regulated either independently of the classical PR or via nongenomic PR-regulated actions (Vanlandingham et al., 2006). Taken together, these findings suggest that synthetic steroids with physicochemical properties similar to those of natural forms can function at the molecular level by mechanisms other than classically mediated transcription. The observation that EIDD-1723 and PROG have comparable functional effects suggests that they may act through some of the same mechanisms, but it is also possible that PROG analogs are acting through a novel receptor mechanism not yet defined. The focus of this study was on comparing the novel analog with PROG's efficacy on the enhancement of functional recovery. The molecular mechanisms of EIDD-1723-mediated neuroprotection will require further attention.

Our results demonstrate that EIDD-1723 is equally as effective as PROG in attenuating edema and lesion volume and promoting motor improvement and cognitive recovery. Importantly, EIDD-1723 is highly soluble and stable in water-based formulations, and can therefore be readily administered in the field by emergency personnel. Like the i.v. route, i.m. administration bypasses the hepatic metabolism, avoiding the first-pass effect and resulting in higher bioavailability. These are crucial properties, as the failure of very high-dose, i.v. PROG in the phase III clinical trials (Skolnick et al., 2014; Wright et al., 2014), in addition to other dosing (Howard et al., 2015) and trial design factors, may have been due in part to the long interval between injury and initial treatment necessitated by formulation requirements. This new analog of PROG will have the same therapeutic benefits of PROG, but without the inherent limitations for administration in emergency situations. The ability of first responders to rapidly administer EIDD-1723 in the field makes it an attractive compound to consider for TBI and/or stroke treatment.

Conflict of interest

IS, DCL, DG, MN and DGS along with Emory University retain patents related to the use of progesterone analogs and its uses but have no financial gains, royalties or licensing agreements from research on progesterone analogs. BW and NT declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.05.017.

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Howard, T.M., Izawa, K., Chen, H., Hidaka, H., Atsumi, M., 2015. Phase III TBI clinical trials with EIDD-1723: trial design factors, may have been due in part to the long interval between injury and initial treatment necessitated by formulation requirements. This new analog of PROG will have the same therapeutic benefits of PROG, but without the inherent limitations for administration in emergency situations. The ability of first responders to rapidly administer EIDD-1723 in the field makes it an attractive compound to consider for TBI and/or stroke treatment.

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References

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