Award Number: W81XWH-10-1-0623

TITLE: Operation Brian Trauma Therapy

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REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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**REPORT DATE (DD-MM-YYYY)**: October 2012  
**REPORT TYPE**: Annual  
**DATES COVERED (From - To)**: 30 September 2011 - 29 September 2012

**4. TITLE AND SUBTITLE**: Operation Brian Trauma Therapy

**5a. CONTRACT NUMBER**: W81XWH-10-1-0623

**5b. GRANT NUMBER**: W81XWH-10-1-0623

**5c. PROGRAM ELEMENT NUMBER**:  
**5d. PROJECT NUMBER**:  
**5e. TASK NUMBER**:  
**5f. WORK UNIT NUMBER**:  

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Pittsburgh, PA  15213

**8. PERFORMING ORGANIZATION REPORT NUMBER**:  

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**:  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland  21702-5012

**10. SPONSOR/MONITOR’S ACRONYM(S)**:  
**11. SPONSOR/MONITOR’S REPORT NUMBER(S)**:  

**12. DISTRIBUTION / AVAILABILITY STATEMENT**: Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**: Please see next page.

**14. ABSTRACT**: Please see next page.

**15. SUBJECT TERMS**: Traumatic Brain Injury, blast, treatment, therapy, biomarker, combat casualty care

**16. SECURITY CLASSIFICATION OF:**  
a. REPORT: U  
b. ABSTRACT: U  
c. THIS PAGE: U

**17. LIMITATION OF ABSTRACT**: UU

**18. NUMBER OF PAGES**: 59

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(include area code)
Operation brain trauma therapy (OBTT) is a multicenter, pre-clinical, drug screening and brain injury biomarker development consortium for TBI. OBTT includes investigators at the Safar Center (University of Pittsburgh), the University of Miami, WRAIR, Virginia Commonwealth University, and Banyan Biomarkers. Three rodent models (controlled cortical impact, parasagittal fluid percussion, and penetrating ballistic-like brain injury) are used for drug screening with the most promising candidates tested in a micropig model. We have completed studies with nicotinamide, erythropoietin (EPO), and cyclosporine-A (CsA), and have just begun testing of simvastatin. Studies with nicotinamide suggest some benefit of 50 mg/kg on motor outcomes, but variable benefit on cognitive outcomes. Studies with EPO did not appear promising. Studies with CsA have been completed; data analysis is ongoing. Studies of the serum brain injury biomarker GFAP from these rats have provided the first ever cross-model biomarker comparison and suggest that GFAP may be useful for drug screening, since nicotinamide treatment significantly reduced serum GFAP levels in two models. A consortium overview was published in Journal of Trauma and numerous abstracts were presented at the 2011 ATACCC, the 2012 NNT congress, and the 2012 MHSRS conference.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Body</td>
<td>1</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>14</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>14</td>
</tr>
<tr>
<td>Conclusion</td>
<td>15</td>
</tr>
<tr>
<td>References</td>
<td>16</td>
</tr>
<tr>
<td>Appendices</td>
<td>18-55</td>
</tr>
</tbody>
</table>
INTRODUCTION

Operation brain trauma therapy (OBTT) is a unique multi-center, pre-clinical, drug screening and brain injury biomarker development consortium for the ultimate translation of the best potential drugs to clinical trials in traumatic brain injury (TBI, Figure 1). OBTT includes investigators at the Safar Center for Resuscitation Research (University of Pittsburgh School of Medicine, Patrick Kochanek, MD, PI; C. Edward Dixon, Co-I), the Miami Project to Cure Paralysis, (University of Miami School of Medicine, W. Dalton Dietrich, site PI; Helen Bramlett, Co-I), the Neuroprotection program at WRAIR (Frank Tortella, site PI; Deborah Shear, PhD, and Kara Schmid, PhD, Co-Is), Virginia Commonwealth University (John Povlishock, PhD, site PI) and Banyan Biomarkers (Kevin Wang, PhD, PI, and Ronald Hayes, PhD Co-I). Three rodent models (controlled cortical impact [CCI], parasagittal fluid percussion injury [FPI], and penetrating ballistic-like brain injury [PBBI]) are used in Pittsburgh, Miami, and WRAIR, respectively, for primary drug screening with the most promising candidates tested in a micropig model at Virginia Commonwealth University. Additional secondary screening of the most promising drugs is also carried out in more complex rodent models with polytrauma, hemorrhage or advanced monitoring, as appropriate. The principle concept and overall hypothesis of OBTT is that clinical TBI is a heterogeneous disease process that involves multiple brain injury phenotypes and that success of an agent tested across multiple established TBI models will identify the best candidates for success in clinical trials. Two types of drugs will be screened, 1) low hanging fruit (drugs already FDA approved for other uses, or otherwise ready for clinical translation) and 2) higher risk but potentially high reward more novel targeted therapies. However, drugs in the latter category should already have at least some track record of success in experimental TBI.

BODY

Administrative overview of accomplishments in year 2 of funding
Safar Center for Resuscitation Research (Patrick M. Kochanek, MD, overall PI)

Year 2 has been a highly productive one for the OBTT consortium. We have now studied three therapies (Nicotinamide, Erythropoietin, and Cyclosporine-A) across the centers and models in over 350 rats, and we are now beginning to test a fourth therapy (Simvastatin). We have also selected therapies 5 and 6 (Levetiracetam and Minocycline), which will similarly be evaluated across models this year. For each therapy, a detailed and comprehensive review of published studies is assembled (please see Appendix 1, manual of operations). Final therapy selection each year has taken place at an annual site PIs investigator meeting at the Congress of the National Neurotrauma Society. The dosing plan for each therapy is developed based on the literature review. For each agent, 4 experimental groups have been used in primary screening, namely, sham, injury plus vehicle, and injury plus treatment at two different doses. The overall approach to primary screening of therapies at the Pittsburgh site is shown in Figure 2A-C as an example. The Morris water maze (MWM) is used to assess cognitive outcome and is the primary outcome parameter across sites. Motor testing is also carried out at each site, but varies depending on the model. In addition, lesion volume and hemispheric and/or cortical tissue loss are also assessed at each site. However, the drug, dose, treatment regimen, and biomarker sampling is identical between sites. The results of some of the work carried out in year two have also been published and/or presented. Please see items 7-15 in reportable outcomes.
The consortium has also held a monthly 1 hour conference call that has included a representative from each site and we have held a very productive face-to-face investigators meeting each year, as indicated above. Therapy selection for the year is one of the agenda items each year. In addition, Dr. Kochanek sent a full report to each of the members of the “Therapy and Oversight Committee” and once again received their input. In addition, our consults on functional outcome assessments (Dr. Robert Hamm) and Biostatistics (Dr. Stephen Wisniewski) were also appraised of our plan and they contributed recommendations to our work. Finally, it is noteworthy that in 2012, Dr. Kochanek was invited to give plenary presentations on OBTT at both the annual congress of the National Neurotrauma Society and the MHSRS. These were well received. He will also presented at the Department of Defense TBI pharmacological in progress review, on October 2nd, 2012, at Fort Detrick and contributed to the Pharmacology working group on October 3rd led by COL Salzer and Dr. Ramon Diaz-Arrastia.

**Primary screening in rodent models of TBI**

*Primary Screening Site 1. Safar Center for Resuscitation Research, University of Pittsburgh School of Medicine (Patrick Kochanek, MD, C. Edward Dixon, PhD)*

An overview of the approach being taken for primary drug screening, biomarker sampling and outcome testing at the Pittsburgh site was previously shown in Figure 2.

**Drug #1 Nicotinamide:**

Treatment or vehicle was administered at 15 min and 24 h after injury—and this identical dosing approach was used at all primary screening centers. The data shown for nicotinamide at all sites is final including 21 day neuropathology. At the Pittsburgh site, the TBI model that is being used for screening is the CCI model in adult rats which was developed by OBTT Co-investigator Dr. C. Edward Dixon, who oversees all of the rat studies using this model in Pittsburgh. The model is well established and expands on the stereotaxic method for inducing traumatic brain injury described by B scout et al. (1998).

**Table 1. Scoring matrix for assessment of therapeutic efficacy across models in OBTT**

<table>
<thead>
<tr>
<th>Site</th>
<th>Neuro Exam</th>
<th>Motor</th>
<th>Cognitive</th>
<th>Neurpath</th>
<th>Biomarker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitt</td>
<td>None</td>
<td>Beam balance (2)</td>
<td>Hidden platform latency (5)</td>
<td>Lesion volume (2)</td>
<td>GFAP</td>
</tr>
<tr>
<td>Miami</td>
<td>None</td>
<td>Cylinder (2)</td>
<td>Hidden platform latency (2)</td>
<td>Lesion volume (2)</td>
<td>GFAP</td>
</tr>
<tr>
<td>WRAIR</td>
<td>Neuroscore (1)</td>
<td>Rotarod (3)</td>
<td>Hidden platform latency (2)</td>
<td>Lesion volume (2)</td>
<td>GFAP</td>
</tr>
</tbody>
</table>

**Table 1. Outcome scoring matrix used for primary screening in rat studies in OBTT.** Note that each therapy tested can generate a maximum of 22 points at each center and thus a 66 point grand total overall. Also note that cognitive outcome is given the greatest weight in the scoring matrix given the importance of this parameter to clinical outcomes for drug development.

**Figure 2.** Protocol overview for primary screening in rats in Pittsburgh.
characterized in Pittsburgh. In general, for all drug studies in the CCI model, ~40 rats are studied with ~10/group, namely, sham, TBI-Vehicle, TBI-50 mg/kg, and TBI-500 mg/kg.

Sensorimotor Function

Beam Balance: In the beam balance test, the TBI + Vehicle and TBI + Nicotinamide (50 mg/kg) groups differed significantly from the Sham + Vehicle group. The TBI + Nicotinamide (500 mg/kg) showed significant improvement on this task, suggesting a benefit from the high dose on nicotinamide on beam balance performance (Figure 3).

Beam Walking: In the beam walking test, the TBI + Vehicle and TBI + Nicotinamide (50 mg/kg) groups differed significantly from the Sham + Vehicle group, demonstrating an obvious functional deficit. In contrast, the TBI + Nicotinamide (500 mg/kg) group did not differ from the Sham group, suggesting a modest benefit from the high dose on nicotinamide treatment on beam walking performance (Figure 4). The motor effects shown are consistent with the published literature on nicotinamide in experimental TBI, where benefit of nicotinamide has been shown on motor function and neuropathology.

Cognitive Function

MWM task: The only statistical group difference in latency to find the hidden platform was between the Sham + Vehicle and TBI + Nicotinamide (50 mg/kg) groups (Figure 5). And surprisingly, the 50 mg/kg dose of nicotinamide worsened performance in the MWM task. There was also no benefit of treatment assessed on probe trial, and also no difference between the experimental groups in swim speed measured on day 19 post injury (data not shown).

Serum biomarker data for drug #1 from the Pittsburgh site are presented later in the report from the Banyan Biomarker site.

Neuropathology

Nicotinamide produced significant beneficial effects on neuropathology in the CCI model, specifically, a reduction in hemispheric tissue loss only at the high dose (Figure 6). There was a trend toward a reduction in contusion volume that did not reach significance. This again appears to mirror the literature, where beneficial effects of nicotinamide on tissue loss have been reported by others. For Tier B primary screening in Pittsburgh, we are using an established mouse model of CCI followed
by 35 min of hemorrhagic shock (CCI+HS) based on the work of Hemerka et al (2012), with minor
modifications. This model is being used to simulate more complex polytrauma insults commonly seen in combat casualty care. We carried out 20 studies (10 vehicle and 10 high dose nicotinamide treated mice) and have followed the mice for 7 d. No benefit was seen on CA1 hippocampal neuronal death for nicotinamide therapy in this model; the primary outcome for the combined injury model.

Drug #2, Erythropoietin (EPO):
Based on a comprehensive review that identified 31 studies in experimental TBI supporting its potential efficacy, EPO was selected by the OBTT consortium as drug #2 for primary screening. Based on that review, two doses were selected, i.e., 5000 or 10,000 IU/kg, by a single IV injection administered at 15 min after injury. This treatment regimen was used at all of the sites. Once again, at the Pittsburgh site, we used four groups, sham, TBI + vehicle, and TBI + treatment at low and TBI + treatment at high doses, with an overall sample size of ~10 rats per group.

Surprisingly, we did not detect any beneficial effect of EPO across any outcome in the CCI model at either dose. This included motor testing, MWM, probe trial, or neuropathology. This was seen despite an excellent and consistent injury effect vs. sham. Also, as discussed later, the serum biomarker data confirmed lack of efficacy. Selected outcomes for EPO in the CCI model are shown in Figures 7 and 8.

Drug #3, Cyclosporine-A. We have just completed testing this agent and are in the midst of data analysis at the Pittsburgh and other sites.

Primary Screening Site 2. Miami Project to Cure Paralysis, University of Miami School of Medicine (W. Dalton Dietrich, PhD, Helen Bramlett, PhD)
At the Miami site, the TBI model used is parasagittal FPI in adult rats, which they have highly characterized.

Drug #1 Nicotinamide:
All animals for study drug #1 (nicotinamide) have been completed surgically at the Miami site. The number of groups are again Sham, TBI-Vehicle, TBI-50 mg/kg, and TBI-500mg/kg, identical to the Pittsburgh site. At the Miami site, all rats receive arterial catheters. Thus this site for all drugs is serving an important additional function, namely, to determine the systemic physiological effects of the doses selected for each therapy. In this regard, at the initiation of the study, the injection of the nicotinamide resulted in a decrease in mean arterial blood pressure (MAP) <90mmHg. Using this important information from the Miami group, we thus adjusted the injection protocol at all injury sites to a slower injection over 15 min and this attenuated this unacceptable drop in blood pressure. Complete physiological data are available (upon request) from the Miami site for each animal in each drug tested however these data are beyond the space considerations for this report.

Sensorimotor function

Sensorimotor testing was performed using the cylinder task (Figure 9) and the rotarod test (Figure 10). The cylinder task is measured as an asymmetry index. An index of 0.5 indicates equal use of the contra- and ipsilateral forelimbs; scores below 0.5 indicate greater use of the forelimb ipsilateral to the injury (i.e., a contralateral limb deficit). Shams exhibited no deficits on this task. All TBI groups had contralateral limb deficits with the TBI-500mg/kg nicotinamide-treated rats showing less of a deficit in the contralateral limb use relative vs. the other TBI groups—again suggesting a benefit of nicotinamide on motor function after TBI, similar to what was observed in CCI. For the rotarod test, sham rats were able to stay on the rotarod slightly longer than the other TBI groups. However, there was no statistically significant effect of treatment at either dose on this task.

Cognitive Outcome

Cognitive function was assessed on using a simple place task (Figure 11) tested over 4 d followed by a working memory test (Figure 12). Shams showed reduced latencies over the 4 d testing period. All three TBI groups had increased latencies vs. sham with the two treated TBI groups exhibiting more cognitive deficits on this task. Thus, nicotinamide treatment did not improve learning and memory on this paradigm—similar to what was seen for CCI and also in the PBBI model (see below). On the working memory task (Figure 12), while sham rats as expected showed the greatest improvement in the delay match-to-place task, rats treated with nicotinamide at the 500mg/kg dose did show improvement on this task as well. The 50 and vehicle treated groups were significantly different than sham, however, the 500 group was not. This was the only evidence for a potential cognitive benefit from nicotinamide treatment—in this case, in the parasagittal FPI model.
Neuropathology

Nicotinamide produced a trend toward a reduction in hemispheric tissue loss and contusion volume, with the trend greatest again at the high dose. However, unlike CCI, this did not reach statistical significance (Figure 13). Similarly, a trend toward a reduction in contusion volume was seen at the high nicotinamide dose vs vehicle, but this again did not reach significant in the FPI model (data not shown).

Serum biomarker data are discussed later in the report on work at the Banyan site.

Drug #2, EPO:
At the Miami site, drug #2, EPO was studied in an identical manner to that used in the other models. An identical dosing and treatment regimen, namely 5000 or 10,000 IU/kg IV as a single dose at 15 min post injury was used. Again, as in all primary screening studies, sham and vehicle treatment groups were performed. Hemodynamic assessments did not reveal any concerns with EPO treatment. As was observed in the CCI model, EPO did not produce any beneficial effect across any outcome in the FPI model at either dose. This included motor testing, MWM, probe trial, and neuropathology. Indeed, there was actually a trend toward worse outcome in the MWM latency task, and the low (5000 IU) EPO worsened working memory vs. both sham and TBI vehicle. Selected outcomes for EPO in the CCI model are shown in Figures 14 and 15. The serum biomarker data again confirmed general lack of efficacy (discussed later).

Drug #3.
Cyclosporine-A. We just completed testing this agent and are in the midst of data analysis at the Miami site.

Primary Screening Site 3. WRAIR (Frank Tortella, PhD, Deborah Shear, PhD, Kara Schmid, PhD).
WRAIR serves as the primary screening center using a military-relevant model of PBBI in rats. The basic PBBI model is used to screen therapies on neurobehavioral (motor and cognitive) and histopathological outcomes, and the advanced (EEG) model will be used to target injury-induced changes in higher level cortical function with the most promising agents. Tier A of the study consists of screening each agent in the PBBI model using 2 doses on neurofunctional (motor and cognitive), neuropathological outcome metrics (compared to appropriate vehicle-treated and sham injury groups), and serum biomarker profiles (Banyan), as described above for the other OBTT sites.
Drug #1 Nicotinamide: Nicotinamide (50 or 500 mg/kg), as carried out in the other OBTT primary screening sites, was delivered via a 10-min IV infusion at 15 min and 24 h post-PBBI. Neuroscore assessments of neurofunctional outcome were conducted (prior to dosing) at 15 min, 24 h, 72 h, 7 d, 14 d, and 21 d post-injury. Motor abilities were assessed on a rotarod task at 7 and 10 d post-PBBI and cognitive abilities were assessed in the MWM task on post-injury d 13-17 (see Shear et al. 2010 for additional details). Again, consistent with the other OBTT primary screening sites, serum blood draws were taken at 4 h and 24 h post-PBBI (prior to administration of the 24 h dose) and at the terminal endpoint were sent to Banyan for further processing of biomarkers results. All rats were euthanized at 21 d post-PBBI for volumetric analyses as performed at the other sites.

Sensorimotor function

WRAIR completed all neurobehavioral assessments for nicotinamide in the PBBI model. The number of rats per group were Sham (n=9), PBBI-vehicle (n=14), PBBI-50 mg/kg (n=15), PBBI-500 mg/kg (n=16). PBBI groups displayed significant motor abnormalities on the rotarod task at both 7 and 10 d post-PBBI. No significant treatment effect was detected on this task (Figure 16).

Cognitive Outcome

Cognitive outcome was assessed on using a spatial learning (hidden platform) task tested over 5 d followed by a retention (missing platform; probe trial) test. All three PBBI groups showed significant cognitive impairment indicated by longer latencies and swim distances to the hidden platform. No significant treatment effect was detected in either the spatial learning paradigm or in the retention (probe trial) task (Figure 17).

Neuropathology

Consistent with the findings in the other outcomes in PBBI, there was no significant benefit of nicotinamide at either dose on lesion volume of hemispheric volume loss (Figure 18).

Biomarker data from serum samples from the rats subjected to PBBI in the drug#1 study revealed a significant reduction in serum GFAP levels at the high treatment dose—please see results from the Banyan site below.

Drug #2 – EPO: EPO (Procrit) was administered via IV infusion at 15 min post-PBBI using the same dosing and treatment protocol that is being used at the other primary screening sites. Outcome metrics were again identical to those used for nicotinamide. WRAIR completed neurobehavioral assessments for EPO in the PBBI model using the same group design (Sham, PBBI-vehicle, PBBI-5000 IU/kg and 10,000 IU/kg).

Sensorimotor function
WRAIR completed all neurobehavioral assessments for EPO in the PBBI model. PBBI groups displayed significant motor abnormalities on the rotarod task at both 7 and 10 d post-PBBI. There was a trend toward improved Rotarod performance in the 5000 IU/kg group which was close to significance (p=0.054). However, no significant treatment effect was detected on this task (Figure 19).

**Cognitive Outcome**
Cognitive outcome was assessed on using a spatial learning (hidden platform) task tested over 5 d followed by a retention (probe trial) test. All three PBBI groups showed significant cognitive impairment indicated by longer latencies to the hidden platform. No significant treatment effect was detected in either the spatial learning paradigm or in the retention task (Figure 20).

**Neuropathology**
Consistent with the findings for other outcomes in PBBI, there was no benefit of EPO at either dose on lesion volume of hemispheric volume loss (Figure 18). Surprisingly, EPO at the low dose showed a trend toward increased tissue loss in the 5000 IU/kg group; this did not reach significance.

Biomarker data from serum samples from the rats subjected to PBBI in the EPO study revealed no effect— please see results from the Banyan site below.

**Drug #3. Cyclosporine-A.** We just completed testing this agent and are in data analysis at the WRAIR site.

Serum Biomarker Development and Application to the primary screening studies

**Banyan Biomarkers (Ronald Hayes, PhD)and the University of Florida (Kevin Wang, PhD)**
For the biomarker studies, a rigorous sampling, shipping, and processing protocol was followed.

**Biomarker sampling processing**
Blood sampling was carried out at 4 h, 24 h and 21 d, as described above. For the early time points, 0.7 mL was obtained. The final time point at sacrifice yielded 2-3 mL of blood obtained from the left cardiac ventricle via a 20-gauge needle. Blood was immediately placed in microcentrifuge tubes and allowed to clot at room temperature for 60 min. Tubes were centrifuged at 5,000xg at room temperature for 5 min. Serum samples were collected, snap frozen on dry ice, and stored at -80°C until shipped. Each sample was
Initial analysis focused on two biomarkers, a glial injury marker glial fibrillary acidic protein (GFAP) (Figure 23) and a neuronal cell body damage marker UCHL1 at the 4 and 24 h time points. Based on prior experience, it was anticipated that the 4 h sample might represent largely the response to primary injury in the various models while the 24 h sample would reflect the evolution of secondary injury— Influenced by both model and potentially by treatment. Thus, several principle analyses were carried out, namely, 1) comparison of biomarker levels across models (injury vehicle vs. sham for the respective models) at 4 h after injury to compare the primary injury, 2) assessment of the effect of treatment on serum biomarker levels at 24 h after injury, and 3) assessment of the effect of treatment on difference between 4 h and 24 h (delta 24-4 h) levels. The 4 h model comparisons shown in Figures 23 and 24 represent unique and novel studies for the field of TBI and an abstract of that specific work was presented at both the NNT Congress and MHSRS Congress. GFAP was increased in all three models and the findings suggested that it might represent an excellent candidate for theragnostic use. In contrast UCH-L1 as assessed at 4 h did not show major injury effects across models, possibly because of its short half-life. Based on this finding, we have focused on GFAP results, with regard to therapeutic effects in OBTT (and in this report) and will add a 1 h sampling time for UCH-L1 in future studies to potentially improve its utility for future studies in OBTT in pre-clinical drug screening.

Effect of Drug #1 (nicotinamide) on serum biomarker levels after TBI across OBTT

We also compared 4 h serum GFAP levels across models in the vehicle treated rats in study 1 (nicotinamide) vs. study 2 (EPO) in order to determine how consistent the injury levels were within each model in the first 2 studies, and to assess the utility of GFAP as a biomarker. We were pleased to see that the increases within each model were very consistent between studies and did not significantly differ within each model from study 1 to study 2 (Figure 25).

Serum biomarker levels – assessment of the effect of nicotinamide.

We used 24 h and delta 24-4 h serum biomarker levels to assess the effect of nicotinamide, EPO, and CsA after TBI across models in OBTT. Given the superior performance of GFAP vs. UCH-L1 discussed above, the data on GFAP represent the point of focus in this report. Figure 26 shows the studies of serum...
Figure 26. Effect of nicotinamide on serum GFAP levels after CCI. CCI produced increases vs. sham in all injury groups at 4 h and 24 h. There was no significant treatment effect, however, in nicotinamide (high dose) treated rats serum GFAP levels at 24 h trended lower vs. the low dose ($P=0.05$) and vehicle groups.

Figure 27. Effect of nicotinamide on serum GFAP levels in the paradagittal FPI model. FPI produced increases vs. sham in all injury groups at 4 h and 24 h. However, there was no treatment effect at either dose.

Figure 28. Effect of nicotinamide on serum GFAP levels after PBBI. PBBI produced increases vs. sham in all injury groups at 4 h and 24 h. There was a significant treatment effect of the 500 mg/kg dose vs. vehicle and low dose treated groups.

GFAP in the CCI model (at the Pittsburgh site) in rats treated with nicotinamide vs. sham and TBI vehicle at the 4 h, 24 h and 21 d time points. At 4 h after injury there were no differences between groups, presumably reflecting a similar level of primary injury across groups. However, at 24 h after injury, in the CCI model, there was a trend toward reduced serum GFAP levels only in the high dose nicotinamide group which reached a $P=0.05$ level vs. the low dose treatment. This appears to corroborate the tissue sparing effect of nicotinamide in the CCI model. Figure 27 shows serum GFAP levels in rats in the parasagittal FPI at the Miami site. Similar to that seen in the CCI model in our studies, there was a significant TBI effect with significant increases in serum GFAP levels vs. sham at both 4 h and 24 h across all treatment groups. However, unlike the CCI model, there was no effect of nicotinamide on serum GFAP levels in the FPI model. Figure 28 shows serum GFAP levels in rats in the PBBI model at the WRAIR site. Once again, there was a significant injury effect across treatment groups at 4 h. However, at 24 h after injury in the PBBI model, high dose (500 mg/kg) nicotinamide significantly reduced serum GFAP levels vs. both the vehicle and low dose treatment groups—suggesting either reduced secondary injury or enhanced clearance by high dose nicotinamide. Given the tissue sparing effects of high dose nicotinamide in the CCI model, it is possible that this drug is reducing secondary injury particularly in astrocytes. These findings also suggest that serum GFAP levels may represent a sensitive marker for injury effect on neuropathology—and possibly a specific aspect of neuropathology. This will be important to follow for future drugs evaluated in our OBTT consortium. Of note, we did not see significant increases in serum GFAP in any of the models at 21 d after injury likely reflecting resolution of damage or a low level of secondary injury by that time point, or possibly closure of the blood-brain barrier to biomarker egress. However, other biomarkers markers of delayed injury (i.e., auto-antibodies) might represent more appropriate injury biomarkers markers germane to processes such as chronic traumatic encephalopathy, and
Figure 29 shows delta 24-4 h GFAP levels across models in our studies of nicotinamide in OBTT. The delta GFAP biomarker levels are being used to evaluate an alternative approach to assess the effects of therapies in primary screening. The results of delta GFAP mirrored those seen with analysis of the 24 h biomarker levels, namely, close to a significant reduction in delta GFAP in the CCI model for high dose nicotinamide treatment (P=0.06 vs. vehicle), a significant reduction for high dose nicotinamide vs. low dose (P<0.03) in the PBBI model along with a trend toward reduced levels in the high dose vs. vehicle (P=0.07) treated. As indicated above, this suggests the possibility that 24 h GFAP levels will represent an excellent serum biomarker for pre-clinical drug testing in all of the future studies in OBTT.

Effect of Drug #2 (EPO) on serum biomarker levels after TBI across OBTT

The serum GFAP levels for the EPO study are presented in a manner identical to those shown for nicotinamide. Figure 30 shows the effect of EPO on GFAP levels in the CCI model at the Pittsburgh site. Consistent with the behavioral and neuropathological data, GFAP levels were increased after injury in all TBI groups vs. sham, however, there was no effect of treatment at any dose on GFAP levels. Similar findings were seen in the FPI model at the Miami site as shown in Figure 31. FPI produced increases vs. sham in all TBI groups at 4 h and 24 h after injury. There was, however, no effect of treatment on GFAP levels at the 2 h and 24 h acute time points. There was, however, a surprising increase in EPO at the high dose vs. low dose groups at 21 d. The significance of the delayed increase in GFAP with EPO treatment is unclear, but could result from trophic effects of this EPO after injury, since GFAP can be induced. It is unclear if this would be viewed as a beneficial or detrimental action, since astrocytes can exhibit beneficial effects, but their proliferation can also contribute to astrocyte scar formation and limitation of plasticity. However, studies in other models are controversial about this point. Vitellaro-Zuccarello et al (Neuroscience 2008) showed that EPO administration attenuates GFAP positive astrocyte proliferation after experimental spinal cord injury while Jing et al (Brain Res, 2009) reported that EPO enhanced survival of GFAP positive differentiated stem cells in model of hippocampal transplantation. Additional study of this unexpected
Figure 31. Effect of EPO on serum GFAP levels after FPI. FPI produced increases vs. sham in all injury groups at 4 h and 24 h. There was, however, no effect of treatment on GFAP levels in acute time points, but a surprising increase in EPO high dose vs. low dose groups at 21 d. The significance of the delayed increase in GFAP with EPO treatment is unclear, however, it could result from trophic effects of this EPO after injury, since GFAP can be induced. Further study of this unexpected finding is needed.

Figure 32. Effect of EPO on serum GFAP levels after PBBI. PBBI produced increases vs. sham in all injury groups at 4 h and 24 h. There was, however, no effect of treatment on GFAP levels in the CCI model at any time point.

Figure 33. Effect of EPO on serum UCH-L1 levels after CCI. High dose EPO surprisingly increased UCH-L1 levels vs. vehicle and low dose groups, but reduced UCH-L1 levels vs. low dose at 24 h. See text for discussion of these findings.

Although we did not see a consistent increase in serum UCH-L1 levels across models at 4 h after injury, and thus have suggested that GFAP might represent a better brain injury biomarker, in a few instances, we received interesting signals from the findings with UCH-L1 in our therapeutic screening studies in OBTT. One example of that is with EPO in the CCI model (Figure 33). We did not see any effect of EPO on UCH-L1 levels at 4 h or 24 h after injury in either the FPI or PBBI models, consistent with the lack of a beneficial effect of this therapy on behavioral and neuropathological outcomes. However, we were surprised to note that in the CCI model, high dose EPO treatment actually increased UCH-L1 levels at 4 h after injury ($P<0.05$ vs both vehicle and low dose treatments). However, curiously, high dose EPO treatment significantly reduced 24 h serum UCH-L1 levels vs. low dose ($P<0.05$) and with a trend toward reduction vs. vehicle ($P=0.05$) treatment. One might postulate that EPO is increasing early post TBI cerebral blood flow (Cherian et al, J Pharmacol Exp Ther, 2007) and thus allowing greater efflux of biomarker levels early after injury, and that this results in some neuronal salvage as assessed at 24 h. Unfortunately, this did not translate into behavioral or neuropathological benefit from EPO in the CCI model in studies at the Pittsburgh site. Finally, consistent with the minor beneficial effects of EPO across models, particularly the lack of effect of EPO on contusion volume or hemispheric tissue loss across models, analysis of Delta-24-4 h levels did not show any significant differences across models (Figure 34). This contrasts the tissue sparing effect of nicotinamide in CCI and trends toward that effect in the other models, which may be coupled to the reductions in serum GFAP seen in PBBI.
and CCI. We will continue to monitor this interesting possibility closely as we test additional therapies in OBTT since it could have important ramifications on biomarker interpretation, development, and utility in drug screening OBTT and in clinical applications.

**Effect of Drug #3 (CsA) on serum biomarker levels after TBI across OBTT**

As indicated above, all of the injuries, blood sampling, and behavioral outcomes have been completed for CsA at each of the sites. In addition, all of the blood samples for biomarker assessments have been shipped to Banyan for drug #3. Assessment of biomarker levels is underway.

**Secondary Screening**

**Large animal model of TBI in micropigs**

Virginia Commonwealth University site (J. Povlishock, PhD)

**Micropig Studies**

As has been outlined in the application and presentations on our consortium, the purpose of this component of the application is to provide a gyrencephalic model of TBI for subsequent screening of those agents found to be most efficacious in the proposed rodent model systems. Specifically, micropigs are subjected to TBI and therein, traumatically-induced microvascular dysfunction and axonal damage is assessed in both sham and drug treated animals.

Consistent with the expectations of this application, seven micropigs have been prepared for the induction of Fluid Percussion Brain Injury (FPBI). Each of the micropigs was equipped with a cranial window placed over the left frontal cortex, with the injury pulse placed over the central occipital parietal domain. Using the cranial windows, vascular responsiveness was assessed prior to injury, with all animals demonstrating the anticipated response to topical acetylcholine application reflected in vasodilation. Following the completion of the vascular functional studies, the animals were subjected to FPBI in the range of 1.9 atm. Prior to and following the injury, the animal’s basic physiologic responses were assessed with no overt systemic physiological response other than a brief alteration in systemic blood pressure. Following injury, the pial vascular functional studies were repeated with the caveat that continued vascular analysis proved difficult in all cases. In three animals, the cranial windows leaked, precluding continued vascular assessment. In two animals, the windows filled with subarachnoid blood due to local microvascular bleeds, while two windows mechanically failed, causing the brain to be exposed to the external environment. At select times postinjury ranging from 1-4 h, the animals were sacrificed, perfused, and their brains removed. In general, the brains appeared unremarkable, although subarachnoid bleeding could be identified in the basal cisterns. Coronal sectioning of the brain revealed no evidence of contusion or intraparenchymal bleeding, a fact further confirmed with additional sections of brain harvested for immunocytochemical analysis. These included the rostral and caudal corpus callosum, subcortical white matter, midbrain, and cerebellum. The histological analyses conducted to date on these samples revealed consistent patterns of APP positive reactive axonal swellings found scattered throughout the corpus callosum, subcortical white matter, and brain stem. The numbers of axons were dramatic and easily quantified. We will continue to use this model system with the caveat that our engineers are redesigning the cranial windows to reinforce window thickness while improving the interface of the window with the cranial vault to preclude window leakage and/or brain subluxation with related bleeding. When the first drug is evaluated in the micropig model, blood sampling parallel to the rat studies will also be used to generate biomarker data, given high level of success in this regard that has been seen for this aspect of the consortium across the rodent models.
KEY RESEARCH ACCOMPLISHMENTS

1. IACUC and ACURO Approval at all sites along with necessary updates
2. Creation and continual updating of a Manual of Operations for the OBTT consortium by Dr. Kochanek
3. Monthly consortium investigator conference calls
4. TBI drug therapy literature review, investigators survey, and selection of the first two therapies to be evaluated by the OBTT consortium
5. Comprehensive review of the TBI literature for the first six drugs, nicotinamide, EPO, CsA, Simvastatin, Minocycline, and Levetiracetam by Dr. Kochanek, with updating of the manual through Simvastain, which is drug #4 and ready for testing (IACUC and ACUROs either submitted or approved at all sites).
6. Publication of a manuscript on the OBTT concept in the Journal of Trauma (1)
7. Presentation of five abstracts on the individual components of OBTT to the 2011 ATACCC meeting. Those abstracts served as the basis of a symposium at the conference.
8. Report sent by Dr. Kochanek on the launching of OBTT to the Therapy and Oversight Committee and Consultants
9. Completion of all experiments for drugs #1 (nicotinamide), #2(EPO), and #3 (CsA)—in primary screening across three rodent models. And complete analysis of all data on drugs #1 and #2, with #3 in process.
10. Investigators meeting held on at the 2011 and 2012 National Neurotrauma Society Meeting
11. Presentation of an afternoon symposium on OBTT by the PI and site PIs at the 2011 ATACCC conference, and a plenary lecture on OBTT by the PI at the 2012 MHSRS conference.
12. Presentation by the PI of a plenary lecture on OBTT at the 2012 annual meeting of NNT society.
13. Presentation of two abstracts by site PIs at the 2012 meeting of the NNT.
14. Re-establishment and continued refinement of the large animal micropig model of FPI TBI at Virginia Commonwealth University
15. Dr. Kochanek is also providing input (in part, representing OBTT) to the US Army for its Neurotrauma, Pharmacology Work Group.

REPORTABLE OUTCOMES (All reportable outcomes since project inception are shown, those from 2012 are shown in bold font)

5. Povlishock, JT. Operation Brain Trauma Therapy: The Virginia Commonwealth University Program. Presented at the Advanced Technology Applications to Combat Casualty Care (ATACCC) Conference in Fort Lauderdale, FL, 2011.
CONCLUSION

The unique multicenter pre-clinical drug screening consortium OBTT has been launched and has successfully screened three drugs across three established rodent models of TBI. In addition, exciting biomarker
applications have also been successfully launched and those data have generated valuable findings. An outstanding collaboration between civilian and US Army investigators has been successfully developed. The consortium data have generated some of the first cross-model comparisons in the field of experimental TBI. The large animal model is being refined for testing of the first promising candidate. We anticipate that we will move a therapy forward to the pig model upon completion of screening the first 6 therapies. The work has been presented at major national meetings in the field and for the DOD and the consortium's findings have been well received. Overall, no significant problems have been encountered.

REFERENCES


APPENDICES

Appendix 1. Manual of operations for OBTT most recent update.
Appendix 2. Peer reviewed publication from year 1 in the *Journal of Trauma*. 
Primary Screening

1. *Modeling and injury protocol*

Drug therapies will be tested in three rat traumatic brain injury (TBI) models, controlled cortical impact (CCI; Pittsburgh), parasagittal fluid percussion injury (FPI; Miami), and penetrating ballistic-like brain injury (PBBI; WRAIR). The protocol to be used will represent the standard approach in each model at each site (see Table 1) with the minor modifications needed to incorporate the following standardized parameters at each site.

**Factors standardized across models**

- Adult, Male, Sprague Dawley rats (specific vendor and weights per site).
- Blood sampling (0.7 mL whole blood) obtained at 30 min*, 4 h, 24 h, and at sacrifice from each rat in primary screening (jugular venous catheter or tail artery sampling method per site). Blood removed will be replaced with an equal volume of sterile normal saline given either IV or SQ to limit hemodynamic consequences of phlebotomy. *Note that 30 min post injury sampling was not initiated until drug 4 studies.*
- Motor function testing performed at each site (specific tools and methods per site).
- Cognitive testing using MWM at each site (specific MWM paradigm per site).
- Euthanasia at 21 days after injury.
- Histopathology includes volumetric analysis (specific approach/ancillary outcomes per site).

**Table 1: Outcome Metrics:**
**Primary Screening**

<table>
<thead>
<tr>
<th>Site</th>
<th>Biomarkers</th>
<th>Neuro Exam /Stress</th>
<th>Motor Function</th>
<th>Cognitive Function</th>
<th>Neuropathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>PITTSBURGH</td>
<td>Rat: Blood Samples (0.7 mL; tail artery): 4 h, 24 h, at sacrifice</td>
<td>Rat: Righting reflex</td>
<td>Rat: Beam balance and beam walking d1-5</td>
<td>Rat: MWM task: 14-20d hidden (14-18d) and visible platform(19-20d) and probe trial (20d)</td>
<td>Rat: Euthanize d21; serial sections volumetric analyses + hippocampal neuron counts</td>
</tr>
<tr>
<td>MIAMI</td>
<td>Rat: Blood Samples (0.7 mL via IV (jugular): 4h, 24h, at sacrifice</td>
<td>Rat: None</td>
<td>Rat: Spontaneous forelimb use, gridwalk task, rotarod: 7d</td>
<td>Rat: MWM task: 13-21d (hidden platform, probe, working memory</td>
<td>Rat: Euthanize d21; serial sections, volumetric analysis + neuron counts &amp; axonal pathology</td>
</tr>
<tr>
<td>WRAIR</td>
<td>Rat: Blood Samples (0.7 mL via IV (jugular): 4h, 24h, at sacrifice</td>
<td>Rat: Neuroscore Exam: 30m, 24h, 72h, 7d, 21d</td>
<td>Rat: Balance Beam/ Rotarod: 7d and 10d</td>
<td>Rat: MWM task : 13-17d (4x/dx5d; 30m ITI; end w/probe trial)</td>
<td>Rat: Euthanize at 21d serial 40 um sections; H&amp;E/Silver</td>
</tr>
</tbody>
</table>

*IV=Intravenous; MWM=Morris water maze; ITI=Inter-trial interval; H&E=Hematoxylin & eosin; WRAIR=Walter Reed Army Institute of Research
2. **Assessment of therapeutic efficacy**

Table 2 shows the scoring approach to evaluation of therapies across rodent model in primary screening studies in OBTT. In rodent screening of therapies in each case the four groups comparison will be sham, TBI + vehicle, TBI + drug (dose 1) and TBI + drug (dose 2). Four categories of outcomes will be scored (neuroscore/motor function; cognitive function, neuropathology [volumetric analyses], and serum biomarkers) and in each model a maximum score of 22 points can be awarded. Cognitive outcome parameters are, by choice, weighted more heavily than other outcomes. Benefit (i.e., scoring the points in a category) requires that the therapy be significantly different than vehicle control at the P<0.05 level. In the case of outliers in a given data set, formal outlier testing is required and awarding of points in any disputed cases will be reviewed with our OBTT statistician, Dr. Steven Wisniewski. If a therapy shows a significantly detrimental effect versus vehicle treatment, a negative score is assigned for that outcome parameter. Of note, the serum biomarkers that are being scored are the neuronal biomarker UCHL-1 and the glial biomarker GFAP. The primary comparison for these biomarkers are 24 h values, and delta between the early primary injury peak at 4 h and the 24 h values which will be potentially mitigated by therapy.

### Table 2. Scoring matrix for assessment of therapeutic efficacy across models in OBTT

<table>
<thead>
<tr>
<th>Site</th>
<th>Neuro Exam</th>
<th>Motor</th>
<th>Cognitive</th>
<th>Neuropath</th>
<th>Biomarker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitt</td>
<td>None</td>
<td>Beam balance (2)</td>
<td>Hidden platform latency (5)</td>
<td>Lesion volume (2)</td>
<td>GFAP 24 h (1) 4-24 h Δ (1) UCHL1 24 h (1) 4-24 h Δ (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beam walk (2)</td>
<td>MWM probe (5)</td>
<td>Hemispheric volume (2)</td>
<td></td>
</tr>
<tr>
<td>Miami</td>
<td>None</td>
<td>Cylinder (2)</td>
<td>Hidden platform latency (5)</td>
<td>Lesion volume (2)</td>
<td>GFAP 24 h (1) 4-24 h Δ (1) UCHL1 24 h (1) 4-24 h Δ (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gridwalk (2)</td>
<td>MWM probe (3)</td>
<td>Hemispheric volume (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Working memory (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WRAIR</td>
<td>Neuroscore (1)</td>
<td>Rotarod (3)</td>
<td>Hidden platform latency (5)</td>
<td>Lesion volume (2)</td>
<td>GFAP 24 h (1) 4-24 h Δ (1) UCHL1 24 h (1) 4-24 h Δ (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MWM probe (3)</td>
<td>Hemispheric volume (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thigmotaxis (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grand total max</td>
<td></td>
<td></td>
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</tbody>
</table>

3. **Approach to treatment**
Drug treatment will be identical between sites and will employ an IV route whenever feasible and optimal. However, strategies involving other routes may be used in selected situations (i.e., nutraceuticals, rehabilitation-related therapies). For each agent, two doses will be screened, again based on the best available literature evidence. Treatments that are selected will be agreed upon unanimously by the site PIs involved in each given study. For each drug screened, four groups will be studied with 10 rats in each group. Specifically, two treatment groups (two doses) along with a single vehicle group and sham group (no treatment) will be studied for each therapy. Results of the primary and secondary outcomes (MWM performance and volumetric analysis, respectively) at each site will not be revealed until all sites have completed work with each agent in primary screening. However, if an agent at a given dose produces greater than 20% mortality, the site PI will notify the other primary screening site PIs since this could trigger reconsideration of dosing at all of the sites.

Specific considerations relevant to each therapy will be generated for each agent that is selected to move forward in primary screening. Thus, the information in this section will build as the program moves forward.

- **Therapy 1: Nicotinamide**

Vitamin B3 has shown dramatic beneficial effects on all aspects of outcome evaluated including function, neuropathology, and blood-brain barrier damage, with several positive reports in TBI, including CCI and FPI (1-3) (Table 3). Most of the reports showing benefit of nicotinamide in TBI are from a single laboratory. Nicotinamide has been shown to attenuate several mechanisms that are important in TBI, including poly-ADP-ribose polymerase activation, inflammation, and replenishing NADPH levels with resultant increases in glutathione. Doses of 50-500 mg/kg have shown efficacy and with a promising 4 h time window (1). Nicotinamide is commercially available as vitamin B3. It represents an example of an agent that could be readily moved forward if found to show benefit across models and could also be used as a nutritional supplement in a pre-treatment approach particularly in light of the ability to provide dietary neuroprotective additives in theater.

Regarding dosing, route of administration and pharmacology, Evidence suggests that nicotinamide rapidly reaches high levels in brain related to the presence of a specific uptake mechanism (4). In addition to the aforementioned key references outlining efficacy of doses ranging between 50 and 500 mg/kg in experimental TBI, two references in the stroke literature are relevant to dosing. Sakakibara et al (5) demonstrated that IV administration is effective in male rats in transient MCAO with administration at 2 h, given immediately before reperfusion. In this study, a variety of rat strains including Fischer, SHR, and diabetic were studied and benefit on infarct volume was seen in all strains. Similarly, in permanent MCAO, nicotinamide (500 mg/kg) given by the IV route at 2 h attenuated infarct volume in both Sprague Dawley and Wister female rats, and reduction in infarct size was larger with IV administration than in prior reports using IP administration (6). This supports the proposed IV use in our studies in TBI. Regarding half-life, it has been reported that nicotinamide has a long half-life when administered PO in humans, where it averaged 9.3 h (7).

For the proposed studies in OBTT, nicotinamide (MW 122.12) will be purchased from Sigma (catalog number N3376). Dosing will be 50 mg/kg or 500 mg/kg given IV at 15 min and 24 h after injury. For administration, the drug should be prepared fresh daily by dissolving it in sterile 0.9 normal saline (NS). The doses of 50 mg/kg or 500 mg/kg will be prepared for delivery for each rat in 1 mL/Kg of sterile NS, respectively. Thus, as an example, for a 400 gram rat, for
each 500 mg/kg dose, 200 mg of nicotinamide would be dissolved in 0.4 ml of sterile NS. Vehicle treated rats will also receive 1 mL/kg of sterile NS. Room temperature saline should be used rather than cold saline to dissolve the nicotinamide, and agitation of the solution may be needed particularly for the 500 mg/kg dosing (personal communication per Dr. Michael Hoane). Once dissolved, the solution should not be refrigerated since cooling to 4 °C can result in precipitation. Each dose should be prepared by an individual who is different from the one performing the surgery and injury and/or carrying out the primary or secondary outcome assessments, namely, MWM or lesion volume analyses. A specific coding system must be carefully developed and used between the technicians preparing the drug, performing, and carrying out the outcome assessments for each rat in the 4 groups particularly given the use of two injections over 2 days and the assessment of outcomes over 21 days.

**Table 3. Therapy 1, Nicotinamide**

**Key references**

<table>
<thead>
<tr>
<th>Drug: Nicotinamide</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study/Author</strong></td>
<td><strong>Dose</strong></td>
</tr>
<tr>
<td>(1) Hoane et al, 2006</td>
<td>50 or 500 mg/kg/IP Given at 15 min and 24 h</td>
</tr>
<tr>
<td>(2) Hoane et al, 2008</td>
<td>50 mg/kg/IP; At 15 min, 4 h, or 8 h post injury followed by 5 subdoses of 50 mg/kg IV at 24 h intervals</td>
</tr>
<tr>
<td>(3) Holland et al, 2008</td>
<td>50 or 500 mg/kg/IP Given at 15 min and 20 h</td>
</tr>
<tr>
<td>(4) Spector</td>
<td>C14 niacinamide</td>
</tr>
<tr>
<td>(5) Sakakibara et al, 2002</td>
<td>IV dosing of 20-750 mg/kg at 2 h effective in rat MCAO model</td>
</tr>
<tr>
<td>(6) Sakakibara et al, 2000</td>
<td>IV dosing of 500 mg/kg</td>
</tr>
<tr>
<td>(7) Bernier et al, 1998</td>
<td>Oral dosing of 6 grams</td>
</tr>
</tbody>
</table>

- Therapy 2: Erythropoietin
Review of the experimental TBI literature suggests that erythropoietin (EPO) is one the most promising future therapies available. A search of PubMed revealed a remarkable 24 studies all showing efficacy of EPO in rodent models of TBI (Table 4). A single center clinical trial of EPO in severe TBI is ongoing at the Baylor College of Medicine (Claudia Robertson, MD, PI), and Dr. Kochanek has discussed this treatment possibility for OBTT with Dr. Robertson. A pleiotropic cytokine involved in erythropoiesis, EPO has a number of beneficial effects that could be important in TBI such as attenuation of glutamate and NO toxicity, anti-apoptotic, antioxidant, and anti-inflammatory effects, stimulation of neurogenesis and angiogenesis, protection of mitochondria and beneficial effects and across many CNS insults such as global and focal ischemia, kainite toxicity, and intracranial hemorrhage (8-32). The exact mechanism of benefit is unclear. Although classical EPO receptors are seen in many cell types in the CNS, these receptors are up-regulated by hypoxia (30), and EPO receptor null mice have a worse outcome than wt after CCI (18), EPO receptors surprisingly do not appear to be needed to mediate the benefit of exogenously administered EPO therapy (19). In the aforementioned 24 studies on TBI, work has included papers in both rats and mice and across models including CCI, FPI, impact acceleration, focal closed head injury, Feeney weight drop, and combined injury (8-31) (Table 4). Studies in large animal models of TBI, however, were not identified.

Route of administration, dosing and therapeutic window appear to be tantalizingly favorable. The studies outlined in Table 4 have suggested that ANY parenteral route of administration shows efficacy including IV, IP or SQ—without obvious differences in this regard. A dose of 5000 IU per kg appear to be classical, with doses of 1000 and 3000 also showing efficacy. Reports testing the higher doses were not identified. The therapeutic window is controversial, with some studies suggesting benefit with first dose as late as 24h (9). However, the most comprehensive study of time window examined 5 min, 3h, 6h, 9h, and 12h dosing and identified 6h as the latest time point for successful initial dosing. Studies have shown benefit from a single dose, two doses, three doses, of daily treatment for 14d (8-31). The initial report of efficacy with EPO in TBI used dosing a 1h and 24h and showed benefit across many outcomes (8). The most detailed study of single vs multiple dosing showed that 3 daily doses were better than a single dose (28). Of note, all of these studies used 5000IU/kg as the dose.

There are some special caveats with regard to EPO therapy in TBI. A concern for potential use of this agent in stroke has resulted from the fact that it increases hematocrit (HCT) and increased mortality (37) in clinical testing. Using single dose regimens in rat TBI models, HCT increased from baseline values of ~45% to between 52 and 60% with increases most prominent on d4-14 after administration (15, 19, 27). An elegant study by Zhang et al (19) from Dr. Chopp’s group showed that benefit was independent of HCT by using post-injury isovolemic hemodilution to normalize HCT. This side effect of EPO may not be a major concern in severe TBI or polytrauma, since HCT is typically reduced in patients suffering these conditions, and some level of erythropoiesis could reduce transfusion risk. For mild TBI, this would not be the case and hyperviscosity could be a concern. Recently, carbamylated EPO analogs (CEPO) that have no effect on HCT but show benefit in TBI have been developed (29). CEPO analogs do not bind to the EPO receptor, yet show similar efficacy in CCI (29). Another EPO analog darbepoietin—which has a longer half-life than EPO, has been tested in CCI and shown to be beneficial. Finally, with special relevance to Banyan, there has been a study of the effect of EPO on serum levels of S100B and IL6 assessed between 6h and 7d after TBI in rats—which showed reductions with treatment (25).

The studies identified in Table 4 are focused on work in TBI models with conventional outcomes—specifically to guide the approach in OBTT. For EPO, there are many papers in other models related to TBI such as hippocampal slices, and intracerebral hemorrhage, among
others, that suggest benefit; they are beyond the scope of this manual of operations (32-34). A recent review on potential benefit of EPO in experimental TBI was published and suggests effects via JAK-2 and downstream effects on NFkB, AKT, and ERK and MAPK pathways, resulting in anti-apoptotic effects (35). However, that review suggests involvement of EPO receptor, which based on recent work, may be incorrect. Finally, a review by Nichol and Cooper (36) discusses relevant issues related to EPO and suggests the need for a multicenter RCT—specifically, the EPO study investigators within the ANZICS Clinical Trials group.

For the proposed studies in OBTT, EPO (PROCRIT, Amgen, preservative free) is likely available through your hospital pharmacy. Prices for PROCRIT vary considerably between vendors. Cost at the UPMC Pharmacy in Pittsburgh is approximately $87 per 10,000 IU/mL vial. Thus, it will cost less than $20 per rat for the low dose and $40 per rat at the high dose. The clinical grade preparation contains tiny amounts of albumin and bicarbonate that would not be expected to have any effect in a TBI model. It must be kept refrigerated. If it is not available to you at a similar price, it can be ordered by us and shipped to you for use. Dosing will be 5000 IU/kg IV or 10,000 IU/kg IV administered at 15 min after injury. Thus, 0.5 mL/kg or 1.0 mL/kg of the 10,000 IU/mL solution is given IV for the low and high dose groups respectively. It can be infused over 5 min safely. The vehicle that we propose is sterile NS. As an example, for a 400 gram rat, for each rat in the 10,000 U/kg high dose group, 0.4 mL of PROCRIT solution (i.e., 1 mL/kg) would be given IV over 5 min. Vehicle treated rats will also receive an equal volume of sterile NS. We will have a single vehicle group using the higher dose volume (i.e., 1 mL/kg). We have experience with the low dose and have used it in preliminary studies in our mouse model of CCI plus hemorrhage, and mice tolerate 5000 IU/kg with no major change in MAP (a modest increase) when it is given the drug during hemorrhagic shock with a MAP of 25-20 mmHg—as tested in our combined injury model. We would suggest using a new vial of PROCRIT each day since it is preservative free.

Thus, for EPO treatment:

Treatments:

PROCRIT—10,000IU/mL vial;

Or

Sterile Normal Saline

Groups

1. Sham (surgery but no treatment)
2. CCI plus Sterile Normal Saline at 1.0 mL/kg given at 15 min after injury
3. CCI plus PROCRIT 0.50 mL/kg (Low dose; equates to 5000 IU/kg) given at 15 min after injury
4. CCI plus PROCRIT 1.00 mL/kg (High dose; equates to 10,000 IU/kg) given at 15 min after injury

Please remember that blood sampling and outcomes must remain identical to the studies carried out with Drug #1, as defined in the modeling and injury protocol.
Table 4. Therapy 2, EPO

<table>
<thead>
<tr>
<th>Study/Author</th>
<th>Drug: EPO</th>
<th>Model/Species</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/Yatsiv et al 2005 (Shohami group)</td>
<td>5000 IU/kg IP in 1 ml of sterile PBS at 1h and 24h vs PBS</td>
<td>Shohami weight drop closed TBI with unilateral insult in mice and rats</td>
<td>TUNEL markedly reduced, axonal injury-Bielschowsky reduced, C3, CD11b, and GFAP reduced</td>
</tr>
<tr>
<td>9/Lu et al 2005 (Chopp group)</td>
<td>5000 IU/kg IP at 24h and then daily for 14d</td>
<td>CCI in rat; chloral hydrate anesthesia</td>
<td>Increased BRDU labeling in ipsi DG</td>
</tr>
<tr>
<td>10/Cheirian et al 2006 (Robertson group)</td>
<td>5000 IU/kg SC vs NS Therapeutic window for single dose at 5min, 3h, 6h, 9h, 12h</td>
<td>CCI in rat</td>
<td>Histo at 2 wks CA1 and contusion volume improved with Rx from 5min-6h. No effect on CA3</td>
</tr>
<tr>
<td>11/Chen et al 2007</td>
<td>5000 IU rh in 4 mL/kg saline IP at 1h 24h, 48h and 72h</td>
<td>Male rat focal wt drop—Feeney; pentobarb anesth</td>
<td>NFKB, ICAM-1, TUNEL, Evan blue, water content all attenuated at 3 days</td>
</tr>
<tr>
<td>12/Ozturk et al 2007</td>
<td>5000IU/kg IP +/- propofol 100mg/kg IP at 10 min</td>
<td>Variant of impact accel in either anesth female Wistar rats</td>
<td>At 24h EPO reduced XO, MDA and NO levels —no additive effect with propofol</td>
</tr>
<tr>
<td>13/Ozisik et al 2007</td>
<td>1000IU/kg rh at 5 min IP</td>
<td>Wistar rats anesth with ketamine + xylazine; Feeney type wt drop</td>
<td>At 24hEPO reduced histo damage score on EM along with increase in BCL2</td>
</tr>
<tr>
<td>14/Verdonc et al 2007</td>
<td>5000IU/kg rh at 30 min—IV in 0.5 mL saline</td>
<td>Male Wistar rats; TBI by impact acceleration</td>
<td>MRI assessments over 6h showed improved ADC and T1 and %BW by EPO</td>
</tr>
<tr>
<td>15/Xiong et al 2008 (Chopp group)</td>
<td>5000IU/kg rh at 6h, 3d, and 7d IP</td>
<td>Male CS7 mice; CCI</td>
<td>50% reduction in lesion volume at 35d; Inc BRDU and neuron counts in DG</td>
</tr>
<tr>
<td>16/Hartley et al 2008</td>
<td>5000IU/kg rh IP at 30 min</td>
<td>Sprague Dawley rats FPI</td>
<td>Reduced lesion volume at 10h</td>
</tr>
<tr>
<td>17/Lieutaud et al 2008</td>
<td>1000, 3000 or 5000IU/kg given IV or IP</td>
<td>Male Sprague Dawley rats FPI</td>
<td>IL-1B and MIP-2 concentrations reduced</td>
</tr>
<tr>
<td>18/Xiong et al 2008 (Chopp group)</td>
<td>NONE</td>
<td>Adult female CS7 mice null for CNS EPO receptor; CCI model- chloral hydrate anesth</td>
<td>EPO null mice surprisingly did not No exacerbation of lesion volume or cell counts in null vs wt; less neurogenesis</td>
</tr>
<tr>
<td>19/Zhang et al</td>
<td>5000IU/kg rh</td>
<td>Male Wistar rat</td>
<td>Hemodilution did not</td>
</tr>
<tr>
<td>Publication</td>
<td>Treatment Details</td>
<td>Outcome</td>
<td>Conclusion</td>
</tr>
<tr>
<td>-------------</td>
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</tr>
<tr>
<td>2008 (Chopp group)</td>
<td>IP d1, d2 and d3; +/- isovolemic hemodilution</td>
<td>CCI; chloral hydrate anesthesia</td>
<td>affect the benefit of EPO on cell loss in DG or CA3 or BDRU</td>
</tr>
<tr>
<td>20/2009 (Chopp group)</td>
<td>1000, 3000, or 5000 IU at 30 min-6h after CCI IP</td>
<td>CCI in Sprague Dawley rats</td>
<td>NONE—focus only on brain mitochondrial function</td>
</tr>
<tr>
<td>21/2009 (Chopp group)</td>
<td>5000 IU/kg IP 6h, 3d and 7d</td>
<td>CCI in EPO null and wt mice—rescued with EPO expression in hematopoietic tissue</td>
<td>Reduced lesion volume, and cell counts in DG and CA3 similar in wt and null with EPO</td>
</tr>
<tr>
<td>22/2009</td>
<td>5000 IU/kg IP at 30 min</td>
<td>Sprague Dawley rat modified Feeney model</td>
<td>Reduced FJB staining at 24 h</td>
</tr>
<tr>
<td>23/Valable et al 2010</td>
<td>5000 IU/kg IV at 30 min</td>
<td>Male Wistar Rat Impact acceleration</td>
<td>NONE</td>
</tr>
<tr>
<td>24/Liao et al 2010</td>
<td>5000 IU/kg IP daily for 7d</td>
<td>Male Wistar rats Feeney wt drop</td>
<td>EPO decreased cortical TUNEL at 72h</td>
</tr>
<tr>
<td>25/Bian et al 2010</td>
<td>1000, 3000, or 5000 IU/kg, or citocline or NS IP immediately after TBI</td>
<td>Male Wistar rats Feeney wt drop</td>
<td>Serum biomarkers S100B and IL6 assessed at 6h, 24h, 3d, 5d, or 7d; reduced by EPO at 3000 and 5000 dose</td>
</tr>
<tr>
<td>26/Zhang et al 2010</td>
<td>5000 IU/kg IP on d1, 2 and 3 after CCI</td>
<td>Male Wistar rat CCI; Chloral hydrate anesth</td>
<td>Biotinylated dextran tracking of corticospinal tract showed plasticity with EPO after CCI</td>
</tr>
<tr>
<td>27/Chauhan and Gatto 2010</td>
<td>5000 IU/kg IP at 6h, 3 and 7d after CCI; Simvastaing 2 mg/kg in feeds</td>
<td>Male C57 mice; CCI; ketamine and xylazine anesthesia</td>
<td>Added benefit for EPO+Simvastatin on BRDU</td>
</tr>
<tr>
<td>28/Xiong et al 2010 (Chopp group)</td>
<td>5000 IU/KG IP at d1 vs d1, 2 and 3</td>
<td>Male Wistar rat CCI; Carbamylation EPO [CEPO]/50 µg/kg IP at either 6h or 6, 24 and 48h</td>
<td>Day 35 histo was improved in both doses but the 3 dose best</td>
</tr>
<tr>
<td>29/Xiong et al 2011</td>
<td>Carbamylated EPO [CEPO]/50 µg/kg IP at 6h or 6, 24 and 48h</td>
<td>CCI in rat</td>
<td>Day 35 histo was improved in both doses but the 3 dose best</td>
</tr>
<tr>
<td>30/Cherian et al 2011 (Claudia Robertson Group)</td>
<td>Darbepoetin alfa (darbEPO) 2.5, 5, 10, 25 or 50 µg/kg SQ at 5 min after</td>
<td>CCI in Long Evans rat</td>
<td>2 wk assessment of contusion volume and CA1 and CA3 cell counts; 25 and 50 µg/kg doses effective</td>
</tr>
<tr>
<td>Therapy 3: Cyclosporine A (CsA)</td>
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</table>

Review of the experimental TBI literature suggests that CsA represents a low hanging fruit therapy that is already in widespread clinical use as an immunosuppressant, and has exhibited beneficial effects on several traditionally important secondary injury mechanisms. Inhibition of post-insult calcium induced mitochondrial permeability transition pore opening (thus maintaining mitochondrial membrane potential) has been suggested to confer benefit in many studies by preserving mitochondrial function and reducing ROS (38, 41, 47) (see Table 5). However, inhibition of calcineurin by CsA may have beneficial effects on learning and memory specifically via blocking the protein phosphatase activity of calcineurin (45). Similarly, immunosuppressive effects, also mediated by calcineurin inhibition, may further confer benefit after TBI (and/or mediate potential side effects).

A total of 17 studies specifically in pre-clinical TBI models were identified by PubMed search (38-54) (Table 5). One of the greatest strengths of the literature on CsA in TBI is the fact that multiple histological outcomes seem to be robustly benefited. Notably, axonal injury has been shown to be attenuated after TBI by CsA treatment by multiple groups (38, 39, 44, 46, 48). Similarly, contusion volume, specifically in cortex, has been shown to be markedly attenuated by CsA, again, by multiple groups (40, 42, 43, 53). Surprisingly, there have been few studies of the effects of CsA on functional outcome after TBI—two studies showing benefit on motor outcomes, and one on MWM performance (45, 50).

With regard to specifics of the studies, all but one used an established TBI model. Sixteen studies showed positive effects, with the only negative study being the one carried out in an

<table>
<thead>
<tr>
<th>Injury; Time window also studied at 5min, 1h, 3h, 6h, 9h, 12h, or 24h</th>
<th>and therapeutic window was 6h.</th>
<th>Also assessed LDBF and NO—and darbEPO increased both CBF and NO levels over 2h after injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>31/Jin et al</td>
<td>5000 IU/kg IP in 4 ml/kg of NS at 30 min</td>
<td>ICR mouse modified impact acceleration TUNEL and %BW at 24 h reduced by EPO Grip test improved by EPO</td>
</tr>
<tr>
<td></td>
<td>Up-regulation of Nrf2 antioxidant pathway; Paper has many errors in Figures—not matching to legends</td>
<td></td>
</tr>
</tbody>
</table>

*Figure 1.* Chemical structure of cyclosporine. The non-immunosuppressive CsA analog NIM811 (see text for details) is nearly identical in structure with the exception of a single substitution of an isoleucine for a leucine moiety in the molecule.
unconventional model of dental drill injury to cortex (49). Most of the studies were carried out in either the impact acceleration model or CCI, with a few in FPI. All but three were carried out in rats, with one in mice (40), one in piglets (54), and one in ewe (48). There has been little study of gender on the efficacy of CsA in experimental TBI with almost all work done exclusively in male animals. There have, however, been many valuable studies of dose response, route of administration, therapeutic window, and brain tissue levels (see below).

Given the goal of OBTT, the IV route of administration is preferred. Based on the literature, it is clearly available for CsA. Some of the early work with CsA showed limited BBB passage. While that is true in uninjured brain, there are data in the impact acceleration model in rats, for example, that show that brain tissue levels of CsA after a 20 mg/kg dose, are similar to those seen after a 10 mg/kg intrathecal dose (46). Most studies have shown efficacy with either 10 mg/kg or 20 mg/kg. The only study showing benefit on cognitive outcome used a surprisingly low (and unconventional) dose of 0.675 mg/kg or 18.75 mg/kg; benefit on MWM was seen with both doses (45). In other studies, 1 or 3 mg/kg were not effective or of only limited efficacy on histology (43, 46, 53). High doses of 150 mg/kg were also not effective on histology (43).

Benefit in the pig model was seen with 20 mg/kg IV given at 5 min and 12 h after injury with the dose diluted in 10 mL of NS. Some studies have shown benefit with IP bolus followed by continuous infusion with an osmotic mini-pump (a 20 mg/kg IP bolus followed by a 10 mg/kg/d infusion for 3 days) (53). Therapeutic window studies suggest that 15 min for the initial treatment is better than 1 h, but efficacy for first dose is seen out to at least 8 h in some studies (43). Several studies showing benefit have used a second dose given at 24 h (see Table 5).

Unlike the use of CsA for immunosuppression, the dose for TBI should likely target permeability transition in mitochondria as the primary endpoint. To this end, Hansson et al (55) reported that the maximal effect inhibiting permeability transition was seen at CsA concentrations of 0.5-1.0 μM. Lower doses of 100 nM or 10 nM or even 5 nM were less effective. The efficacy of CsA depended on the calcium concentration and other factors. Brustovetsky and Dubinsky (56) also used successfully 1μM on CNS mitochondria. However, in dog cardiac mitochondria, 0.2 μM CsA produced only partial benefit (57). Those studies were carried out in vitro and CsA is highly bound to RBCs and lipoproteins—so direct extrapolation is complex. Nevertheless, the terminal half-life of CsA in rats of 7.5-12 h suggests that the q 24 h dosing is reasonable at doses of 10 or 20 mg/kg. Based on Molpeceres et al, (58) whole blood plasma levels will still be >1 μM with 10 mg/kg at 24 h and a 24 h re-dosing time is proposed. Tanaka et al (59) confirm this. The issue of brain penetration/kinetics in rat TBI models is unclear. There are, data on total (not free CyA) brain levels in animal models or controls. Friberg (60) achieved ~2 μM in brain 45 min after a 20 mg/kg IV dose in rats (they also provided drug administration details… diluted it CsA 6-fold with NS). Lemaire (61) showed 0.85 μM and 9.9 μM at 2 h after IV administration of 10 and 30 mg/kg respectively. Tanaka (62) showed 6 mg/kg and 30 mg/kg CyA IV had very high peak levels and 24 troughs of ~0.3 and ~2 μg/mL respectively (0.5 μM = 0.6 μg/mL).

In controls, CyA is subjected to saturable distribution in brain (is affected by efflux transporters at the BBB) and this results in dose-level nonlinearity at dose >3mg/kg IV in rats so levels go up in brain disproportionately to dose (the Tanaka paper [59] is an excellent description of this). CNS toxicity (ataxia) has been observed in rats at oral doses of 50 mg/kg likely due to this
nonlinearity—we should watch for this given the potential for increased brain penetration after TBI. The pharmacokinetics are also sex-specific and vary according to species. For pigs, dosing may need to be re-evaluated—q12 h administration may be needed.

Overall, 10 and 20 mg/kg IP are clearly most supported in the current literature. It is unclear what percent of the 24 h dosing interval will produce levels >0.5-1 μM free CyA levels in the injured brain. Without an injury, studies above show total levels will likely exceed this level for most of the 24 h dosing interval, but free levels will not for the entire interval. However, our levels will resulting from IV administration will almost certainly be higher than previous TBI studies using IP dosing given the low IP bioavailability and the injury will likely increase brain penetration relative to the control animal studies above so we should not require greater doses.

Thus, for CsA treatment in OBTT

Groups

1. Sham (surgery but no treatment)
2. CCI plus vehicle (4 mL/kg) given by slow infusion over 5 min starting at 15 min and 24 h after injury
3. CsA 10 mg/kg IV first dose at 15 min after injury and second dose at 24 h after injury
4. CsA 20 mg/kg IV first dose at 15 min after injury and second dose at 24 h after injury

Recall that the second dose is administered after the 24 h blood sample is obtained for biomarker levels.

Cyclosporine A

Stock solution = SandImmune® Injection (cyclosporine injection, USP), 5 mL sterile ampule, $15.94/vial from hospital pharmacy

Each mL contains:

- 50 mg Cyclosporine, USP
- 650 mg Cremophor® EL (liquid, polyoxyethylated castor oil)
- 32.9% Alcohol by volume.

Dosing solution preparation on day of experiment:

Rat experiments: Dilute 1 mL of stock with 9 mL of sterile NS to yield a total volume of 10 mL at 5 mg/mL (1:10 dilution)

This diluted stock has 24 h expiration.

- For 10 mg/kg dose, administer 2 mL/kg (eg. for 300 g rat, give 0.6 mL) by slow IV infusion over 5 min
- For 20 mg/kg dose, administer 4 mL/kg (eg. for 300 g rat, give 1.2 mL) by slow IV infusion over 5 min
**Vehicle-control**

**Stock solution preparation (for 5 mL final volume):**

- 3250 mg Cremophor EL (Sigma C5135-500g). Although it is a liquid, measure by weight in glass vial.
- Add 1.65 mL ethanol (absolute, 99%, Spectrum #E1028-500mL).
- Add 0.25 mL sterile normal saline.

**Dosing solution preparation or the vehicle on day of experiment:**

Take 1 mL of stock solution, dilute with 9 mL of sterile NS to yield a total volume of 10 mL.

Sterile filter (Millipore Millex GV, 0.22 mM, 33 mm sterile syringe filters, SLGV033RS).

This stock has 24 h expiration.

- Administer 4 mL/kg (eg. for 300 g rat, give 1.2 mL) by slow IV infusion over 5 min

### Table 5. Therapy 3, Cyclosporine A (CsA)

#### Key references

<table>
<thead>
<tr>
<th>Drug: CsA</th>
<th>Study/Author</th>
<th>Dose</th>
<th>Model/Species</th>
<th>Outcomes</th>
<th>Function</th>
<th>Other Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>38/Okonkwo and Povlishock 1999</td>
<td>10 mg/kg (Sandoz) intracisternal in PEG/sterile saline/camphor (25 mg CsA/mL vehicle) at 30 min before injury</td>
<td>Male, Sprague Dawley Rat impact acceleration</td>
<td>EM, APP immunohistochem &amp; physiology</td>
<td>CsA reduced mito damage on EM and reduced APP+ profiles</td>
<td>NONE</td>
<td>Suggested mechanism was by preventing Ca-mediated opening of mito PT pore</td>
</tr>
<tr>
<td>39/Buki et al, 1999</td>
<td>10 mg/kg (Sandoz) intracisternal in PEG/sterile saline/camphor (25 mg CsA/mL vehicle) at 30 min after injury</td>
<td>Male, SD Rat impact acceleration</td>
<td>Reduction in spectrin proteolysis, neurofilament compaction, and APP immunohistochemistry</td>
<td>NONE</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td>40/Scheff and Sullivan, 1999</td>
<td>Dose response studies of 150 mg/kg, 40 mg/kg or 20 mg/kg in olive oil or polyethlated castor oil and saline Treatment either immediately before or 15 min after injury. There was a subsequent injection at 24 h. All dosing was IP.</td>
<td>C57 mouse CCI Gender not defined</td>
<td>Reduction in cortical lesion volume at 7 days after injury with either pre or post 20 mg/kg or pre 40 mg/kg. More modest reduction with pre 150 mg/kg</td>
<td>NONE</td>
<td>Compared to post treatment with 0.5, 1.0, or 10 mg/kg of FK 506 in CCI model in SD rat—which showed no significant reduction in lesion volume</td>
<td></td>
</tr>
<tr>
<td>41/Sullivan et al(1999)</td>
<td>20 mg/kg in polyethylene glycol/sterile saline and cremophor oil IP at 15 min after injury.</td>
<td>Male, SD rat—CCI</td>
<td>Beneficial effects on isolated mitochondria swelling, Ca accum, membrane potential, ROS</td>
<td>NONE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42/Sullivan et al, 2000</td>
<td>20 mg/kg IP bolus 15 min post injury</td>
<td>Male, SD rat CCI</td>
<td>74% reduction in lesion volume at 7 days with</td>
<td>NONE</td>
<td>Note—full 7 days of treatment in</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Methodology</td>
<td>Outcome</td>
<td>Description</td>
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</tbody>
</table>
| 43/Sullivan et al, 2000 | EXP1) 40, 20, 10, 5, or 1 mg/kg in polyethylene glycol/sterile saline and cremophor oil IP at 15 min after injury with a subsequent IP injection at 24 h  
EXP2) Time window 15 min, 1 h, 6 h or 24 h for first dose with a subsequent 24 h dose. | Male, SD rat  
Lateral FPI or CCI | Dose response for %cortex damaged at 7d showed that the 20 mg/kg dose was best with a 50-60% in cortical damage.  
Time window showed 15 min treatment was better than 1h and 6h showed no effect and 24h slight effect | NONE | A BBB study was also done to assess the effect of treatment on BBB permeability assessed by endogenous IgG—about a 50% reduction in BBB perm |
| 44/Suehiro and Povlishock, 2001 | CsA plus hypothermia  
ICV treatment Hypothermia for 1 h at 32°C, CsA 10 mg/kg before re-warming | Male, SD rat, impact acceleration | Hypo+CsA attenuated the rebound in APP+ profiles seen after hypo + rapid re-warming alone | NONE |
| 45/Alessandri et al, 2002 | Dosing for the Cognitive outcome studies  
CsA 0.125 mg/kg/h IV infusion for 3h beginning 1h before FPI (total dose 0.375 mg/kg)  
OR 6.25 mg/kg/h infusion for 3h beginning 1h before FPI (total dose 18.75 mg/kg)  
Sandimmun diluted to a working solution of 0.125 mg/mL or 6.25 mg/mL with sterile saline | Male, SD rat, Lateral FPI | NONE | IV infusions used  
Parallel microdialysis & O2 consumption studies done  
Microdialysis showed robust ~6-fold increase in brain penetration of CyA after FPI  
Effects on calcineurin in addition to effects on mito proposed |
| 46/Okonkwo et al, 2002 | 3, 10, 20, 30, or 50mg/kg IV OR 10 mg/kg IT, or vehicle IV starting immediately after TBI over 1 h IV drugs by microinfusion pump; Vehicle was a solution of polyethylene glycol, cremophor E1 and sterile saline | Male, SD rat, impact acceleration | 10, 20, or 30 mg/kg were effective in reducing APP+ axons.  
10 mg/kg IV showed the maximal efficacy. | Nice brain tissue level study shows that a 20 mg/kg IV yields similar brain tissue levels as a 10 mg/kg IT |
<p>| 47/Signoretti et al, 2004 | 10 mg/kg IT using the approach described by Okonkow et al, 30 min after TBI | Male, Rat impact acceleration | None | Description of the procedures a bit unclear on several points related to |</p>
<table>
<thead>
<tr>
<th>IV treatment groups used 20 or 35 mg/kg/h for 1.5 h</th>
<th>NAA, ADP, and ATP levels in brain tissue better than 20 mg/kg</th>
<th>treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>IT CsA given at 10 mg/kg at 30 min after injury in polyethylene glycol/sterile saline and cremophor oil with assessments at either 2 or 6 h post TBI</td>
<td>Ewe impacted with a stun gun in the left temporal region</td>
<td>None</td>
</tr>
<tr>
<td>Male Wistar rats—rotating dental drill injury</td>
<td>APP mRNA and immunohistochemistry at 2 or 6h after injury APP mRNA reduced ~15% and 30% by CsA at 2h and 6h respectively. More dramatic reductions in APP immuno by CsA Note: The model produces widespread DAI and vascular damage</td>
<td>None</td>
</tr>
<tr>
<td>20 mg/kg IP repeated at 24 h</td>
<td>No benefit of treatment-on survival of calretinin or parvalbumin staining neurons or area of damage—indeed CsA worse in some cases</td>
<td>None</td>
</tr>
<tr>
<td>Comparison of treatment with CsA 20 mg/kg vs 10 mg/kg NIM811 (non-immunosuppressing CsA analog) both IP</td>
<td>Male CF1 mice CCI</td>
<td>Similar effect of CsA and NIM811 on spectrin breakdown and volume of silver positivity</td>
</tr>
<tr>
<td>Treatment with 20 mg/kg at either 15 min or 1h after TBI</td>
<td>Male SD rat midline FPI</td>
<td>Similar beneficial effect of both CsA and NIM811 on motor function and composite neuroscore</td>
</tr>
<tr>
<td>IP CsA 20 mg/kg CsA 10 mg/kg in Chitosan microspheres implanted into the craniectomy site to produce timed release</td>
<td>Male SD rat, Feeney wt drop model</td>
<td>None</td>
</tr>
<tr>
<td>Both treatments reduced lipid peroxidation and mito damage</td>
<td>None</td>
<td>Touted in contusion as a way to give CsA without side effects</td>
</tr>
<tr>
<td>IP 20 mg/kg CsA at 1, 3, 4, 5, 6, or 8h after injury followed by 10 mg/kg/day by osmotic minipump for 3 d</td>
<td>Male SD rat CCI</td>
<td>None</td>
</tr>
<tr>
<td>Cortical tissue sparing at 3 d after injury seen across a wide therapeutic window out to 8h. Trend toward best at 1 h post injury</td>
<td>20 mg/kg IP suggested to mimic blood levels in the 2.5 mg IV infusion over 2h used in patients and 10 mg/kg/d infusion n rat suggested to mimic the 5 mg/kg IV 72 h infusion in humans</td>
<td>Improvements in</td>
</tr>
</tbody>
</table>
Simvastatin chemical structure

Therapy #4: Simvastatin

The 3-hydroxy-3-methylglutaryl coenzyme A (HMGA) reductase inhibitor Simvastatin reduces serum cholesterol but also has potent inhibitory effects on neuro-inflammation and possible effects on brain edema, Akt, CBF and trophic factor production. At the time the grant proposal was submitted, one study used systemic administration of statins (63) and suggested that Atorvastatin had similar benefits on Rotarod but a somewhat greater reduction in neuronal death than Simvastatin. In addition, in that study Atorvastatin was favored over Simvastatin related to its longer half life and active metabolites. Both are FDA approved and represent, thus, prime low hanging fruit candidates.

With regard to oral dosing, Lu et al (66) reported that Simvastatin reduced CA3 cell death and improved MWM performance after CCI in rats. The MWM findings were limited to an effect on % time in target; latencies were not reported. Simvastatin exhibited greater benefit than Atorvastatin, although both showed benefit.

A total of 14 studies were identified with Simvastatin in TBI—8 published after submission of the grant proposal. One study on spinal cord injury in rats is also noteworthy. In two studies in 2007, Mahmood et al (64, 65) in the group of Dr. Michael Chopp reported a small benefit of Simvastatin on motor score after CCI in female Wistar rats. A reduction in CA3 cell death and an increase in BDNF levels were also seen in one of the reports. In subsequent reports by the Chopp group, also in CCI in rats, beneficial effects were noted with treatment on Brdu labeling, blood vessel formation, VEGF, Akt, eNOS, P-FOXO1, P-IκB, GSK3 and CREB expression (66-68). In one of the papers, Wu et al (68) reported a reduction in TUNEL, although they noted variable effects on cytokines with a surprising increase in TNF by treatment (70). In the studies by the Chopp group, in general, a dose of 0.5 or 1 mg/kg daily beginning on day 1 after injury and continued for 14 d was used—and 1 mg/kg was usually best. Wu et al from the Chopp group also reported beneficial effects of Simvastatin (1 mg/kg/PO for 14d) on a variety of parameters focused on angiogenesis via AKT related effects on eNOS (80).

In studies by other groups, Chen et al (69) used a Feeney weight drop model in rats and used a much higher dosing regimen 37.5 mg/kg po at 1 h and 6 h. A small effect on TUNEL was seen, but beneficial effects on Rotarod, % brain water (at 24 h) and cytokines were reported. No appreciable effect on BBB was seen. Beziaud et al (79) also used this high dose of 37.5 mg/kg at 1 h and 6 h after lateral FPI in rats, and demonstrated significant effects on brain water, BBB, and other inflammatory markers vs vehicle. Abrahamson et al (71) reported a beneficial effect
of Simvastatin (3 mg/kg PO daily, first dose at 3 h post CCI) on probe trial, but no effect on MWM latency in murine CCI using mice genetically modified to express human Aβ. Reduction in Aβ deposition was also seen. Chauhan et al (73) also studied CCI in mice using a different 2 mg/kg oral dosing regimen—specifically, with the drug incorporated in the feedings. They reported once again a beneficial effect on probe trial without improvement in latency. A beneficial effect on axonal injury (axonal marker SMI 312) was also noted.

Not all studies with Simvastatin have been positive. Chen et al (74) used the parasagittal FPI model in rats and doses of 25, 37.5, 50, 75 or 100 mg/kg PO at 1h and 6h after injury and reported a reduction in edema, but no consistent effects on neuroscore, beam walking or lesion volume. No obvious dose-response was seen; possibly the 37.5 mg/kg dose was best. Overall the effects were modest. Similarly, Indraswari et al (78) reported that Simvastatin at 1 or 5 mg/kg PO given in divided doses twice daily was not effective in improving Rotarod performance after CHI in C57BL6 mice. This contrasted Resuvastatin (Crestor) which showed benefit on Rotarod, MWM, and hippocampal neuron counts at the same dose. This study would argue for Crestor rather than Zocor treatment. Lee et al (72) studied the effect of Simvastatin (20 mg/kg SQ d 1-3 and 5 mg/kg SQ d 4-7) in rats after spinal cord injury. No reduction in lesion volume was observed and pellet retrieval was actually worse in treated vs vehicle.

Our WRAIR group has been studying an IV formulation of Simvastatin in the PBBI model in rats at doses of 0.001, 0.01, 0.1, and 1.0 mg/kg (81). They dissolved Simvastatin using a combination of 100% Ethanol/1,2 Propanediol/sterile (nonpyrogenic) H₂O and gave 10 min IV infusion at 30min, 6h post-PBBI and every 24h out to 10d (all treatments given IV). There was no benefit on Rotarod from 7-10d post-PBBI. However, SIM dose-dependently protected against cognitive deficits in the MWM task with D-R curves across a wide dosing range; and a striking D-R curve in probe trial. Shortening the dosing regimen to 4 DPI (n=6/group) failed to show benefit so chronic treatment is required. Simvastatin IV in those studied did not result in any increase in mortality rates but "bloody" urine was detected immediately after the first 10 min IV infusion (30 min post-PBBI) in a number of 1.0 mg/kg (highest dose) SIM rats and several 0.1 mg/kg (2nd highest dose) SIM rats. No additional bloody urine was detected following subsequent infusions.

Regarding dosing, route of administration and pharmacology, in general, an oral (gavage) dosing regimen of between 1-3 mg/kg daily for up to 14d has shown the most benefit in experimental TBI. A dose of 0.5 mg/kg is in general less effective. Several studies have used much higher doses, but they have shown more variable results. SQ administration has been effective in one study at 20 mg/kg. Most of the oral administration studies have used a 14d treatment regimen, the two aforementioned parenteral studies used shorter regimens of 3d or 1 dose. Most of the studies have been carried out in CCI in rats or mice and two in modified weight drop, one in mouse and one in rat, and one study, where the smallest effect was seen was carried out in FPI in mice.

Finally, surprisingly, Simvastatin has been shown to enhance LTP in C57BL/6 mice (75). None of the studies in experimental TBI have included naïve controls treated with the Simvastatin or any other statin; thus it is unclear whether the cognitive enhancement that has been shown with these agents after TBI represents an effect specific for TBI or nonspecifically enhanced cognitive function.

Given the mission of OBTT to test low hanging fruit—drugs that have shown promise using treatment regimens that have in general been established in experimental TBI by other
laboratories, we had several options, 1) use the doses (1 vs. 3 mg/kg) and route of administration (PO gavage) taken in the majority of studies, with treatment beginning at 3h and continuing daily for 14d, 2) use the IV approach taken by Shear, 3) use a modified approach with acute IV therapy as used by Shear, but then chronic PO therapy out to at least 10-14d or even to the completion of testing, 4) use the higher dosing regimen of 37.5 mg/kg suggested by Laskowitz or Beziaud with acute administration only, or 5) consider Rosuvastatin rather than Simvastatin based on the work of Indraswari et al (78). Dr. Chopp suggested that we use Simvastatin in preference to Atorvastatin.

Subsequent to submission of our grant proposal, several papers on Simvastatin were published. One of the most relevant describes work with Atorvastatin and Simvastatin in Alzheimer disease (AD). In murine AD models Simvastatin has good BBB permeability, while Atorvastatin does not cross intact BBB (76, 77). Curiously, both Simvastatin and Atorvastatin reduced neurofibrillary tangles (NFTs) in murine AD models (76). Simvastatin (20 mg/kg/d) for 1 mo attenuated NFTs and microglial burden in aged Tau transgenic mice, and 8 mo of treatment (30 mg/kg/d) improved T-maze function. Similarly, 5 mo of treatment with Atorvastatin (0.01% in the diet) reduced NFTs and attenuated microglia burden in hyper-cholesterolemic mice. Peripheral effects on inflammation may thus play a role. Sierra et al (77) compared 9 statins with regard to BBB penetration, lipophilicity, HMG CoA reductase inhibition, and protection vs. neurodegeneration from Tau and concluded that Simvastatin was best. How these findings translate to TBI is unclear, but the BBB permeability of Simvastatin may be important. Based on this information and extensive discussion by our team, we will take the following approach:

Oral gavage treatment with Simvastatin in rat for OBTT; 1 or 3 mg/kg PO with first dose at 3 h after injury and subsequent daily doses for 14 d.

Dosing solution preparation:
1. Prepare a stock solution of methylcellulose (M0512 Sigma) 3% in distilled water.
   Note: Dissolving methylcellulose requires some care as it is only soluble in cold water, yet attempting initial dispersion in cold water will fail as a gel rapidly forms upon hydration, causing it to clump. The best way to dissolve it is to first disperse the powder in hot water (eg. 80°C), then cool it down with additional water while stirring to allow for dissolution. For example, disperse 3 g in 20 mL of 80°C distilled water. While mixing using a stir bar, add cold water to a total volume of 100 mL.

2. Prepare a 20 mg/mL stock solution of Simvastatin (S6196 Sigma) in 100% undenatured ethanol (eg. dissolve 5 mg in 250 μL of ethanol). Prepare this solution daily and store refrigerated at 4°C.
3. Add 60 μL of the stock solution of Simvastatin in ethanol to 2.45 mL of distilled water and vortex well. The solution will precipitate turning cloudy.
4. Add 0.5 mL of the 3% methylcellulose solution
5. Vortex, invert, and mix thoroughly until the dosing suspension is homogenously suspended.
6. This final solution is 0.4 mg/mL of simvastatin in 0.5% methylcellulose.

To make stock for the 1 mg/kg doses:

7. Prepare a stock solution of methylcellulose (M0512 Sigma) 3% in distilled water.
   Note: Dissolving methylcellulose requires some care as it is only soluble in cold water, yet attempting initial dispersion in cold water will fail as a gel rapidly forms upon hydration, causing it to clump. The best way to dissolve it is to first disperse the powder in hot water (eg. 80°C), then cool it down with additional water while stirring to allow for dissolution. For example, disperse 3 g in 20 mL of 80°C distilled water. While mixing using a stir bar, add cold water to a total volume of 100 mL.

8. Prepare a 20 mg/mL stock solution of Simvastatin (S6196 Sigma) in 100% undenatured ethanol (eg. dissolve 5 mg in 250 μL of ethanol). Prepare this solution daily and store refrigerated at 4°C.
9. Add 60 μL of the stock solution of Simvastatin in ethanol to 2.45 mL of distilled water and vortex well. The solution will precipitate turning cloudy.
10. Add 0.5 mL of the 3% methylcellulose solution
11. Vortex, invert, and mix thoroughly until the dosing suspension is homogenously suspended.
12. This final solution is 0.4 mg/mL of simvastatin in 0.5% methylcellulose.
2. Prepare a 60 mg/mL stock solution of Simvastatin (S6196 Sigma) in 100% undenatured ethanol (eg. dissolve 15 mg in 250 uL of ethanol). Prepare this solution daily and store refrigerated at 4°C.

3. Add 60 µL of the stock solution of Simvastatin in ethanol to 2.45 mL of distilled water and vortex well. The solution will precipitate turning cloudy.

4. Add 0.5 mL of the 3% methylcellulose solution

5. **Vortex, invert, and mix thoroughly** until the dosing suspension is homogenously suspended.

6. This final solution is 1.2 mg/mL of simvastatin in 0.5% methylcellulose.

**To make stock for vehicle controls:**

1. Obtain undenatured ethanol.

2. Add 60 µL of the undenatured ethanol to 2.47 mL of distilled water and vortex well. The solution will precipitate turning cloudy.

3. Add 0.5 mL of the 3% methylcellulose solution

4. **Vortex, invert, and mix thoroughly** until the dosing suspension is homogenously suspended.

5. This final solution is 0.5% methylcellulose.

**Administration (technique):**

7. Be sure the dosing solution is well-mixed and homogenous.

8. Draw of 2.5 ml/kg (eg. 1 mL for a 400 g rat) of the desired dosing solution stock through the oral gavage needle into a syringe to account to fill any dead space in the gavage needle.

9. To administer: The rat is firmly restrained (grasped by the loose skin of the neck and back) to immobilize the head and maintained in an upright (vertical) position. The gavage tube is passed through the side of the mouth, following the roof of the mouth, and advanced into the esophagus and toward the stomach. After the tube is passed to the correct length, inject the dose solution. Do not aspirate or attempt to empty the dead space in the gavage needle as this will result in an inaccurate dose.

**Note:** An analogous description of the procedure for drug administration in mice is available for viewing on Youtube for reference: [http://www.youtube.com/watch?v=AAXeMAaEjb8&feature=player_embedded](http://www.youtube.com/watch?v=AAXeMAaEjb8&feature=player_embedded)

10. The length for depth of administration in rat is again based on distance to last rib. The gavage tube that is currently used by Ed Dixon's team is provided below:

   - Cosh Healthcare  Tel: 770-939-2007
   - Popper animal feeding needle - curved
   - 16/102mm guage x 3.00mm dia: 01-290-11C, 12/pk (16ga x 4”)
   - 1 pk 319.08

**Administration (Dosing):**

1. The first dose will be administered at 3 h after injury.

2. Subsequent dosing will be carried out daily for 14 d

3. The dose will be administered after functional outcome testing is completed each day

Groups
1. Sham (surgery but no treatment)
2. CCI plus Vehicle at 3 h after injury and daily thereafter for 14 d
3. CCI plus Simvastatin 1 mg/kg at 3 h after injury and daily thereafter for 14 d
4. CCI plus Simvastatin 3 mg/kg at 3 h after injury and daily thereafter for 14 d

**Table 3. Therapy**

<table>
<thead>
<tr>
<th>Study/Author</th>
<th>Dose</th>
<th>Model/Species</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drug: Simvastatin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63/Wang et al 2007 (Laskowitz group)</td>
<td>20 mg/kg/d SQ for 3 d; First dose 30 min post TBI; Simvastatin alkaline hydrolysis</td>
<td>Closed Head Injury/Mouse</td>
<td>Decreased neuronal death by FJB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Benefit on Rotarod</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Atorvastatin and Simvastatin similar effects; Atorvastatin ↓ FJ+ neurons;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Simvastatin slightly better Rotarod; ↑LDF with Simvastatin</td>
</tr>
<tr>
<td>64/Mahmood et al 2008 (Chopp group)</td>
<td>0.5 or 1 mg/kg PO for 14 d beginning on d 1 after TBI</td>
<td>CCI in female Wistar rats</td>
<td>Modestly better neuroscore—1.0 &gt; 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A number of other studies but Simvastatin plus stem cells were studied</td>
</tr>
<tr>
<td>65/Mahmood et al 2009 (Chopp group)</td>
<td>0.5 or 1 mg/kg PO for 14 d began d 1</td>
<td>CCI in female Wistar rats</td>
<td>Improvement in CA3 neuronal survival at 3 mo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slight ↑ neuroscore—1.0 &gt; 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Similar neuroscore data as ref #2; higher BDNF levels at 3 mo</td>
</tr>
<tr>
<td>66/Lu et al 2007 (Chopp group)</td>
<td>1 mg/kg PO for 14 d beginning d 1</td>
<td>CCI in male Wistar rats</td>
<td>Greater CA3 survival; and nearly identical for Simvastatin and Atorvastatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MWM greater % time in target quadrant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Simvastin better than Atorvastatin in MWM; no latencies given; Brdu ↑ new blood vessels ↑ with both statins</td>
</tr>
<tr>
<td>67/Wu et al 2008 (Chopp group)</td>
<td>1 mg/kg PO for 14 d beginning on d 1 after TBI</td>
<td>CCI in male Wistar rats</td>
<td>MWM ↑ % time in target quadrant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MWM data, no sham &amp; no latencies; VEGF; Akt, eNOS, P-FOXO1, and P-IκB all ↑</td>
</tr>
<tr>
<td>68/Wu et al 2008 (Chopp group)</td>
<td>1 mg/kg PO for 14 d beginning on d 1 after TBI</td>
<td>CCI in male Wistar rats</td>
<td>20-60% ↓ TUNEL+ in hippo; 60% ↓ in caspase 3 activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Modestly better neuroscore</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enhanced Akt, GSK3 and CREB</td>
</tr>
<tr>
<td>69/Chen et al 2009</td>
<td>37.5 mg/kg po at 1 h and 6 h</td>
<td>Feeney weight drop in male Wistar rats</td>
<td>Tiny reduction in TUNEL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Improved rotarod at 24 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NFKb, cytokines ↓ vs vehicle; brain water ↓; little Δ in BBB</td>
</tr>
<tr>
<td>70/Li et al 2009 (Chopp group)</td>
<td>1 mg/kg PO for 14 d began d 1</td>
<td>CCI in male Wistar rats</td>
<td>Minimal effect on neuroscore</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IL1 ↓, TNF↑, IL6 unchanged by Rx; CD68 &amp;GFAP ↓ by Rx</td>
</tr>
<tr>
<td>71/Abrahamson et al 2009 (Dixon group)</td>
<td>3 mg/kg PO daily, first dose at 3 h post CCI</td>
<td>CCI in mouse</td>
<td>Rx ↑ only probe trial</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reduced Aβ peptide deposition; Rx ↑ synaptophysin in CA3</td>
</tr>
<tr>
<td>72/Lee et al 2010</td>
<td>20 mg/kg SQ d 1-3 and 5 mg/kg SQ d 4-7</td>
<td>Spinal cord injury in SD rats</td>
<td>No effect on lesion volume</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Little effect; ↓ pellet retrieval</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Also studied minocycline and saw similar lack of effect</td>
</tr>
<tr>
<td>73/Chauhan et al 2010</td>
<td>2 mg/kg feed weight (very unclear)</td>
<td>CCI in C57 male mice</td>
<td>Improvement in axonal marker SMI 312 in dentate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No effect on MWM latencies; ↑ probe trial</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Also studied EPO and combined EPO plus Simvastatin</td>
</tr>
<tr>
<td>74/Chen et al 2008</td>
<td>25, 37.5, 50, 75 or 100 mg/kg PO at 1h and 6h</td>
<td>Moderate parasagittal FPI in male SD rat</td>
<td>No effect on lesion volume</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Variable neuroscore &amp; beam walking</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brain water ↓—37.5 best on BW; No clear dose response; did</td>
</tr>
</tbody>
</table>

**Key References**
<table>
<thead>
<tr>
<th>Reference</th>
<th>Treatment</th>
<th>Time Point</th>
<th>Outcome</th>
<th>Combined Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>75/Mans et al, 2010</td>
<td>Atorvastatin or Simvastatin added to hippocampal slice bath</td>
<td>LTP</td>
<td>2 hr but not 20 min of treatment enhanced LTP and increased Akt phosphorylation</td>
<td></td>
</tr>
<tr>
<td>76/Boimel et al, 2009</td>
<td>Simvastatin 20-30 mg/kg/day Atorvastatin 0.01% of the diet</td>
<td>Tau transgenic murine models of AD</td>
<td>Simvastatin and Atorvastatin both effective in reducing NFTs in brain and microglial burden</td>
<td></td>
</tr>
<tr>
<td>77/Sierra et al, 2011</td>
<td>Comparison of 9 statins</td>
<td>Assessment of BBB perm, lipophilicity, HMGCoA reductase activity, and neuroprotection vs Tau</td>
<td>Simvastatin suggested to be the best statin for chronic neuroprotection vs Atorvastatin &amp; others</td>
<td></td>
</tr>
<tr>
<td>78/Indraswari et al, 2011</td>
<td>Comparison of Simvastatin (Zocor) to Rosuvastatin (Crestor); 5 d gavage in 100 μLNS beginning at 1 h postTBI then twice daily in divided doses of 1 or 5 mg/kg/d</td>
<td>C57BL6, closed head injury model</td>
<td>Simvastatin tested for Rotarod and not effective unlike Rosuvastatin which was effective at 1 mg/kg begun at 1 h &amp; 5 mg/kg begun as late as 3 h. NEGATIVE study for Simvastatin but it was only tested in Rotarod and in a 5d regimen. Discussion suggests statins oxidative stress, cytokines, NADPH oxidase, thrombogenic genes &amp; Tangiogenesis &amp; plasticity via PKB</td>
<td></td>
</tr>
<tr>
<td>79/Beziaud et al, 2011</td>
<td>Simvastatin 37.5 mg/kg gavage 1 and 6 h after TBI</td>
<td>Male SD Rat Lateral FPI</td>
<td>None</td>
<td>Claudin, MPO, MMP9, ICAM-1, and other outcomes almost all also favorably effected</td>
</tr>
<tr>
<td>80/Wu et al, 2011</td>
<td>Simvastatin 1 mg/kg gavage beginning 1d after TBI and continued for 14d</td>
<td>Adult male Wistar rat CCI</td>
<td>Increased angiogenesis in cortex and hippo</td>
<td>Improved foot fault Also increased VEGF receptor and AKT mediated phosphorylation of eNOS</td>
</tr>
<tr>
<td>81/Shear et al, 2012</td>
<td>Simvastatin IV at doses of between 0.001 and 1.0 mg/kg given at 30 min, 6 h and daily IV</td>
<td>Rat PBBI model</td>
<td>Benefit on MWM but no benefit on Rotarod</td>
<td>Histology pending Poster presentation at 2012 NNT</td>
</tr>
</tbody>
</table>

4. **Biomarker sampling processing**

Blood sampling will be carried out as described above. For the early time points, 0.7 mL will be obtained as described above. The final time point at sacrifice will include sampling of 2-3 mL of blood obtained from the left cardiac ventricle via a 20-gauge needle. Blood will be placed immediately in microcentrifuge tubes (1.5 mL Eppendorf brand, colorless) and allowed to clot at room temperature for 60 min. Tubes will be centrifuged at 5,000xg at room temperature for 5 min. The serum is collected into 1.5 mL microcentrifuge tubes or cryotubes (screw cap-type
preferred) with “Tough-tag label” (freezer proof) – use with permanent fine black marker for label writing. Serum samples after labeling will be snap frozen on dry ice and stored at -80°C in waxed cardboard freezer boxes (#C5520)/81 cell divider (#CD81) (http://www.crystalgen.com/wax.php) until used or shipped. Each sample will have a code number for the specific rat followed by a -4 h, -24 h, or final (-F) designation. Also note that any sampling for biomarkers that coincides temporally with drug dosing should be done prior to drug administration. For example, if a drug dose is scheduled for 24 h, obtain the biomarker sample first and then administer the drug. Samples will be shipped (FedEx overnight on Monday or Tuesday) in large Styrofoam boxes (taped) with extra dry ice that can last 3 days (~4-6 kg). Prior to shipping please notify Banyan and provide tracking number.

Shipping contact: Ms. Olena Glushakova, 386-518-6762; oglushakova@banyanbio.com
Backup: Danny Johnson, 386-518-6763; djohnson@banyanbio.com

5. Publications and presentations (Guidelines)

Manuscripts

1. Manuscript submissions of work supported by OBTT will be discussed collectively by the group and each will be addressed on a case by case basis.
2. Manuscript submissions of work supported by OBTT will include, as authors, each of the site PIs and co-investigators, and others as appropriate for their contribution to the work.
3. Manuscript submissions of work supported by OBTT need to be approved by the five PIs of OBTT (Kochanek, Dietrich, Hayes, Povlishock, and Tortella). In cases where there is a difference of opinion on publication where a unanimous decision cannot be reached by the site PIs, Dr. Kochanek will make the final decision.
4. If studies using an OBTT defined drug were carried out independent of OBTT funding, the results of those studies can be published unrestricted by OBTT guidelines.
5. Operation Brain Trauma Therapy should appear in the title of any manuscript submitted that results from work supported by OBTT.
6. Manuscript submissions of work supported by the OBTT grant will acknowledge support from the US Army, W81XWH-10-1-0632.

Abstracts

1. Abstract presentations at scientific meetings by investigators at individual sites are encouraged from work supported by OBTT funding. Abstract submissions should adhere to the following four guidelines;
   a. Data cannot be submitted or presented until the code has been broken for the full OBTT group.
   b. Operation Brain Trauma Therapy should appear in the title of the abstract from each of our groups on a given therapy if the studies were done as part of OBTT and if allowed in the abstract submission, support for the US Army, W81XWH-10-1-0632 should be acknowledged (on both the abstract and poster).
c. If studies using an OBTT defined drug were carried out independently, the results of those studies can, of course, be reported unrestricted by OBTT guidelines.

d. Author inclusion should be appropriate for the work done at the site or sites involved for each given submission. Authors of abstracts should receive a copy of the abstract prior to submission for review and comment.

e. The PI at each OBTT site should receive a copy of all abstracts submitted, whether or not their site is part of the work.

6. Results: The outcome tables for each therapy are presented in order of study as an appendix at the end of this document.

References


# Appendix: Outcome table with findings and overall score for each therapy

## Drug 1. Nicotinamide

<table>
<thead>
<tr>
<th>Site</th>
<th>Neuro Exam</th>
<th>Motor</th>
<th>Cognitive</th>
<th>Neuropath</th>
<th>Biomarker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitt</td>
<td>None</td>
<td>Beam balance (2)</td>
<td>Hidden platform latency (5)</td>
<td>Lesion volume (2)</td>
<td>GFAP 24 h (1) 4-24 h Δ (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beam walk (2)</td>
<td>MWM probe (5)</td>
<td>Hemispheric volume (2)</td>
<td>UCHL1 24 h (1) 4-24 h Δ (1)</td>
</tr>
<tr>
<td>Pitt: Site max total</td>
<td>0</td>
<td>4</td>
<td>10</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Pitt: Nicotinamide 50 mg/kg</td>
<td>0</td>
<td>0,0</td>
<td>-5, 0</td>
<td>0,0</td>
<td>0,0,0,0</td>
</tr>
<tr>
<td>Pitt: Nicotinamide 500 mg/kg</td>
<td>0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0</td>
<td>0,0,0,0</td>
</tr>
<tr>
<td>Miami</td>
<td>None</td>
<td>Cylinder (2)</td>
<td>Hidden platform latency (5)</td>
<td>Lesion volume (2)</td>
<td>GFAP 24 h (1) 4-24 h Δ (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gridwalk (2)</td>
<td>MWM probe (3)</td>
<td>Hemispheric volume (2)</td>
<td>UCHL1 24 h (1) 4-24 h Δ (1)</td>
</tr>
<tr>
<td>Miami: Site max total</td>
<td>0</td>
<td>4</td>
<td>10</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Miami: Nicotinamide 50 mg/kg</td>
<td>0</td>
<td>0,0</td>
<td>0,0,0</td>
<td>0,0</td>
<td>0,0,0,0</td>
</tr>
<tr>
<td>Miami: Nicotinamide 500 mg/kg</td>
<td>0</td>
<td>0,0</td>
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<td>0,0</td>
<td>0,0,0,0</td>
</tr>
<tr>
<td>WRAIR</td>
<td>Neuroscore (1)</td>
<td>Rotarod (3)</td>
<td>Hidden platform latency (5)</td>
<td>Lesion volume (2)</td>
<td>GFAP 24 h (1) 4-24 h Δ (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MWM probe (3)</td>
<td>Lateral ventricle volume (2)</td>
<td>UCHL1 24 h (1) 4-24 h Δ (1)</td>
</tr>
<tr>
<td>WRAIR: Site max total</td>
<td>1</td>
<td>3</td>
<td>10</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>WRAIR: Nicotinamide 50 mg/kg</td>
<td>0</td>
<td>0</td>
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A Novel Multicenter Preclinical Drug Screening and Biomarker Consortium for Experimental Traumatic Brain Injury: Operation Brain Trauma Therapy

Patrick M. Kochanek, MD, FCCM, Helen Bramlett, PhD, W. Dalton Dietrich, PhD, C. Edward Dixon, PhD, Ronald L. Hayes, PhD, John Povlishock, PhD, Frank C. Tortella, PhD, and Kevin K. W. Wang, PhD

T

raumatic brain injury (TBI) is a leading cause of morbidity and mortality in Operation Iraqi Freedom largely due to the emergence of blast-injury from attacks with improvised explosive devices (IEDs) along with continued importance of ballistic injury.1,2 The pathology resulting from these insults is complex, spans the spectrum from mild-to-severe TBI and is often complicated by polytrauma, hemorrhagic shock (HS), and burns.1–3 Current therapy of severe TBI includes supportive care, but brain-oriented therapy is limited to approaches targeting intracranial pressure (ICP) such as mannitol or surgical decompression.4 In blast-induced TBI, vasospasm, neuronal death, cognitive disability, and axonal injury are key targets. There is also no current therapy for mild TBI, which represents a source of morbidity and may be linked to posttraumatic stress disorder (PTSD).

Research to date in the field of TBI has focused on the study of pathomechanisms. This National Institutes of Health–driven approach has helped to identify and characterize many mechanisms of secondary damage. However, focus on mechanism has not led to the rapid advancement of new therapies to the bedside. This approach also has not encouraged cross-talk between laboratories and has unfortunately failed to build consensus in the field regarding the efficacy of new therapies and move them forward to clinical trials. A recent search of the terms “TBI” and “therapy” on PubMed produced over 21,000 citations including many positive results. Yet, few therapies have been tested in Phase III clinical trials and no new therapy for TBI has emerged.

As with the need for new therapies, there is a parallel need for the development of serum biomarkers of brain injury. Research has identified several potential biomarkers through clinical studies and limited work in TBI models.5–7 The performance of biomarkers of brain injury across the key contemporary TBI models, species, injury levels, and secondary insults such as hypoxemia or HS; however, has not been systematically evaluated. Studies of the ability of serum biomarkers to confirm neuroprotection are also lacking, and a comparison of the effect of therapies on conventional outcomes (function/neuropathology) versus serum biomarker levels remains to be carried out. Given that specific serum biomarkers have been developed to identify specific aspects of brain injury such as neuronal death, axonal injury, or glial injury, there is the potential to better understand the effect of therapies on these various cellular components. Such studies could advance the potential utility of biomarkers in both their translation to clinical application and utility in drug screening.

This review article discusses a consortium called operation brain trauma therapy (OBTT) that was recently established in attempt to address both the need for novel therapies and biomarkers in TBI. OBTT was designed to serve as a high-throughput therapy screening research consortium that identifies the most promising therapies and compares them across a spectrum of the state-of-the-art models and injury levels. The most promising therapies will be moved up the phylogenetic scale to a large animal model and ultimately to clinical trials.

WHY HAVE THERAPIES IN EXPERIMENTAL TBI FAILED TO TRANSLATE TO CLINICAL EFFICACY?

A key question in designing a research consortium to evaluate new therapies for TBI is “why have therapies failed to translate from the lab to the clinical in TBI?” Many reasons have been suggested to explain this failure, ranging from the...
complexity of the disease, difficulties in stratifying and evaluating outcome in humans with TBI, lack of knowledge of brain pharmacodynamics for new therapies, the fact that patients with TBI are already treated with various drugs and interventions that are not incorporated into preclinical studies, and the fact that the timing of drug administration in pre-clinical models is often chosen for proof-of-concept and maximal effect rather than clinical relevance. A common criticism of pre-clinical work in TBI has been the lack of a “proven definitive model” that has demonstrated success in translating a therapy from the bench to the bedside. However, it is being increasingly recognized that TBI, particularly severe TBI, is an extremely complex and heterogeneous disease. The importance of heterogeneity in TBI was highlighted in a recent review by Saatman et al. who have suggested the need to consider the myriad forms of TBI such as contusion, diffuse axonal injury, diffuse swelling, and subdural hemorrhage, and their combinations, as individual conditions, that may require individualized therapy including stratification in clinical trials. This suggests that optimized preclinical therapeutic screening will require the use of multiple models. This concept represents a key element of the approach that was developed for OBTT.

**COMPONENTS OF OBTT**

Figure 1 outlines the components of the consortium including the institutions, principal investigators (PIs), and models that will be used. Therapies will be screened first in rodents across a spectrum of established TBI models in two species, mice and rats, using clinically relevant paradigms. Outcomes in the primary screening models will include lesion volume, neuronal death, and axonal injury along with motor and cognitive outcomes (including Morris water maze performance). Drugs that show benefit in the first phase of primary screening in established TBI models will move to testing in more complex TBI models, namely, those with superimposed secondary insults such as HS or an inflammatory insult (IL-1β infusion). Therapies showing benefit in these screening models will also be studied across injury levels from mild to severe and will also be evaluated for effects on brain edema and cerebral blood flow (CBF) after TBI. The most promising therapies will be evaluated in a large animal TBI model—specifically, midline fluid percussion injury (FPI) in micropigs. Outcomes in the micropig model will include assessments for axonal injury, cerebrovascular dysfunction, and ICP—outcomes that have been shown to be highly relevant to blast injury, where axonal injury, vasospasm, and malignant brain swelling have been recently described. The time line for therapy development in this multiteried approach includes screening of each drug in the primary models (controlled cortical impact [CCI], parasagittal FPI, and penetrating ballistic-like brain injury) in rats over a 2- to 3-month period at each center, secondary evaluation of promising agents in the more complex rodent models such as TBI plus hemorrhage over a 6-month period, and finally, advancement of the single most promising agent tested each year to the micropig TBI model. Serum biomarker assessments will also be incorporated into the study designed.
with serial assessments layered upon the therapy screening (for biomarker assays to be used, Table 1). OBTT will then deliver agents that are either U.S. Food and Drug Administration (FDA) approved (for other uses) or in clinical development that are found to be effective across models for rapid clinical translation to the Defense Advanced Projects Agency Prevent Blast program and other investigative teams working with blast TBI models, and the clinical TBI and PTSD consortia that have been established by the US Army. OBTT will, for the first time, allow a direct comparison of therapies across TBI models in multiple centers. It will also include comparison of therapies across highly relevant combat casualty care scenarios (TBI plus polytrauma).

OBTT includes five internationally recognized centers:
1. the Safar Center for Resuscitation Research, University of Pittsburgh School of Medicine (Patrick Kochanek, MD; overall PI);
2. the Miami Project to Cure Paralysis, University of Miami School of Medicine (W. Dalton Dietrich, PhD; site PI);
3. the Neuroprotection program at Walter Reed Army Institute of Research (Frank Tortella, PhD; site PI);
4. Virginia Commonwealth University (John Povlishock, PhD; site PI); and
5. Banyan Biomarkers Inc. (biomarker core directed by Ronald Hayes, PhD).

**APPROACH TO DRUG SELECTION IN OBTT**

In OBTT, we will consider testing therapies that could be implemented at any point in the continuum of care for the TBI victim in combat casualty care (Fig. 2). However, the majority of the expertise of the screening centers, along with the experimental TBI field as a whole, is in the setting of acute neuroprotection. Severe TBI produces direct parenchymal disruption (primary injury) and sets into motion many secondary injury processes (Fig. 3) including disturbances in CBF resulting in a cascade of mechanisms related to ischemia, excitation, oxidative stress, mitochondrial failure, proteolysis, and disturbances in cell signaling, among other mechanisms, triggering neuronal death cascades from necrosis, apoptosis, and autophagy.17 Cascades contributing to brain swelling are also produced and can result in intracranial hypertension.17 Axonal, dendritic, and synaptic damage also occur18 as does inflammation, which may either contribute to secondary injury or signal the regenerative response.17,19,20

**TABLE 1. Proposed Biomarkers to be Used for Operation Brain Trauma Therapy**

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<tr>
<th>Biomarkers</th>
<th>Characteristics</th>
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<td>UCH-L1</td>
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Figure 2. Schematic of the continuum of care for severe TBI in combat casualty care. In OBTT, potential therapies that could be translated to clinical care are considered for use at any point along this continuum. CASH, combat advanced support hospital; OR, operating room; ICU, intensive care unit; Rehab, rehabilitation facility.

Figure 3. Schematic outlining the mechanisms involved in the evolution of secondary damage after TBI (see text for details). Primary injury (green), CBF disturbances (purple), excitation and oxidative stress (gray), cell death cascades (black), brain swelling (blue), axonal, synaptic and dendritic damage (red), and inflammation (yellow) occur simultaneously and interact to exacerbate damage and initiate repair. The secondary injury mechanisms culminate in neuropathological damage and/or behavioral deficits. Many of these important secondary injury mechanisms and outcome targets will be assessed in the various models used in the consortium. See text for details. BBB, blood-brain barrier; CBV, cerebral blood volume; EAA, excitatory amino acid; $\text{O}_2^-$, superoxide anion; AA, arachidonic acid; PKC, protein kinase C; ER, endoplasmic reticulum.
Two key therapeutic targets have served as the primary endpoints in testing of therapies in experimental TBI, namely some aspect of neuropathology (contusion volume, cortical lesion volume, neuronal death, or axonal damage), and functional outcome (neuroscore, motor testing, cognitive testing). Review of the experimental TBI literature identifies a number of therapies that have favorably affected these two outcome categories in individual laboratories. Many of these therapies reduce lesion volume by at least 20%, and some as much as 60% in experimental TBI. Various therapies targeting mitochondria and neuro-inflammation have shown robust effects in various individual laboratories. In contrast, therapies targeting neurotransmitter systems have generally shown the greatest effects on cognitive outcome reviewed in ref. Nevertheless, for just these two targets, neuropathology and functional outcome, there are a number of promising acute therapy candidates.

PROMISING THERAPEUTIC STRATEGIES FOR TRANSLATION IN TBI

Two categories of drugs have been identified for screening in OBTT. These include (1) “low hanging fruit” representing agents that are FDA approved for other uses and/or otherwise readily available that have shown promise in experimental TBI in multiple published reports and (2) novel but potentially high impact therapies that have a more limited publication track record. A number of agents in each of these categories are provided below, based on literature review and suggestions of the site PIs and overall PI of the consortium. In addition (Table 2), these drugs are also classified with regard to their putative primary mechanistic targets. Each of these targets, if appropriately addressed, has potential to reduce secondary damage and improve functional outcome.

We anticipate that a number of these therapies will be evaluated by the consortium; however, the specific drugs to be tested and the sequence of testing are currently being debated by the consortium investigators. An oversight committee will also evaluate and contribute recommendations and review results annually. This list does not, in any way, reflect a complete menu of potential agents for evaluation in OBTT, rather it reflects selected promising therapies across a number of categories. The following brief discussions of these therapies provide insight into the basic rationale for therapy selection by the consortium investigators.

TABLE 2. Putative Secondary Injury Mechanisms Targeted by Therapies Being Considered for Testing by the Operation Brain Trauma Therapy consortium

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Italic, low hanging fruit therapies; boldface, high risk-high reward therapies; ×, therapeutic target for the drug shown; DHA, docosahexanoic acid; CBF, cerebral blood flow; BBB, blood brain barrier.
be used as a nutritional supplement in a pre-treatment approach particularly in light of the ability to provide dietary neuroprotective additives in theater.

**Choline**

Chronic pre- and posttreatment with the nutritional supplement choline may offer substantial benefit for TBI. Rats fed a diet supplemented with 2% choline exhibited improved functional outcome, reduced contusion volume, and reduced neuro-inflammation at 2 weeks after injury.23 Chronically after TBI there is a well-recognized reduction in high affinity choline uptake sites; thus, choline may represent a prototype agent for both chronic pre-treatment to attenuate neuroinflammation, and as rehabilitation therapy to serve in neurotransmitter replacement. Cytidine diphosphate-choline, in a posttreatment approach, is in clinical trials.

**Atorvastatin**

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, also referred to as “statins,” not only reduce serum cholesterol but also have potent inhibitory effects on neuroinflammation and possible effects on CBF and trophic factor production. They confer benefit in experimental TBI.24-25 Atorvastatin, simvastatin, and lovastatin all show promise after TBI in rats. Impressive benefit was seen with atorvastatin therapy by Wang et al.24 where improved performance on rotarod and Morris water maze, reduced hippocampal neuronal death, and attenuated microglial proliferation and cytokine production in the brain were seen after TBI. Equal doses (20 mg/kg) of atorvastatin outperformed simvastatin. Atorvastatin is FDA approved and a logical candidate to study in our consortium. Either pre- or posttreatment paradigms could be evaluated.

**FK506**

There have been several reports of beneficial effects of the FK 506 (Tacrolimus) in experimental TBI.26,27 It is an immunophilin ligand that inhibits the protein phosphatase calcineurin. Benefit from this agent has been shown mostly in models focusing on axonal injury—which could be a special relevance to blast TBI.26,27 Immunomodulatory effects of this agent could also contribute to potential benefit after TBI.28 Another factor that raises the interest in cross model evaluation of this agent is that it may have fewer propensities to initiate seizure activity than another promising calcineurin antagonist cyclosporine A28—which is currently in clinical trials for TBI. However, unlike cyclosporine A, FK 506 does not appear to inhibit mitochondrial permeability transition. FK 506 is an FDA-approved immunosuppressant. Because the related agent, cyclosporine A, is already in clinical trials for TBI it would be logical to explore this agent in our consortium.

**Minocycline**

The tetracycline antibiotic minocycline has potent anti-inflammatory actions in brain related to its ability to inhibit microglial activation/proliferation. Specifically, P38 kinase activation and proliferation of microglia in culture is attenuated by minocycline.29 Many reports have confirmed inhibition of microglial proliferation by minocycline in stroke, cerebral hemorrhage, and other models.30-32 Minocycline has shown benefit after TBI in mice,32 where it also reduced IL-1β levels in brain. A dose of 45 mg/kg at 30 minutes after TBI, and continued every 12 hours for 3 days decreased lesion volume. A therapeutic window of 2.5 hours has been reported.30 Minocycline is FDA approved for other uses, and available for clinical trials. It is a prototype multifaceted anti-inflammatory candidate that could be rapidly translated into clinical trials. Its antimicrobial effects could also be of benefit in polytrauma.

**Lithium**

Lithium treatment down-regulates pro-apoptotic mechanisms33,34 and upregulates cell survival factors and markers of plasticity.35,36 It also improves synaptic plasticity, as measured by enhanced long-term potentiation in the hippocampus37 and spatial memory and retention in a T-maze paradigm.38 As evidenced by recent publications on lithium,37,38 there is renewed interest in its therapeutic potential. Thus, lithium has multiple targets that may favorably influence both acute and chronic TBI pathophysiology. This, together with the fact that lithium is approved for human use as a treatment for bipolar disorder, makes it an excellent translational research candidate for the treatment of TBI-induced cognitive and affective dysfunction.

**Rolipram**

The type IV phosphodiesterase (PDE) inhibitor rolipram has shown benefit in experimental TBI and spinal cord injury.39,40 It blocks PDE-IV-mediated breakdown of cAMP resulting in increased PKA activation, enhancing cell survival pathways, and inhibiting pro-inflammatory NFKb activation.39 Rolipram and other PDE IV and V inhibitors have also shown promise in reducing memory impairment in dementia and improving CBF and are in clinical trials in dementia.41 Although nausea and vomiting can be limiting with the use of rolipram in conscious patients, if beneficial, second generation type IV PDE inhibitors, with better side effect profiles, are in clinical trials for lung injury.

**Aniracetam**

This agent is an allosteric potentiator of AMPA-specific glutamate receptors and has shown promise in improving cognitive outcome across central nervous system (CNS) injury models including ischemia and TBI.42-44 Transmitter supplementation (norepinephrine and dopamine) is often used in TBI rehabilitation, but a similar strategy has not been developed for glutamatergic neurotransmission. AMPA receptor desensitization is seen chronically after TBI.42 Thus, aniracetam may serve that purpose. It also enhances glucose availability and acetylcholine synthesis, pathways that are similarly disturbed in TBI.42 It has an exceptionally broad therapeutic window being equally effective whether started at 24 hours or 11 days after injury in rats.42 Daily oral doses of 25 to 50 mg/kg were used. It will thus serve as a prototype for potential use as a rehabilitation therapy that is focused on functional rather than neuropathological outcomes. It also has shown benefit in models of anxiety and insomnia,45,46 both of
which are behavioral sequelae of TBI. It is available as an over-the-counter nutritional supplement.

**Pentostatin**

Adenosine is an endogenous neuroprotectant with CBF promoting, anti-excitotoxic and anti-inflammatory properties. Local injection of the adenosine-2A (A2a) receptor agonist CGS 21680 increases CBF after CCI in rats.47 Adenosine A1 receptor activation confers anti-excitotoxic effects and A1 receptor knock-out mice develop lethal status epilepticus after CCI.48 Adenosine effects at A2a and A3 receptors may also be anti-inflammatory.49,50 Thus, adenosine augmentation could be useful in TBI. A limitation of systemic use of adenosine agonists (i.e., acadesine, ATL-146e) is hypotension. An alternative strategy is to enhance or sustain local increases in adenosine level in brain, where they occur after TBI.51 Administration of the adenosine deaminase inhibitor pentostatin, which is 10 times more potent than EHNA53 is effective in many ischemia models, and at low doses (0.2–2.0 mg/kg).54,55 It has not been tested in experimental TBI but is FDA approved and used in cancer therapy. It could have multiple benefits including reversal of vasospasm, anti-inflammatory actions, and anti-excitotoxic effects. Each of these mechanisms is felt to be important in blast and penetrating TBI.

**Progesterone**

A large body of research from several laboratories supports the putative beneficial effects of progesterone in experimental TBI is reviewed in ref. 56. Favorable effects across several mechanisms have been shown including excitotoxicity, inflammation, and brain swelling, among others.57,58 Two single-center clinical trials have suggested beneficial effects on ICP and outcome, and a large multicenter randomized controlled trial is currently underway.59,60 Progesterone thus represents a logical agent for evaluation by our consortium which could provide additional insight into issues such as efficacy across injury severity and complex secondary insults.

**Docosahexanoic Acid**

Fish oil, or one of its constituents, docosahexanoic acid (DHA), has recently been shown to confer beneficial effects in experimental TBI.61–63 Notably, attenuation of axonal injury by DHA has been suggested to represent the major target of its protective effects. Given the important role of axonal injury recently shown in blast TBI14,15 and the ability to provide this agent as a nutritional supplement, DHA given either as pre- or posttreatment represents a logical candidate to consider for testing in OBTT.

**POTENTIAL HIGH RISK-HIGH REWARD THERAPIES FOR TBI**

**XJB-5–125**

XJB-5–125 is a nitroxide, with multifaceted effects against oxidative stress, that is conjugated with a gramicidin S fragment.64 The gramicidin S fragment exhibits high affinity for the inner mitochondrial membrane, greatly increasing its ability to concentrate in mitochondria, enhancing specificity. XJB-5–125 protects cells against apoptosis.65 The mechanism(s) of the nitroxide component’s protective effects may be associated with its superoxide dismutase mimicking activity, radical scavenging effects, or its electron acceptor propensities preventing superoxide generation during dysregulated electron transport. These properties suggest a special opportunity for XJB-5–125 in TBI.66 XJB-5–125 also exhibits beneficial systemic effects in HS and may be valuable in blast polytrauma. A library of hemigramicidin tempol conjugates has been developed.64,65

**Anti-ASCab**

Recent work67 suggests a pivotal role of a molecular platform NALPIII inflammasome consisting of caspase-1, caspase-11, and apoptosis-associated speck-like protein containing a caspase-activating recruitment domain (ASC) that is assembled in neurons subjected to experimental spinal cord injury. Treatment with an antibody against ASC (Anti-ASC), either intraperitoneal (IP) or intravenous (IV), produced pluri-potent anti-inflammatory effects (against IL-1β, IL-18, and caspase-1) with tissue sparing and functional improvement. Effects on multiple pathways make this an attractive highly novel strategy.

**Necrostatin-1**

Recent work has identified a novel cell death pathway called “necroptosis” that involves the Fas/TNF receptor pathway but exhibits both a necrotic phenotype and autophagy.68 A subsequent study revealed that this pathway is involved after TBI in mice subjected to CCI. Improvements in functional outcome, cell permeability, and inflammation were seen with treatment with necrostatin-1, a specific inhibitor of necroptosis. Necrostatin-1 is commercially available (Biomol) and a family of necrostatins has been developed. This new cell death pathway is a worthy potential therapeutic target for exploration by our consortium.

**Poloxamer-188**

Recent studies in CNS injury models have shown marked benefit of surfactant poloxamer-188.69–70 It has multifaceted effects against apoptosis, necrosis, and cell membrane injury. It attenuates P38-MAP kinase-mediated apoptosis, blunts neuro-inflammation, attenuates axonal injury, and exhibits a unique membrane resealing effect.71 It attenuated lesion volume after intracerebral hemorrhage in rats.69 It is FDA approved as an indirect food additive in a variety of products, has been in clinical trials with IV use in conditions such as sickle cell disease72 and is commercially available. A limitation of this intriguing agent is that it has shown efficacy in brain injury only with intracisternal administration; thus, we have characterized this agent speculative despite FDA approval for other uses.

**Anti-CD11dab**

Recent studies in FPI have revealed a 50% reduction in lesion volume in rats treated with an antibody to the alpha chain CD11d of the integrin heterodimer CD11d/CD18.73 In addition, a marked reduction in CD68 immuno-positive inflammatory cell influx into brain was seen with treatment. It
was effective despite a 30 minutes delay in treatment. It was also shown to be highly effective in experimental spinal cord injury.\textsuperscript{74} Unlike more broad-spectrum therapies targeting the B2 integrin family, this approach may offer greater selectivity against infiltrating inflammatory cells, blood-brain barrier injury, and tissue destruction.

**GENERAL APPROACH TO BIOMARKER SCREENING IN OBTT**

Banyan Biomarkers, Inc., has established state-of-the art capability and expertise in tooling and configuring Good Laboratory Practice-level sandwich ELISA assays for a variety of serum biomarkers including glial fibrillary acid protein, an astrocyte marker, ubiquitin C-terminal hydrolase-L1, a neuronal marker, and α-II spectrin degradation products, among others.\textsuperscript{6,7,76–78} Proposed brain injury biomarker assays to be run are outlined in Table 1. Assessment of serial samples across models in OBTT will be used both to compare the biomarker profile produced in each of the models and to probe the ability of these biomarkers to be used to assess therapeutic efficacy. OBTT thus represents a unique opportunity to examine biomarkers across simple and complex models, injury levels, and species in experimental TBI.

**DISCUSSION**

Despite the unique potential of the consortium, there are a number of potential challenges and limitations to the approach that is proposed in OBTT. First, in the 1990s, the National Institutes of Health/National Institute of Neurologic Disorders and Stroke sponsored a multicenter preclinical drug screening consortium in the field of spinal cord injury called Multicenter Animal Spinal Cord Injury Study (MASCIS).\textsuperscript{79} The approach made important contributions to modeling and outcomes assessments in experimental spinal cord injury, but did not bring new therapies to clinical trials. We selected our overall consortium design to benefit from the experience of the MASCIS consortium. Specifically, a stumbling block in MASCIS resulted from the plan to have all centers learn and use the same experimental model to test therapies. It became difficult for centers to replicate the benefits of methylprednisolone across the consortium sites. This led to a prolonged period of model development and validation. As previously discussed, we have designed our consortium to use the established models at each site to reflect the heterogeneity in clinical TBI, take advantage of the established track records for each of the models at each site, and limit the many well-recognized challenges in model development and modification.

Second, an important facet of TBI in combat casualty care is repeated injury, particularly repeated mild TBI.\textsuperscript{13} Given that few established models of repeated TBI exist,\textsuperscript{80} addressing this important issue would require considerable model development which we believe is currently outside the scope of our consortium.

Third, it is often suggested that given the multifaceted nature of TBI, combination therapy will be required.\textsuperscript{81} Although results of some studies have challenged this notion,\textsuperscript{82} although the use of combined therapy with the most promising agents identified in years 4 or 5 could be included in our ultimate consortium plan, our primary goal is to advance individual agents to clinical trials.

Fourth, we recognize that all therapies may not produce a simple linear pathway from primary to secondary screening. An agent may be effective only in mild TBI, or only in the advanced TBI plus HS and polytrauma models. Such an agent would not be dismissed as ineffective; rather, it could suggest the need for clinical testing either in mild TBI or in polytrauma. Similarly, an agent showing benefit only in the FPI models might suggest a predominant effect on axonal injury since that mechanism is highlighted in FPI. Our approach could, thus, produce a paradigm shift in the field of TBI and suggest the need for therapies targeting specific types of injury, rather than across all injuries.

Finally, we recognize that PTSD is an important therapeutic target in combat casualty care that may in some cases be linked to TBI.\textsuperscript{83} However, given the expertise of the individual members of the consortium, we are focusing on traditional outcomes that have been developed in experimental TBI. We will, of course, communicate our findings with the most promising agents to laboratories studying PTSD in civilian and blast TBI models.

**CONCLUSION**

We have launched a multicenter preclinical drug and biomarker screening consortium, OBTT, for the field of TBI. This approach is unique and has a specific focus on drug development for TBI in combat casualty care. We believe that as it develops, the findings of this consortium have the potential to provide special insight for field of experimental and clinical TBI.

**ACKNOWLEDGMENTS**

The authors thank Kenneth Curley, MD, and Col. Dallas Hack for helpful discussions.

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