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Introduction

Synergy in Science and Resources

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The Department of Defense (DoD) Combat Casualty Care Research Program (CCCRP) is a requirements-driven platform that applies investment to a spectrum of topics in military-relevant trauma and injury.[1,2] Unlike many medical research programs or institutes, the CCCRP plans and programs research topics that are aligned to established gaps in care with an emphasis toward delivery of knowledge and materiel (devices and therapeutics), solutions, and accelerated translation. As a major focus area of the program, traumatic brain injury receives considerable attention and investment directed across the spectrum of medical research—discovery, basic, pre-clinical translational, and human subjects. Each of these domains is steered with the intent to improve the diagnosis and treatment of mild, moderate, and severe brain injury. Operation Brain Trauma Therapy (OBTT) and the articles in this special issue of the *Journal of Neurotrauma* represent the output from one line of effort stemming from the military's trauma research program. With OBTT, the effort was unique and aimed at integrating the expertise of civilian scientists in order to improve the understanding of medications and circulating biomarkers in the early and acute phases of moderate and severe brain injury.

To appreciate the achievements of OBTT and the capability it provides the military and civilian trauma communities, it is important to understand the founding strategy of the consortium and context surrounding the findings reported in this publication. Foremost, OBTT is one of several efforts spearheaded by the DoD trauma research program in the pre-clinical translational focus area of brain injury. OBTT was not designed to be the only means by which to achieve knowledge pertaining to therapeutic strategies in pre-clinical models. Exuberance of superb investigators notwithstanding, OBTT was not necessarily intended to provide encompassing and immediately transformative results. Instead, the military's strategy with OBTT rests in its unique opportunity to coordinate expertise from three nationally recognized laboratories, including the Army's Walter Reed Army Institute of Research. In establishing the consortium to endeavor with three validated models of traumatic brain injury (TBI)—parasagittal fluid percussion injury (FPI), controlled cortical impact (CCI), and penetrating ballistic-like brain injury (PBBI)—the CCCRP attempted to achieve unity of effort and efficiency of resources. Stated another way, linking the military's own laboratory with the University of Miami and the Miami Project to Cure Paralysis and the Safar Center for Resuscitation Research at the University of Pittsburgh School of Medicine provided an opportunity for synergy in science.

Additional context for this publication can be found in the main objectives of the consortium, which were focused and pragmatic. In a resource limited environment, OBBT chose to make the most of established, ''up and running,'' rodent models to: (1) select potential therapies among existing pharmacologics; (2) implement an evidence-based, clinically relevant, and concise pharmacological approach; (3) assess the medications in three distinct models of moderate and severe TBI, and (4) evaluate for effects in either one or more of the models across the consortium. Although the medications evaluated in the OBTT network – nicotinamide, simvastatin, erythropoietin (EPO), cyclosporine-A (CsA), and levetiracetam – did not ''perform'' to anticipated standards, the objectives of the consortium were summarily met and important information was gained; both as it pertains to the drugs and emerging biomarkers and to the integration of the scientific effort.

The network and scientific results reported in this publication constitute a pre-clinical, research capability achieved through a unique military–civilian partnership. Now established, this capability has the potential to evaluate different dosing strategies of these same or other pharmacologics or to characterize other brain resuscitation and preservation strategies. This type of capability can also be extended to include different pre-clinical models including ones of mild brain injury or those incorporating polytrauma and hemorrhagic shock (rodent or porcine). Importantly, and as a common iterative step, the capability achieved in OBTT stands to inform and hone subsequent research performed in more translatable models including those in the nonhuman primate.

With this context, the investigative teams of the OBTT network are to be commended for their dedication and expert accomplishment. The articles in this issue exemplify a tremendous amount of intricate work aimed at advancing the diagnosis and management of TBI. The effort as a whole is an apt tribute to civilians and military members who have sustained this type of injury and the overall effort to improve survival and outcomes. However, the work is not complete and the reader of this journal is encouraged to ''dig into'' the issue and consider with us its strengths, weaknesses, meaning, and implication for future study. The organizers

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of this initiative also provide the OBTT strategy and effort as a case study of planned and integrated pre-clinical research and thank the *Journal of Neurotrauma* for featuring this issue. By continuing to maximize military–civilian partnerships in the area of trauma and injury research, the CCCRP hopes to be efficient with resources and effective with science to narrow high priority gaps in patient care.

Acknowledgment

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army, Department of the Air Force, or the Department of Defense.

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Original Articles

Approach to Modeling, Therapy Evaluation, Drug Selection, and Biomarker Assessments for a Multicenter Pre-Clinical Drug Screening Consortium for Acute Therapies in Severe Traumatic Brain Injury: Operation Brain Trauma Therapy

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Abstract

Traumatic brain injury (TBI) was the signature injury in both the Iraq and Afghan wars and the magnitude of its importance in the civilian setting is finally being recognized. Given the scope of the problem, new therapies are needed across the continuum of care. Few therapies have been shown to be successful. In severe TBI, current guidelines-based acute therapies are focused on the reduction of intracranial hypertension and optimization of cerebral perfusion. One factor considered important to the failure of drug development and translation in TBI relates to the recognition that TBI is extremely heterogeneous and presents with multiple phenotypes even within the category of severe injury. To address this possibility and attempt to bring the most promising therapies to clinical trials, we developed Operation Brain Trauma Therapy (OBTT), a multicenter, pre-clinical drug screening consortium for acute therapies in severe TBI. OBTT was developed to include a spectrum of established TBI models at experienced centers and assess the effect of promising therapies on both conventional outcomes and serum biomarker levels. In this review, we outline the approach to TBI modeling, evaluation of therapies, drug selection, and biomarker assessments for OBTT, and provide a framework for reports in this issue on the first five therapies evaluated by the consortium.

Key words: biomarker; controlled cortical impact; fluid percussion; micropig; neuroprotection; penetrating ballistic-like brain injury; rat; therapy

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Introduction

The importance of traumatic brain injury (TBI) is now being recognized in both civilian and military settings over the range of injury severity. Given the magnitude of the problem, new therapies are needed across the continuum of care—from the field to rehabilitation. It is well known that secondary injury is the therapeutic target in TBI; however, the injury mechanisms that have been identified are multifactorial, time dependent, and highly complex. Few therapies have been shown to be successful.

In the setting of severe TBI, guidelines-based acute therapies currently in use are focused on the reduction of intracranial hypertension to limit brain swelling and optimize cerebral perfusion with agents such as hypertonic saline, mannitol, or barbiturates,[1] while chronic therapies are used, such as neurotransmitter replacement in rehabilitation with agents such as amantadine.[2] In cases of moderate or mild TBI, even less evidence is available, and therapy is largely empiric.[3] Therapies can be applied early or late after injury, but it has long been suggested that the most potentially efficacious approach would be to limit secondary damage early in its evolution after TBI.[4]

Historically, for TBI therapy development, a number of drugs and approaches have been shown to be efficacious in pre-clinical models (reviewed in [5–7]); however, for acute therapies, no agent has successfully translated from bench to bedside. Most pre-clinical work has focused on severe TBI. Several highly promising acute therapies such as mild-moderate hypothermia,[8] magnesium,[9] tirilazad,[10] polyethylene glycol-conjugated superoxide dismutase,[11] nimodipine,[12] and progesterone,[13] among others, exemplify this situation. Recently, a few other agents shown to have efficacy in experimental TBI have also shown promise with acute administration in early clinical trials in TBI such as N-acetyl cysteine.[14] Definitive studies remain to be carried out or completed, however.

The failure of translation of acute therapies to clinical success in TBI has been the subject of considerable discussion.[15] Some have suggested that it might be wise to defer randomized controlled clinical trials (RCTs) in TBI until comparative effectiveness trials have been performed to understand/optimize current clinical management before testing new therapies.[16] Another suggestion to explain this failure is that the available TBI models do not replicate the clinical condition; however, the recent successful trial of amantadine in TBI represents translation of a therapy from the controlled cortical impact (CCI) model[17] to a successful clinical trial,[2] supporting both the concept that RCTs can be successful and that our current models have potential utility for translation. That work in CCI was recently confirmed in the fluid percussion injury (FPI) model in rats.[18]

Another explanation put forth to explain the failure of translation of therapies to successful clinical trials includes the concept of the need for alternative strategies to the National Institutes of Health (NIH)-driven single molecular mechanism approach to therapy development—i.e., test therapies targeting multiple mechanisms (dirty drugs) or combination therapies. One concept that has emerged with considerable support, however, is that TBI represents more than a single disease and thus to show translation, therapies either need to be effective across multiple models or be translated in the context of a specific clinical phenotype, such as translating from CCI to contusion or penetrating ballistic-like brain injury (PBBI) to gunshot wound.[19]

In light of these concepts and supported by the United States Army, we assembled a pre-clinical therapy screening consortium in severe TBI called Operation Brain Trauma Therapy (OBTT) with the specific goal of identifying promising acute therapies that show success across multiple pre-clinical TBI models. The overall approach taken in OBTT was to assemble a consortium of established pre-clinical TBI investigators using a menu of rodent models, select promising therapies, test them across models using a screening approach, and move promising therapies up the phylogenic scale to testing in a newly developed large animal model—namely, fluid percussion injury (FPI) in micropigs.[20] In addition, given the special opportunity that OBTT represents, it was decided that it would be valuable to integrate the use of serum biomarkers of brain injury across the models in parallel theranostic applications, notably using biomarkers that are currently in clinical development.

An initial brief overview of OBTT was presented shortly after the consortium was launched.[20] In this special issue of the *Journal of Neurotrauma*, we present eight articles including (1) this manuscript providing a more detailed description of the OBTT consortium including the underpinnings of its design, composition, models, outcomes, overall approach to therapy testing, therapy scoring, biomarker applications, and rationale for drug selection and administration, (2–6) five individual reports focused on the results of screening of the first five therapies tested in OBTT across the consortium, including nicotinamide (Shear and colleagues),[21] erythropoietin (EPO, Bramlett and colleagues),[22] cyclosporine A (CsA, Dixon and colleagues),[23] simvastatin (Mountney and colleagues),[24] and levetiracetam (Browning and colleagues),[25] (7) an article demonstrating the utility of serum biomarkers as applied in OBTT both to compare the screening models and provide insight into reproducibility of the models and relationships between circulating biomarker levels and both behavioral and histological outcomes (Mondello and colleagues),[26] and finally, (8) an article summarizing the findings and discussing future directions for the consortium (Kochanek and colleagues).[27]

Lessons Learned from the NIH-Sponsored Multicenter Animal Spinal Cord Injury Study (MASCIS)

In the 1990s, a seminal program that comprised a multicenter pre-clinical drug screening consortium in spinal cord injury (SCI) was formed and supported by the NIH.[28,29] That consortium took the logical approach of using a single standardized rat model and battery of outcomes across a number of sites to screen therapies in SCI. Each center involved was thus trained at a central site to use a single SCI model (weight drop). Subtle differences in the execution of various aspects of the model across centers were seen, however, and although that work contributed importantly to model and outcome tool development in the field of SCI, a menu of therapies was not ultimately compared by the consortium. New therapies were thus not brought to clinical trials.

We used that information to help guide the approach taken by our OBTT TBI consortium for pre-clinical therapy testing and development. First, we similarly selected highly experienced centers and research teams; however, we specifically chose to use the models that were already established at the various sites without changing any of the key elements of the models. Thus, injury severity, anesthesia, and other aspects of the models were not altered from the established practice at each site, and no training was involved. This approach was taken in to avoid the unavoidable pitfalls associated with concurrent model development and therapy testing, potentially allowing us to determine if a given therapy performs with varying efficacy across models. Such an approach might also identify a highly potent therapy—if one were to show significant benefit across substantially differing models.

We also chose to use the established outcomes at each site, ensuring, however, some consistent threads across models, such as the use of both motor and Morris water maze (MWM) tasks as behavioral outcome targets, and assessment of lesion volume and tissue loss in the injured hemisphere (CCI and PBBI) or cortex (FPI) as histological screening targets. We recognized that such an approach to histological assessment was restrictive. We thought, however, that lesion volume and hemispheric or cortical tissue loss represented reasonable first approaches to screening therapies.

More sophisticated approaches such as assessments of neuronal death and/or axonal injury could follow in additional studies and/or other models for the most promising therapies, or in the case where a very specific outcome target was deemed to be essential. Details of each of these outcomes were allowed to differ at the sites, keeping in step with the methods already used at each center and recognizing the different levels of injury that each model produced could importantly influence the specifics of the assessments that might be required to detect therapeutic effects.

In contrast to our relatively ''flexible'' approach taken with the models and outcomes, all aspects related to the therapies (such as dosing, timing, route of administration, timing of blood sampling, and timing of sacrifice [21 days]) were rigorously held consistent across sites. This approach has allowed for direct comparisons of the treatments across models for behavioral, histological, and biomarker outcomes—facilitating cross-model comparisons of both the models themselves and also of therapeutic efficacy.[26,27]

Components of the OBTT Consortium

TBI centers and models in primary screening

In addition to assembling a team of highly experienced centers and investigators to perform the screening, the centers within OBTT were also selected specifically to produce a diverse menu of models in rats for ''primary screening'' of therapies. Figure 1 shows the three primary screening models in rats that are being used in OBTT. The models, which include parasagittal FPI, CCI injury, and PBBI in rats represent established models with the strongest possible track record for pre-clinical investigation for acute therapies in severe TBI—the specific focus of OBTT.[20,30–39] They are models in which behavioral and histopathological outcomes have been routinely used in publications on drug testing. As will be illustrated in the articles that follow in this issue of the *Journal of Neurotrauma*, although OBTT is focused largely on severe TBI, the models within OBTT cover a range of injury levels within the severe and moderate-severe spectrum, which was the goal of OBTT.

The parasaggital FPI model represents the least severe injury within OBTT, while the PBBI model represents the most severe model, based on assessment of both behavioral deficits and histological end-points, such as MWM deficit and hemispheric tissue loss. This will become quite clear across the articles in this issue that describe the testing and cross-model comparisons in OBTT. Parasaggital FPI has a significant diffuse injury component, with a relatively small focal injury at the gray/white junction.[30,31] Studies in that model are being performed by Drs. Helen Bramlett and W. Dalton Dietrich at the University of Miami, Miami Project to Cure Paralysis.

The CCI model produces a substantial contusional injury, but also has been shown to have fiber tract injury across the corpus callosum and injury to more remote brain regions such as the hippocampus and striatum ipsilateral to impact.[40,41] CCI is intermediate in injury level within the primary screening models used in OBTT as assessed by these outcomes. Studies in the CCI model are being performed by Dr. C. Edward Dixon, who is one of the inventors of the model, and published on its first use in rats.[32–34] Studies in the CCI model within OBTT are being performed at the Safar Center for Resuscitation Research, University of Pittsburgh School of Medicine.

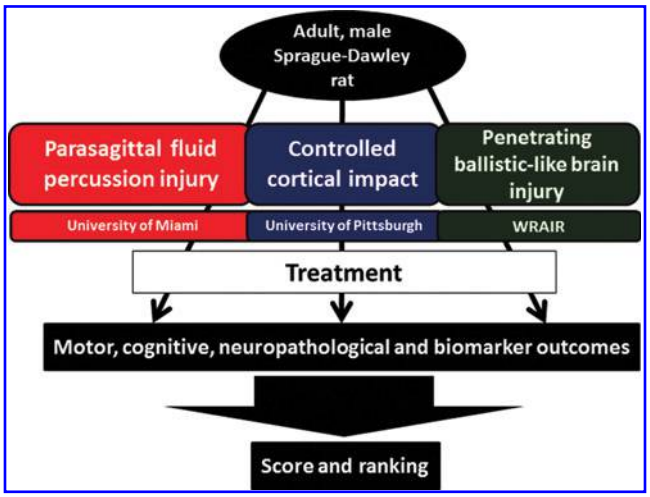

FIG. 1. Models used for primary screening or therapies in Operation Brain Trauma Therapy. For initial screening of therapies, adult male Sprague-Dawley rats are used across the models, which include parasagittal fluid percussion injury, controlled cortical impact, and penetrating ballistic-like brain injury. All treatments are administered after injury using clinically relevant post-injury approaches tailored to each given therapy, and the dosing paradigms, route of administration, and timing and duration of treatment are identical across centers and models. Motor and cognitive testing, neuropathology, and biomarker outcomes are assessed at each site. The details of the tools used to assess these outcomes at each center, however, are site specific. Nevertheless, there is considerable overlap for the outcome tools between centers as described in Tables 1 and 2. A total score is calculated for each therapy at each site using a 22-point matrix (Table 2), and an overall score is generated by summing the three total scores. Please see text for additional details. WRAIR, Walter Reed Army Institute of Research.

The PBBI model produces a cavitary lesion mimicking ballistic injury and represents a model that has considerable relevance in combat casualty care, particularly given the recent resurgence in interest in the treatment of penetrating TBI.[35–39] Studies in the PBBI model are being carried out by Drs. Deborah Shear, Frank Tortella, and Major Kara Schmid, at the Walter Reed Army Institute of Research.

Numerous aspects of intracranial dynamics, cerebrovascular physiology, and extracerebral physiology have been documented in each of these models and in the FPI model, for each drug study in OBTT, an arterial catheter is placed and relevant physiological monitoring is performed including assessment of mean arterial blood pressure (MAP), brain and body temperature, and blood gases. This is done to ensure that therapies do not produce unwanted or confounding systemic side effects in the early post-TBI period.

One of the unique aspects of OBTT is the ability of the consortium to perform direct cross-model comparisons including study of both conventional outcomes and serum biomarker levels. Key

aspects of the valuable insight generated by those studies are described in this issue as outlined in the article by Mondello and coworkers,[26] which focuses on cross-model comparisons and provides insight into reproducibility of the models and relationships between circulating biomarker levels and both behavioral and histological outcomes.

Secondary screening of therapies: advanced models

Therapies that demonstrate promising effects may also receive additional screening in more advanced models, as deemed appropriate for the specific therapeutic mechanisms that are being targeted. In both FPI and CCI, secondary insults can be superimposed to generate models that mimic the commonly encountered scenarios seen in combat casualty care, where polytrauma, hypoxemia, hypotension, hemorrhage, and/or inflammation often accompany TBI.[42,43] In FPI this entails addition of an interleukin-1β infusion,[44] while in CCI, the second insult incorporates severe hemorrhage.[45–47]

Both of these secondary insult models are established and, in some cases, they have been used to test therapies.[48–51] Highly promising therapies will also be subjected to more extensive testing focused on electrophysiological end-points using an advanced version of the PBBI model, once again as deemed appropriate based on the pathomechanism that is being targeted by a given therapy.

Secondary screening of therapies: studies in a large animal model of TBI

Finally, additional screening of promising therapies will also be performed at the Medical College of Virginia by Dr. John Povlishock, using a recently established micropig model of FPI and that screening will focus on axonal injury and also consider cerebrovascular end-points, and the glial response. That model will thus use outcomes that differ from the primary screening models in rats, which focus on behavior and volumetric analyses. The large animal micropig model also incorporates into OBTT an animal with a gyrencephalic brain, which may be important for optimal clinical translation.

Taken together, these models replicate all of the relevant aspects of severe TBI and thus are well served for therapeutic screening in OBTT to bring the best possible therapies to clinical trials. Scoring of therapies is discussed later in this article.

Administrative Components of OBTT and Rules of Operation

On establishment of the consortium, and based on the plans outlined in the funded grant application, a series of conference calls were orchestrated to move the consortium forward. The principal investigator (PI, PMK) launched efforts to create a manual of standard operating procedures (MSOP) and to finalize the approach to therapy selection. These two efforts are discussed below.

A MSOP was created to guide the day-to-day operations of OBTT. It is a working and evolving document that includes details of the models with regard to the specific outcome metrics used in each case and the approach to scoring of outcomes in primary screening of drugs to compare therapeutic efficacy across models/sites. The outcome metrics in each model in primary screening from the MSOP are shown in Table 1.

The MSOP also includes a description of the overall approach to treatment for OBTT, a *PubMed* literature review for each therapy that is tested including a table of key references for each therapy, and a detailed treatment plan on drug acquisition, preparation, dosing, and administration. In each case, this information is prepared by the PI (PMK). In addition, the MSOP also outlines the approach to blood sampling and processing for biomarker assessments across the models. The MSOP also contains preliminary prepublication outcome tables with findings of the consortium as they become available initially in draft form and the overall score for each therapy as it evolves (ultimately to final form), as seen in each of the articles that follow. The MSOP is updated regularly.

In addition to the MSOP, a second important administrative aspect of OBTT relates to the execution of a monthly conference call that features one or more representatives from each participating site. At these calls, the status of the studies of each therapy under evaluation is presented and discussed, and the results of outcomes that have recently been completed are also discussed. Joint planning for future therapies is similarly carried out. Problems are also discussed.

Approach to Therapeutic Testing

Quantifying therapeutic efficacy in primary screening

The investigators within OBTT jointly developed an approach to scoring of therapies using a 22-point system for each model, with heaviest weight on cognitive outcome (Table 1). This approach ensured an equal number of total points for each model, taking into

Table 1. Primary Screening: Outcome Metrics at Each Site

Site	*Biomarkers*	*Neuro exam*	*Motor function*	*Cognitive function*	*Neuropathology*
Miami	Rat: Blood samples (0.7 mL) via IV (jugular): 4h, 24h, at sacrifice	Rat: None	Rat: Cylinder task, grid-walk task, 7d	Rat: MWM task: 13–21d (hidden platform d13-16, probe d17, working memory d20–21	Rat: Euthanize d21; serial sections for volumetric analyses
Pittsburgh	Rat: Blood samples (0.7 mL; tail artery): 4 h, 24h, at sacrifice	Rat: None	Rat: Beam balance and beam walking d1–5	Rat: MWM task: 14-20d hidden (14–18d) and visible platform(19–20d) and probe trial (20d)	Rat: Euthanize d21; serial sections for volumetric analyses
WRAIR	Rat: Blood samples (0.7 mL) via IV (jugular): 4h, 24h, at sacrifice	Rat: Neuroscore: 30m, 24h, 72h, 7d, 21d	Rat: Rotarod: 7d and 10d	Rat: MWM task: 13-17d (4x/dx 5d; 30m ITI; end w/probe trial d19)	Rat: Euthanize 21d serial sections for volumetric analyses

IV, intravenous; MWM, Morris water maze; ITI, intertrial interval, WRAIR, Walter Reed Army Institute of Research.

account the differences between both models and centers in the various established outcomes that each used. Given the importance of cognitive outcome in successful recovery after clinical TBI and the fact that MWM performance was used at each site, the various MWM performance parameters were given the highest weight in evaluating therapeutic efficacy in screening across the centers. All outcomes that were assessed, however, contributed to the overall score at each site.

Specifically, as shown in Table 1, motor testing in the early post-injury phase, lesion volume at 21 days after injury, hemispheric or cortical tissue loss at 21 days after injury, and biomarker values (the 24 h value and the delta between 4 h and 24 h after injury for each biomarker) were also scored in a weighted fashion using a scoring matrix developed by our consortium investigators (Table 2). A final overall score is then calculated for each therapy to be used for prioritizing therapies to be advanced to additional screening in rodents and/or testing in our large animal model.

Minimizing bias across sites during therapeutic screening

Given that the rate of progress varied at each site for each therapy, to limit any potential bias related to emerging or completed findings at one or more of the screening sites on other sites, experimental findings for each category of outcomes (behavior, histopathology, and biomarkers) are simultaneously revealed to the group by e-mail by the overall PI (PMK). For example, for a given therapy, results of all of the behavioral outcomes are not provided to the overall PI until all of the behavioral evaluations are completed at all of the sites. The overall PI monitors progress at each site on studies regularly by e-mail. Once all of the behavioral testing and data evaluation are completed, the findings are first e-mailed by each site PI to the overall PI (PMK).

The results are then assembled and then e-mailed simultaneously to each of the site PIs. A draft preliminary overall score is then generated by the overall PI for that outcome for the given therapy, and those results are incorporated into the MSOP. This approach precludes negative or positive findings from influencing in any way the results for a given category of outcomes at the other sites. Concerns with regard to any given therapy or specifics of protocol design are discussed on a monthly conference call, however, to optimize that final protocol used across the sites and identify problems as soon as possible. In some

Table 2. Scoring Matrix for Assessment of Therapeutic Efficacy Across Models in Operation Brain Trauma Therapy

Site	*Neuro exam*	*Motor*	*Cognitive*	*Neuropathology*	*Serum biomarker*
Drug:					
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform path length (2) MWM probe (2) Working memory latency (2) Working memory path length (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)
Miami total	N/A	4	10	4	4
Miami Dose 1 Dose 2					
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)
Pittsburgh total	N/A	4	10	4	4
Pittsburgh Dose 1 Dose 2					
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)
WRAIR total	1	3	10	4	4
WRAIR Dose 1 Dose 2					
Grand total					
Dose 1					
Dose 2					

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin carboxy-terminal hydrolase L1; Δ, delta; N/A = not applicable; WRAIR = Walter Reed Army Institute of Research.

( ), point value for each outcome within each model.

cases, for the therapies that have been studied, pilot experiments were conducted at a site with the proposed dosing regimen to ensure that the approach did not produce unwanted side effects. This approach has been successful.

Approach to Therapy Selection and Testing

Therapy selection

A vast number of therapies could be tested by OBTT, and thus a practical approach to therapy selection was needed. Based on the funded grant application and recognizing the desire to try to move new therapies promptly to clinical trials, priority was given (1) to therapies that had promising published pre-clinical data specifically in TBI, preferably from multiple independent sites, and (2) to therapies that were already approved by the Food and Drug Administration or in use for other indications.

Such therapies were considered ''low hanging fruit'' and given the highest priority. A listing and brief discussion of these therapies was presented previously.[20] As outlined in the manuscripts that follow, this category of drug was chosen for the first five therapies selected for primary screening by OBTT. In addition, based on the funded grant application, a second category of therapies deemed ''higher risk but potentially high reward'' would also be considered for screening within OBTT, but with a somewhat lower priority.

A literature review of potential therapies was performed by the overall PI that included multiple *PubMed* searches along with input from (1) all of the members of each research team at each site, (2) the scientific advisory board, and (3) programs at the Congressionally Directed Medical Research Programs (CDMRP). Thus, after performing the relevant general searches related to the topics of TBI, head injury, treatment, and therapy to identify promising therapies, specific searches were performed on agents identified and also those recommended for consideration into the list of therapies to be considered by the individuals mentioned above.

The focus of those reviews was specifically on pre-clinical research in TBI, although some studies in other models deemed to be of high relevance were also included. Notably, pre-clinical studies in other models that performed extensive pharmacokinetic evaluations in rodents of a therapy that was being advanced or seriously considered for testing by OBTT were also reviewed.

For the most promising agents, the overall PI assembled evidence tables containing the relevant articles. Therapies identified that had the largest number of supporting publications, those showing the largest beneficial effects on the aforementioned outcomes relevant to primary screening, and/or therapies already in clinical use but that remain controversial in TBI were assembled and presented to the site PIs and co-investigators in a document e-mailed by the overall PI to each investigator before the annual OBTT investigators meeting that is held at the National Neurotrauma Society Symposium. A secret ballot vote was taken before the Symposium. The results of the vote were then presented by the overall PI to the site PIs at the OBTT investigators meeting at the Symposium, and after additional discussion, three therapies each year are selected and prioritized.

The review of therapies also identified drugs or treatments currently in clinical trials and/or having failed in previous or recent clinical trials. The initial approach outlined in the grant application indicated that therapies currently in the midst of large multicenter RCTs on TBI would not be given high priority for testing in OBTT—given its goal of identifying new potential therapies to bring to clinical trials. Ongoing study of a given therapy in a single center clinical trial was not deemed to reduce priority because a positive assessment in OBTT might represent additional evidence toward a decision in support of a large multicenter RCT for that therapy. Therapies that had failed previous RCTs (single center or multicenter), however, were appropriately reduced in priority, although not necessarily dismissed. Once a therapy was selected by the consortium, the evidence table for that agent was incorporated into the MSOP, and a detailed protocol for drug administration was crafted as discussed below.

Treatment protocols for each therapy

For each therapy selected for primary screening by OBTT, the principal factor guiding the approach to treatment across the consortium has been the published literature on that therapy in pre-clinical TBI models. Given that the goal of OBTT is to advance as promptly as possible the most promising therapies, our approach has been to take maximal advantage of the published literature on each therapy to shape our study design—with modifications of previously successful approaches largely limited to attempt to maximize clinical relevance. In studies where published evaluations of dose response were performed, that information was carefully reviewed and also used by the consortium to select the dose, dosing interval, treatment duration, and route of administration. When published pre-clinical studies on a given therapy were performed at multiple sites, in general the findings viewed as the strongest on beneficial effects on multiple outcomes were used to select the doses used.

For most therapies selected, we chose to test two doses given at a treatment interval relevant to the therapy, replicating previous successful studies, whenever possible. In addition to two doses, we also included a sham group (preparatory surgery and anesthesia but no injury or treatment) and a vehicle group (injury plus vehicle treatment—with the vehicle administered in a fashion identical to treatment). We specifically chose not to include treated sham groups in this phase of testing given the fact that the goal of primary screening in OBTT was to identify promising therapies. Agents that are positive in primary screening will be subjected to additional testing that would more fully address issues related to off-target effects and dose response, among others. Drug administration is blinded at each site, animals are randomized to treatment group, and outcome evaluation (including both behavioral and histological) is also blinded.

For timing, interval, and duration of dosing, once again whenever possible, the published pre-clinical literature showing the most promising effects on outcomes is used. It has been, however, necessary in some cases to modify treatment approaches based on logistical factors relevant to the OBTT consortium. For each drug, we also consult with two faculty members in the University of Pittsburgh School of Pharmacy (Samuel Poloyac, PharmD, PhD, and Philip Empey, PharmD, PhD) who are experts in the area of drug metabolism in pre-clinical and clinical brain injury,[52,53] and who reviewed the pre-clinical and clinical literature for each agent tested to aid in arriving at acceptable timing, interval, and duration of dosing, along with providing information on drug preparation. In each case thus far, the vehicle was either purchased or prepared in a manner mimicking the test drug including composition and volume. In addition, for each therapy tested to date, the drug was purchased in identical formulation and in most cases by the overall PI from a single vendor, and then distributed to the individual sites.

For route of administration, given the stated focus of OBTT on severe TBI, it is deemed to be important to maximize relevance to both combat casualty and clinical care, and when acute

administration is planned for primary screening, the intravenous route is selected if possible. Pilot studies were often performed to ensure that we did not encounter problems related to drug preparation such as solubility, and/or problems related to acute side effects such as hypotension at the proposed dose. In several cases, authors of successful published work on a given therapy selected for use in OBTT were contacted, and they generously provided additional detail on dosing and/or drug preparation.

Biomarkers and Biomarker Sampling

As with therapies, a wealth of potential serum biomarkers of brain injury could be selected for monitoring of injury and theranostic effects across the consortium.[54–65] Our goal in designing our approach, however, was to use biomarkers that had the greatest potential for translation to clinical use. To this end, in the grant proposal that was funded, we partnered with Banyan Biomarkers LLC, and biomarker selection and sampling were guided by three affiliated scientists (RH, SM, and KW). The biomarkers chosen were based on previous success in published clinical trials[54,55,59,64,65] among others and pre-clinical studies in rodent models.[57]

Based on that work, two prototype serum biomarkers were selected—the astrocyte marker glial fibrillary acidic protein (GFAP) and the neuronal marker ubiquitin carboxy-terminal hydrolase L1 (UCH-L1). Additional information on these two biomarkers and the rationale supporting their selection for the studies in OBTT is provided in the article that is specifically focused on biomarkers in OBTT in this issue (Mondello and colleagues).[26]

Timing of blood sampling for biomarker assessments was also based on published clinical and pre-clinical reports[54–65] and included samples at 4 h, 24 h, and 21 days (final) after injury. It was thought that this spectrum of samples would (1) allow for comparison of the initial injury across models (4 h values), (2) facilitate assessment of theranostic effects of the various therapies that were screened (based on both the 24 h biomarker value and the delta between the 4 h and 24 h values in each rat), and (3) define whether or not increases in blood biomarker levels had resolved by 21 days after injury.

For the 4 h and 24 h time points, blood was obtained either from an indwelling vascular catheter (Miami and WRAIR sites) or by tail artery puncture (Pittsburgh site), while for the final time point, 2–3 mL was obtained by cardiac puncture across the sites. Once again, the approach taken with regard to sampling was selected to minimize changes in any of the models at each site—i.e., catheter placement was already part of the standard protocol at the Miami and WRAIR sites but was not in Pittsburgh. In cases where blood sampling coincided temporally with drug administration, the blood sample was obtained first.

A detailed blood sampling and processing protocol was crafted and included in the MSOP and carefully followed at each study site. After collection, all samples were processed using an identical protocol across sites and stored at −70°C until study completion and then shipped to Banyan Biomarkers LLC for assessment in a blinded fashion.

In addition to their value in contributing to prioritizing the individual therapies in OBTT, the blood biomarker measurements also allow for comparison of the three pre-clinical models, correlations between serum biomarkers and the other conventional behavioral and histopathological outcomes, and assessments of model stability across the studies—a parameter rarely formally assessed in pre-clinical studies. The biomarker data relevant to treatment effects are presented in the article addressing each therapy,[21–25] while the biomarker assessments made in cross-model comparisons and assessments of model stability and correlations between circulating biomarker levels and both behavioral and histopathological outcomes are presented in a separate article focused on these unique biomarker applications.[26] As will become evident in the articles that follow, the biomarker data generated by the OBTT consortium are quite unique and highly informative about biomarkers in the models studied.

Therapies Selected for Primary Screening

Based on the criteria discussed previously, five therapies were selected as the initial drugs to be evaluated in primary screening by the OBTT consortium—namely, nicotinamide, EPO, CsA, simvastatin, and levetiracetam. These five therapies represent agents that would be readily translatable to clinical trials if shown to be efficacious across OBTT. They are also drugs that have either a considerable body of support in the published literature for pre-clinical studies or support for clinical use in other applications. Details on the rationale, background, and evidence for each of these therapies are presented in the article devoted to each therapy that follow in this issue of the journal. The evidence tables for each of these therapies from the OBTT MSOP are based on the data collected and reviewed at the time that each of the drugs was tested by the consortium. The evidence tables are included in each of the respective articles on therapy. The results of testing for each therapy are presented in the article that follow.

Limitations

There are numerous aspects of therapeutic testing that could not be addressed in OBTT, at least in the primary screening studies that are reported here. For example, important gender-based differences in therapeutic efficacy have been reported for a number of drugs.[66] Given that OBTT is a screening consortium and that the majority of cases of TBI, particularly those in combat casualty care, occur in males, however, we chose to use male rats for all of the primary screening studies. For therapies with substantive beneficial effects in screening, we will certainly consider additional testing in female rats.

Similarly, we chose to study severe TBI rather than mild TBI. Given that at the time of submission of our grant proposal, there was little pre-clinical work done in the area of drug testing in mild TBI, it was a logical choice. Indeed, the recent comprehensive report of the Defense Neurotrauma Pharmacology Workgroup on the state of pre-clinical therapeutic testing in mild TBI revealed that huge gaps persist.[66] We also recognize the emerging importance of repetitive injury.[60] We thought, however, that it was important given the seminal nature of OBTT, to begin by studying single insults.

We also did not propose testing combination therapy in our initial studies of drug screening, although it is possible that if two promising therapies are identified, we may try combining them in a definitive study. Such an approach has been taken by individual laboratories.[67]

Finally, it is important to recognize that the failure to demonstrate beneficial effects of a given therapy by the work of OBTT does not in any way refute published work, nor is it a goal of our consortium. Many nuances of study design are involved such as differences in strain of rat, vendor, injury level, timing of drug administration, vehicle, differences in various aspects of selected outcome tasks, differences in tissue sampling, and many other confounding factors. The overriding goal of OBTT is simply to

screen as many therapies as possible across a spectrum of models, using the published literature to provide clues to study design to identify the most beneficial therapies among those screened. Our hope is to advance one of more therapies to successful clinical trials in the heterogeneous setting of TBI.

Alternatively, we might find that no individual therapy is highly protective across models, but individual therapies show potent effects in one or two models, depending on the mechanisms that agent targets. Such a finding would support the notion that clinical TBI therapy will need to be based on the injury phenotype in a precision or personalized medicine fashion.

Conclusions

We have provided an overview of the approach to modeling, evaluation of therapies, and drug selection for the multicenter preclinical drug screening consortium for acute therapies, OBTT in TBI. This article thus sets the stage for seven articles that follow, including those addressing the findings for each of the first five therapies that have been screened by the consortium,[21–25] the biomarker-based comparisons of the models, including their severity, stability, and relationships between serum biomarker levels and conventional outcomes,[25] and finally, a article on the vision of the OBTT consortium for future drugs to be evaluated and possible modifications of our approach based on the lessons learned.[27]

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Author Disclosure Statement

Dr. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Dr. Hayes is an employee and receives salary and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and, as such, all of these persons may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

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Levetiracetam Treatment in Traumatic Brain Injury: Operation Brain Trauma Therapy

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Abstract

Levetiracetam (LEV) is an antiepileptic agent targeting novel pathways. Coupled with a favorable safety profile and increasing empirical clinical use, it was the fifth drug tested by Operation Brain Trauma Therapy (OBTT). We assessed the efficacy of a single 15 min post-injury intravenous (IV) dose (54 or 170 mg/kg) on behavioral, histopathological, and biomarker outcomes after parasagittal fluid percussion brain injury (FPI), controlled cortical impact (CCI), and penetrating ballistic-like brain injury (PBBI) in rats. In FPI, there was no benefit on motor function, but on Morris water maze (MWM), both doses improved latencies and path lengths versus vehicle ($p < 0.05$). On probe trial, the vehicle group was impaired versus sham, but both LEV treated groups did not differ versus sham, and the 54 mg/kg group was improved versus vehicle ($p < 0.05$). No histological benefit was seen. In CCI, there was a benefit on beam balance at 170 mg/kg ($p < 0.05$ vs. vehicle). On MWM, the 54 mg/kg dose was improved and not different from sham. Probe trial did not differ between groups for either dose. There was a reduction in hemispheric tissue loss ($p < 0.05$ vs. vehicle) with 170 mg/kg. In PBBI, there was no motor, cognitive, or histological benefit from either dose. Regarding biomarkers, in CCI, 24 h glial fibrillary acidic protein (GFAP) blood levels were lower in the 170 mg/kg group versus vehicle ($p < 0.05$). In PBBI, GFAP blood levels were increased in vehicle and 170 mg/kg groups versus sham ($p < 0.05$) but not in the 54 mg/kg group. No treatment effects were seen for ubiquitin C-terminal hydrolase-L1 across models. Early single IV LEV produced multiple benefits in CCI and FPI and reduced GFAP levels in PBBI. LEV achieved 10 points at each dose, is the most promising drug tested thus far by OBTT, and the only drug to improve cognitive outcome in any model. LEV has been advanced to testing in the micropig model in OBTT.

Key words: biomarker; controlled cortical impact; excitotoxicity; fluid percussion; Keppra; neuroprotection; penetrating ballistic-like brain injury; post-traumatic seizures; rat; therapy

Introduction

Increasing attention is being paid to the heterogeneous spectrum of traumatic brain injury (TBI). In the United States, ~1.5 million TBIs occur each year across the injury severity spectrum.[1] Much effort has been devoted to ameliorating the secondary injury that occurs in an attempt to reduce morbidity and mortality. Unfortunately, many treatments that show promise in the pre-clinical setting fail to translate to meaningful patient improvements.

Treating such a diverse group of injuries will likely necessitate either a highly potent therapy or a personalized medicine approach with different therapies and modalities targeted to the injury type to optimize patient recovery. Testing potential drug candidates across

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multiple models of TBI may increase the likelihood of finding robust therapies able to bridge bench with bedside. With this goal in mind, the Operation Brain Trauma Therapy (OBTT) consortium was founded to identify and rigorously test therapies for severe TBI.

Levetiracetam (LEV) was selected as the fifth OBTT therapy. Despite limited pre-clinical TBI data, it was compelling because of its ability to manage post-traumatic seizures via novel mechanisms, its low toxicity, and its increasing empirical clinical use after severe TBI. It is a second generation antiepileptic drug (AED)—structurally unique from other AEDs.[2] LEV possesses antiepileptic, antiepileptogenic, and neuroprotective properties. While it is known to bind to synaptic vesicle protein 2A (SV2A), the precise downstream mechanism(s) of action have not been fully elucidated. SV2A may impact SNARE complex formation and alter synaptic vesicle fusion.[3,4] LEV decreases glutamate mediated excitatory transmission via interactions with SV2A, modulation of neurotransmitter release (effects on γ-aminobutyric acid [GABA] turnover, and Zn^{2+} induced suppression of pre-synaptic inhibition), and effects on calcium signaling.[5] It also up-regulates expression of glial glutamate transporters.[6]

There were limited pre-clinical studies of LEV in TBI—most pre-clinical work focused on rat models of epilepsy. Klitgaard and associates[7] tested a range of doses (17–1700 mg/kg intraperitoneal [IP]) in a variety of rat models of epilepsy and found that the dosage efficacy depended on the seizure induction agent.[7] Doses of 17 mg/kg IP abolished pilocarpine-induced seizures, 54 mg/kg abolished kainite induced seizures, and 170 mg/kg abolished benzodiazepine antagonist-induced seizures. Toxicity appeared only with an extremely high dose (1700 mg/kg) when rats displayed impaired rotarod performance. They suggested potent antiepileptogenic activity in kindling models with inhibition of disease progression. Loscher and colleagues[8] used a chronic rat seizure kindling model and reported that 54 mg/kg IP blunted kindling for weeks after treatment despite a half-life of 2–3 h in rats.[8] This suggested that LEV might limit the development of post-traumatic epilepsy.

Wang and coworkers[9] performed the first study of LEV in a pre-clinical TBI model.[9] They studied two intravenous (IV) LEV doses (18 or 54 mg/kg) versus fosphenytoin in a mouse model of TBI (a single dose at 30 min after TBI). The 54 mg/kg dose provided maximal benefit on motor testing and 18 mg/kg provided maximal benefit on hippocampal neuronal death at 24 h (54 mg/kg also provided benefit). In contrast, fosphenytoin proved detrimental. After OBTT began studies with LEV, Zou and colleagues[6] reported that daily IP LEV (50 mg/kg) in rats after controlled cortical impact (CCI) improved motor and Y-maze performance and reduced hippocampal neuronal death and contusion volume versus saline control.

Post-traumatic seizures and subclinical status epilepticus worsen TBI outcomes and have been associated with hippocampal atrophy.[10] Phenytoin is the most common choice for acute seizure prophylaxis, although there is controversy regarding this choice. Darrah and colleagues[11] found increased hippocampal cell loss in animals treated with chronic phenytoin, and Szaflarski and associates[12] found that LEV resulted in fewer undesirable side effects and improved long-term outcome in patients. Similar results have also been seen in patients with Alzheimer disease, a disease that carries an increased risk of seizures and epilepsy. A retrospective observational study by Vossel and coworkers[13] reported improved treatment outcomes (better seizure control with fewer adverse effects) in patients treated with LEV versus patients treated with phenytoin.

Given LEV's encouraging findings and concern about potential adverse effects of phenytoin, OBTT chose to study LEV across its three rat models. We used a single IV dose 15 min after TBI, based on Wang and coworkers.[9] We chose a low dose (54 mg/kg) that previously conferred benefit in TBI and a high dose (170 mg/kg) based on work in epilepsy in rats.[7]

Methods

Methods will be described briefly given that this is the fifth in a series of articles published by the OBTT consortium in this issue of the *Journal of Neurotrauma*. For additional detail on the individual models, please see the first therapy article in this issue.[14]

Adult male Sprague-Dawley rats (300–350 g), cared for in accordance with the guidelines set forth by each site's Institutional Animal Care and Use Committee, the United States Army (ACURO), and the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals,* were housed in temperature-controlled rooms (22°C) with a 12-h light/dark cycle and given access to food and water *ad libitum*, except as noted in Methods.

Animal models

Fluid percussion brain injury (FPI) model—Miami. Rats were anesthetized (70% N_2O/30% O_2, 1–3% isoflurane) 24 h before injury and surgically prepared for parasagittal FPI as described previously.[15] A right craniotomy was performed, and a plastic injury tube was placed over the exposed dura. The scalp was sutured closed, and rats returned to their home cage. After fasting overnight, the rats were anesthetized, tail artery and jugular vein catheters were placed, the rat was intubated and underwent a moderate FPI. Blood gas levels were measured from arterial samples 15 min before and 30 min after moderate FPI.

FPI served as our sentinel model for assessing the effects of therapies on acute physiological parameters including hemodynamics and blood gases, and the 30 min time point provided an assessment of the effect of TBI and treatment at 15 min after drug administration. After TBI, the rats were returned to their home cages with food and water *ad libitum.* Sham rats underwent all procedures except for the FPI.

CCI Model—Pittsburgh. Rats were anesthetized (2–4% isoflurane in 2:1 N_2O/O_2), intubated, and placed in a stereotaxic frame. A parasagittal craniotomy was performed, and rats were impacted with the CCI device (Pittsburgh Precision Instruments, Inc.) at a depth of 2.6 mm at 4 m/sec.[16] The scalp was sutured closed, and rats were returned to their home cages. Sham rats underwent all procedures except for the CCI.

Penetrating ballistic-like brain injury (PBBI) model—Walter Reed Army Institute of Research (WRAIR). PBBI was performed as described previously.[17] Anesthetized (isoflurane) rats were placed in a stereotaxic device for insertion of the PBBI probe into the right frontal cortex at a depth of 1.2 cm. The pulse generator was activated, and the elliptical balloon was inflated to a volume equal to 10% of the total brain volume. After probe withdrawal, the craniotomy was sealed with sterile bone wax, and wounds were closed. Sham rats underwent all procedures except for the PBBI probe insertion.

Drug administration

LEV (500 mg/5 mL vial, clinical grade for IV use) was purchased from West-Ward Pharmaceuticals (Eatontown, N.J.) and refrigerated until use. A new vial was used each day. Rats in the treatment groups received either 54 mg/kg (LEV-Low) or 170 mg/kg (LEV-High) dissolved into sterile physiologic saline to comprise a total IV injection volume of 2 mL (<10 mL/kg). This was given beginning at 15 min after injury via slow infusion over a 15 min period.

The dosing regimens were chosen based on previous pre-clinical studies as stated above.[7,9]

Rats in the vehicle groups (TBI-Vehicle) received 2-mL injections of sterile physiologic saline given beginning at 15 min after injury again via slow infusion over a 15 min period. Sham operated rats received no treatment or vehicle. The drug was prepared at each site by a person who did not perform the injury, behavioral testing, or histopathological analysis. The group sizes for each site are summarized in Table 1.

Biomarker serum sample preparation

Blood samples (0.7mL) were collected at 4 h and 24 h post-injury and again on day 21 before perfusion for histological analysis. Blood withdrawals for the FPI and PBBI model were taken from an indwelling jugular catheter at 4 h and 24 h after TBI and via tail vein at identical time points after CCI. At the terminal end point for all models (21 days), blood samples were taken via cardiac puncture. Blood was prepared as described previously for serum in FPI and PBBI and plasma in CCI.[18] All samples were shipped via FedEx priority overnight (on dry ice) to Banyan Biomarkers, Inc., for analysis of biomarker levels.

Outcome metrics

The approaches to outcome testing, scoring, and specific outcome methods and metrics are described in detail in the first article within this issue.[19] These outcomes include (1) sensorimotor, (2) cognition, (3) neuropathology, and (4) biomarkers.

Sensorimotor methods

FPI model. The spontaneous forelimb or cylinder test was used to determine forelimb asymmetry as described previously.[20] The grid walk task was use as well to determine forelimb and hindlimb sensorimotor integration. Assessments occurred on post-injury day 7.

CCI model. Two sensorimotor tests, the beam balance and the beam walking tasks, were used as described previously on the first 5 consecutive days after CCI.[21]

PBBI model. A modified neuro examination was used to evaluate rats at 15 min, 1, 7, 14, and 21 days post-injury.[22] Further motor coordination and balance assessments used the fixed-speed rotarod task on days 7 and 10 post-injury.[14]

Cognitive testing. All sites used the Morris water maze (MWM) to assess cognition. Spatial learning was assessed over ~13–18 days post-injury. Primary outcomes included path latency (all sites), path length (only FPI), and thigmotaxis (only PBBI). Probe trial was performed at all sites to gauge retention of platform location after its removal. The Miami site also tested working memory on days 20 and 21, and both the Pittsburgh and WRAIR sites used a visible platform task on days 19–20. Detailed descriptions of cognitive testing are described in accompanying articles.[14,19]

Histopathological assessments. After behavioral testing, rats were anesthetized and perfused with 4% paraformaldehyde (FPI and PBBI) or 10% phosphate-buffered formalin (CCI). Brains were processed for paraffin embedding or frozen sectioning. Coronal slices were stained with hematoxylin and eosin for lesion volume (all sites) and cortical (FPI) or hemispheric (CCI and PBBI) tissue volume as described previously.[14] Both lesion volume and tissue volume loss were expressed as a percent of the contralateral (''noninjured'') hemisphere (CCI and PBBI) or as a percent of the contralateral cortex (FPI). In FPI, lesion volume and tissue volume loss were expressed as a percent of the contralateral cortex given the small lesion size and established standard protocol in Miami.

Biomarker assessments. Blood levels of neuronal and glial biomarkers, namely ubiquitin C-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) were measured by enzyme-linked immunosorbent assay (ELISA) at 4 h and 24 h after injury. Please see Mondello and associates[18] and Shear and colleagues[14] for a more detailed description of the ELISA and biomarker-related methods used in these studies.

Primary outcome metrics for the biomarkers consisted of (1) evaluating the effect of drug treatment on blood biomarker levels at 24 h post-injury and (2) the effect of drug treatment on the difference between 24 h and 4 h (delta 24–4 h) levels. We chose these two primary outcomes for different reasons: 24 h post-injury represents an optimal time window for evaluating any substantial effects of a drug on biomarker levels. On the other hand, the delta 24–4 h accounts for the initial severity of the injury while allowing each rat to serve as its own control.

GFAP and UCH-L1 levels at 1 h after TBI were also assessed as an exploratory method (based on previous work by the OBTT consortium) to determine whether the performance of UCH-L1 was further optimized with earlier sampling given its short half-life. The results of these exploratory 1 h sampling assessments are not part of the OBTT scoring matrix, were performed for future potential investigations, and are thus not reported in this article.

OBTT outcome scoring matrix

To determine therapeutic efficacy across all models, a scoring matrix summarizing all of the primary outcome metrics (sensorimotor, cognition, neuropathology [lesion volume, cortical volume]) and biomarker (24 h and delta 24-4 h) assessments was developed. A maximum of 22 points at each site can be achieved. Details of the OBTT Scoring Matrix are provided in the initial companion article in this issue.[19]

Statistical analysis

Behavioral and histological parameters and biomarker measurements were assessed for normality, and data are expressed as mean ± standard error of the mean or median (interquartile range), as appropriate. Physiological data, contusion and tissue volumes, and probe trial were analyzed using a one-way analysis of variance (ANOVA). One-way ANOVA or repeated measures ANOVA was used to analyze motor tasks as appropriate, depending on the

Table 1. Summary of Experimental Group Sizes for Traumatic Brain Injury/Levetiracetam Study

Group	*Sham*	*TBI-Vehicle*	*TBI-54 mg/kg*	*TBI-170 mg/kg*	*N*
FPI—Miami	12	11	12	12	47
CCI—Pittsburgh	10	10	10	10	40
PBBI—WRAIR	9	12	11	11	43

TBI, traumatic brain injury; FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury; WRAIR, Walter Reed Army Institute of Research.

specifics of the data collection. Repeated measures ANOVA was also used to analyze data for the hidden platform and working memory tasks.

Post hoc analysis, when appropriate, used the Student-Newman Keuls (SNK) or Tukey test. Comparison of biomarker concentrations among the groups in each TBI model was performed using the Kruskal–Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U* and Bonferroni correction. Delta 24–4 h biomarker levels in injured groups were calculated in each rat as the difference between 24 h and 4 h biomarker concentrations.

All statistical tests were two-tailed and a p value <0.05 was considered significant. Statistical analysis was performed using SAS (SAS version [9.2] of the SAS System, © 2002–2008 by SAS Institute Inc., Cary, NC) or Sigmaplot v.11.0 (Systat Software, Inc., Chicago, IL).

Results

Physiological parameters

Physiologic data (mean arterial blood pressure, PaO_2, $PaCO_2$, and blood pH) were recorded pre- and post-TBI in the FPI model (Miami) and are provided in Table 2. All physiologic parameters remained within normal range with no significant differences between groups, and there appeared to be no treatment effect on acute physiology or blood gases.

Sensorimotor parameters

FPI model. Rat performance on the cylinder task is shown in Figure 1A. The TBI-vehicle and LEV-high dose groups were impaired vs. sham at 7-days post injury. However, one-way ANOVA was not significant between groups ($p = 0.344$) and thus there was no significant improvement on this task vs. vehicle with either dose, although there was a trend toward improvement in the low-dose LEV group.

Results of the grid walk task are shown in Figure 1B. Fore- and hind limbs were independently assessed for foot-faults and expressed as a percent of total steps for each limb. One-way ANOVAs for contralateral and ipsilateral forelimb and hindlimb were also not significantly different between groups.

CCI model. On beam balance testing, two-way repeated measures ANOVA revealed a significant group main effect for beam balance latencies over 5 days post-injury ($p < 0.05$) (Fig. 1C). The LEV-high dose group displayed significant motor benefit on beam balance testing ($p < 0.05$ vs. vehicle) scoring full points (+2) for this parameter in the outcome matrix. The LEV-low dose group showed a trend toward improvement versus TBI-vehicle, and sham differed from vehicle but not low dose. Thus, LEV-low dose received half of the point value (+1) for this intermediate benefit on this outcome. In contrast to beam balance results, the results for the beam walking task revealed no treatment effect (Fig. 1D). Two-way repeated measures ANOVA revealed a significant group main effect ($p = 0.001$) for beam walking latencies over 5 days post-CCI; however, all injury groups performed significantly worse versus sham.

PBBI model. *Post hoc* analysis of neuroscore assessments revealed significant abnormalities in all injured groups versus sham that persisted throughout the 21 day evaluation period post-PBBI ($p < 0.05$) regardless of therapy (Fig. 1E).

The rotarod task was used to evaluate motor and balance coordination on days 7 and 10 (Fig. 1F, G). Repeated-measures ANOVA for four groups at three different speeds revealed a difference between injured rats and shams ($p < 0.05$). Motor impairment was evident across all injured groups. The primary outcome, mean motor score per testing day, revealed a significant injury effect on day 7 ($p < 0.05$) with no improvement in either therapy group versus sham (Fig. 1G). Mean motor score is the rotarod parameter that can generate points in the OBTT scoring matrix. Ancillary analysis of individual testing days showed surprisingly that on day 10, the high dose group performed worse than sham ($p < 0.05$). Nevertheless, PBBI rats showed no overall significant sensorimotor improvement when treated with either dose of LEV—which resulted in no points for this task on the OBTT scoring matrix—however, as indicated above, there was a potential detrimental effect seen on day 10 in the high dose group.

Cognitive testing

FPI model. All groups showed improvement over time manifested by decreasing mean latency during hidden platform testing (simple place task) (Fig. 2A). Two-way repeated measures ANOVA was significant for time ($p < 0.05$) and group ($p < 0.05$). A trend toward improvement with LEV emerged, but this was not

Table 2. Effects of Levetiracetam on Fluid Percussion Injury Physiology

Group	*Sham*	*TBI-Vehicle*	*TBI-54 mg/kg*	*TBI-170 mg/kg*
Pre-TBI				
pH	7.43 ± 0.01	7.43 ± 0.01	7.43 ± 0.01	7.43 ± 0.01
pO_2 (mm Hg)	149.2 ± 9.79	149.9 ± 7.32	147.4 ± 6.29	154.8 ± 2.92
pCO_2 (mm Hg)	38.77 ± 0.56	40.1 ± 0.87	41.53 ± 0.75	40.12 ± 0.72
MAP (mm Hg)	118.52 ± 3.58	120.64 ± 3.63	120.06 ± 2.40	116.82 ± 2.96
Brain temp (°C)	36.6 ± 0.03	36.7 ± 0.06	36.6 ± 0.04	36.7 ± 0.05
Body temp (°C)	36.7 ± 0.08	36.8 ± 0.07	36.9 ± 0.06	36.7 ± 0.05
Post-TBI				
pH	7.44 ± 0.01	7.44 ± 0.01	7.43 ± 0.01	7.44 ± 0.01
pO_2 (mm Hg)	146.7 ± 9.77	141.2 ± 7.27	145.7 ± 7.63	158.17 ± 4.54
pCO_2 (mm Hg)	37.42 ± 0.49	37.96 ± 0.76	39.10 ± 0.78	38.03 ± 0.61
MAP (mm Hg)	114.47 ± 3.18	111.70 ± 2.09	110.29 ± 3.62	106.73 ± 3.61
Brain temp (°C)	36.7 ± 0.05	36.7 ± 0.04	36.6 ± 0.03	36.7 ± 0.04
Body temp (°C)	36.9 ± 0.06	36.8 ± 0.07	36.8 ± 0.05	36.7 ± 0.06

TBI, traumatic brain injury.

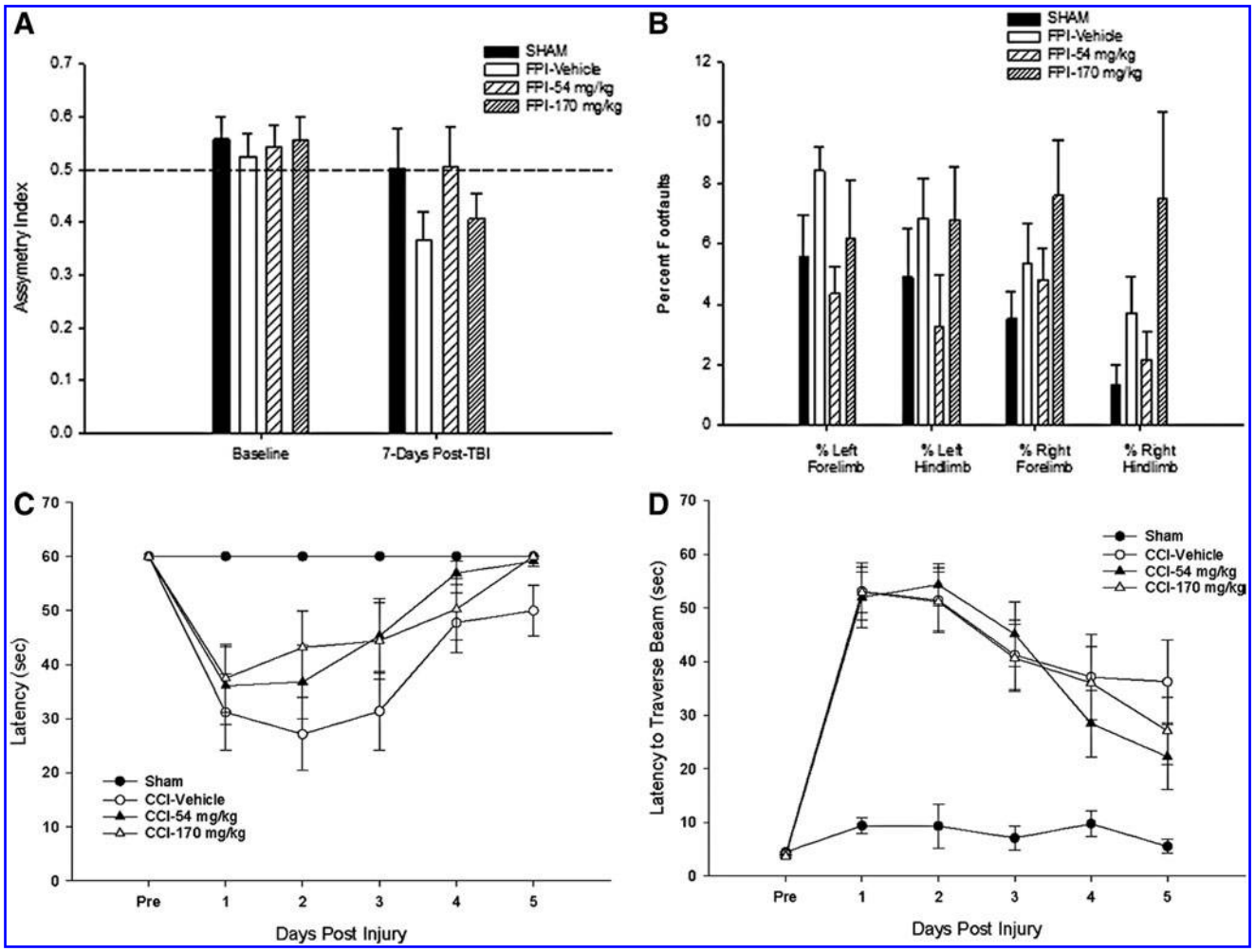

FIG. 1. Sensorimotor outcome. Fluid percussion injury (FPI) model (**A,B**): Bar graphs show the results of (**A**) spontaneous forelimb assessment and (B) the gridwalk task. Controlled cortical impact (CCI) model (**C,D**): Line graphs show the results of the beam balance and walking task: (C) the total time each animal remained on the elevated beam and (D) the mean time taken to traverse the beam. Penetrating ballistic-like brain injury (PBBI) model (**E-G**): Graphs showing results from (E) neuroscore evaluations, and (F,G) the fixed-speed rotarod task. In FPI, neither dose of levetiracetam (LEV) improved sensorimotor outcomes. In CCI, however, the LEV high dose group significantly improved beam balance performance versus vehicle ($p < 0.05$). The LEV low dose group did not differ from sham in contrast to both the vehicle and high dose groups (both $p < 0.05$ vs. sham). In PBBI, LEV did not improve neuroscore or rotarod performance versus vehicle (*$p < 0.05$ vs. sham). See text for details. Data represent group means ± standard error of the mean.

significant ($p = 0.089$). *Post hoc* analysis (SNK), however, revealed that sham, low dose, and high groups performed significantly better than vehicle ($p < 0.05$), and thus both doses achieved full points (+2) for this task in the OBTT scoring matrix. Sham also performed significantly better than both dosage groups ($p < 0.05$).

Similar to the mean latency data, sham and both dosage groups displayed improved MWM path length—i.e., decreased mean path length after TBI (Fig. 2B). Again, both doses received full (+2) points for this outcome in the OBTT scoring matrix. In the vehicle group, rats exhibited longer path lengths versus sham on all testing days. Two-way repeated measures ANOVA was significant for time ($p < 0.05$) and group ($p < 0.05$) because the path length decreased for all groups over time.

There was also an improvement with LEV administration ($p < 0.05$). Again, *post hoc* analysis revealed that sham, low dose, and high dose LEV groups performed better than the vehicle group ($p < 0.05$), and thus full points were awarded for treatment at both doses on this task. The results of working memory are shown in Figures 2C, D. All groups improved by the second trial, and although not significant, LEV treated rats showed a trend toward improved latency and path length versus vehicle.

CCI model. For the hidden platform MWM task (Fig. 2E), two-way repeated measures ANOVA for average latency revealed a significant group main effect ($p = 0.028$). *Post hoc* analysis revealed significant differences in both vehicle ($p < 0.05$) and high dose groups ($p < 0.05$) versus sham. The low dose group showed improvement and did not display a significant difference versus sham ($p = 0.4$) indicating intermediate benefit of LEV generating half (+2.5) of the total points for this task in the OBTT scoring matrix.

PBBI Model. Repeated-measures ANOVA for latency to locate the hidden platform (Fig. 2F) was significant for group ($p < 0.05$). *Post hoc* analysis, however, revealed significant differences between sham and all injured groups ($p < 0.05$) and no significant treatment effect. On repeated-measures ANOVA,

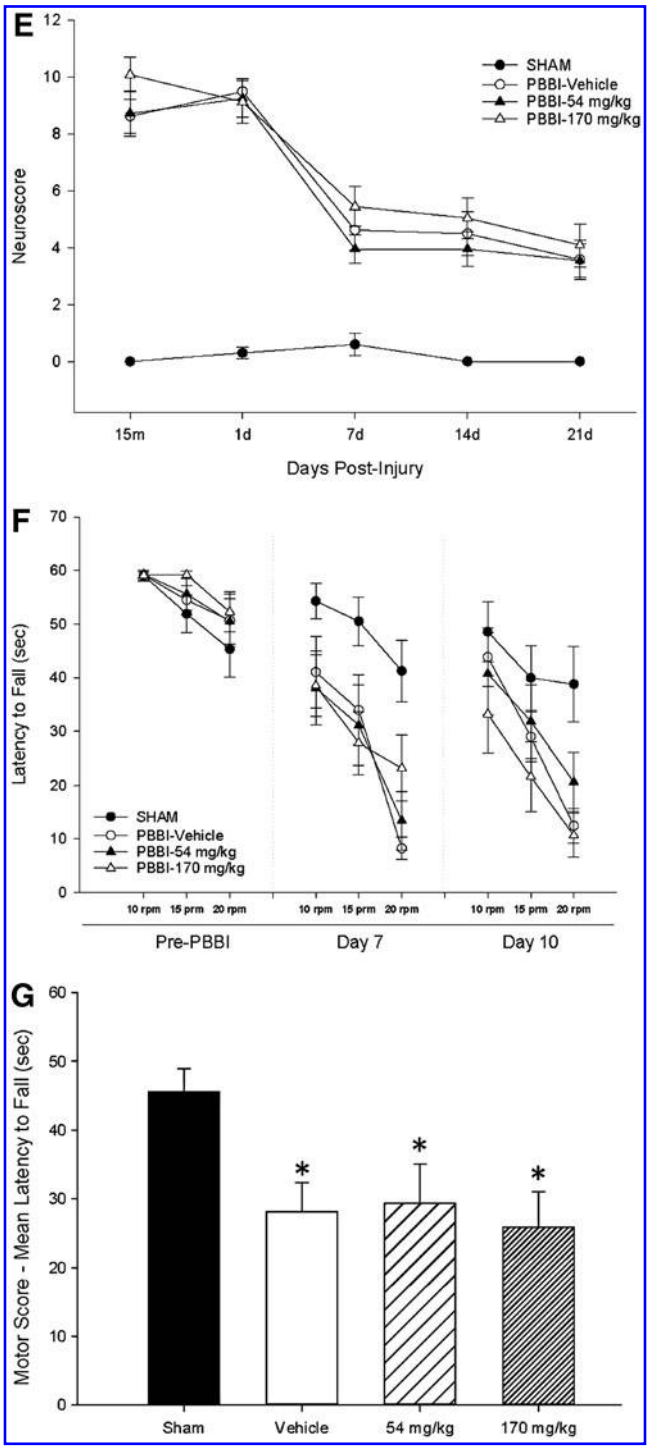

FIG. 1. *(Continued)*

thigmotaxic behavior (Fig. 2G) was significant for group ($p < 0.05$); *post hoc* analysis showed that all injured rats spent more time circling maze periphery versus sham ($p < 0.05$), and again there was no treatment effect for LEV on this behavior.

Pooled analysis of therapeutic effects

For ease of comparison of the findings, we present a pooled analysis of the four key outcomes in OBTT—namely, average latency to find the hidden platform, probe trial, lesion volume, and tissue loss (Fig. 2H, I and 3A, B, respectively).

Cognitive outcomes. Figures 2H, I show the effect of LEV treatment across all models in OBTT for average latency across days and probe trial, respectively. In FPI, average latency (Fig. 2H) was significantly improved versus TBI vehicle in both LEV treated groups ($p < 0.05$). In FPI, on probe trial (Fig. 2I), TBI vehicle was impaired versus sham, while low dose LEV was improved versus TBI vehicle (both $p < 0.05$). Thus, low dose LEV received full (+2) points in the scoring matrix for this parameter on FPI. High dose LEV was not significantly different versus vehicle on probe trial, but high dose was also not significantly different from sham, and thus it received half of the point value (+1) for this outcome in the scoring matrix.

These findings are consistent with benefit on cognitive outcome for both doses of LEV in FPI. In CCI, average latency to find the hidden platform was significantly increased versus sham for both the TBI vehicle and the high dose LEV group, but not the low dose. Again, partial benefit of low dose LEV was suggested in CCI. In CCI, probe trial performance did not differ between groups (Fig. 2I); there was no group effect ($p = 0.2$, one-way ANOVA). In PBBI, average latency to find the hidden platform was increased in all injury groups versus sham, but there was no treatment effect (Fig. 2H). In PBBI, probe trial testing (Fig. 2I) revealed that while all injured groups spent less time searching the target (missing platform) zone versus sham, there was no treatment effect. Thus, in contrast to FPI and CCI, LEV did not appear to confer any cognitive benefits for outcomes tested in PBBI.

Histopathological outcomes. Cross model comparisons of gross histopathological measurements are shown for FPI, CCI, and PBBI in Figures 3A, B. Lesion volume analysis in the FPI model revealed no significant difference between groups ($p = 0.187$). There was a significant group effect ($p < 0.05$) for cortical tissue loss, and all injured groups displayed significantly more cortical loss versus sham. There was no treatment effect in FPI, however.

In the CCI model, although lesion volumes did not differ significantly between injured groups ($p = 0.077$), there was a trend toward reduced lesion volumes with increasing doses of LEV (Fig. 3A). Hemispheric tissue loss, however, displayed a significant group effect between sham and all CCI injured groups ($p < 0.05$), and there was a marked and significant reduction in tissue loss in the group treated with high dose LEV versus vehicle ($p < 0.05$, Fig. 3B). No treatment effect was seen in PBBI for either lesion volume or hemispheric tissue loss. Thus, on histological assessment, treatment with high dose LEV produced significant benefit in CCI, but not FPI or PBBI.

Biomarker assessments

Circulating biomarker concentrations from the study of the effect of LEV in OBTT were made with blood samples collected from 127 rats of the 130 rats in this study. Sampling was unsuccessful in three rats. Effects of LEV on post-injury TBI biomarker (UCH-L1 and GFAP) levels are shown in Figures 4A–C and 5A–C.

FPI model. A Kruskal-Wallis test revealed a significant main effect on GFAP levels at both 4 h ($p < 0.05$) and 24 h post-injury ($p < 0.05$), with all injured groups showing significant increases in GFAP versus sham (Fig. 4A). Delta 24–4 h GFAP levels did not differ between TBI vehicle and TBI treatment groups for either

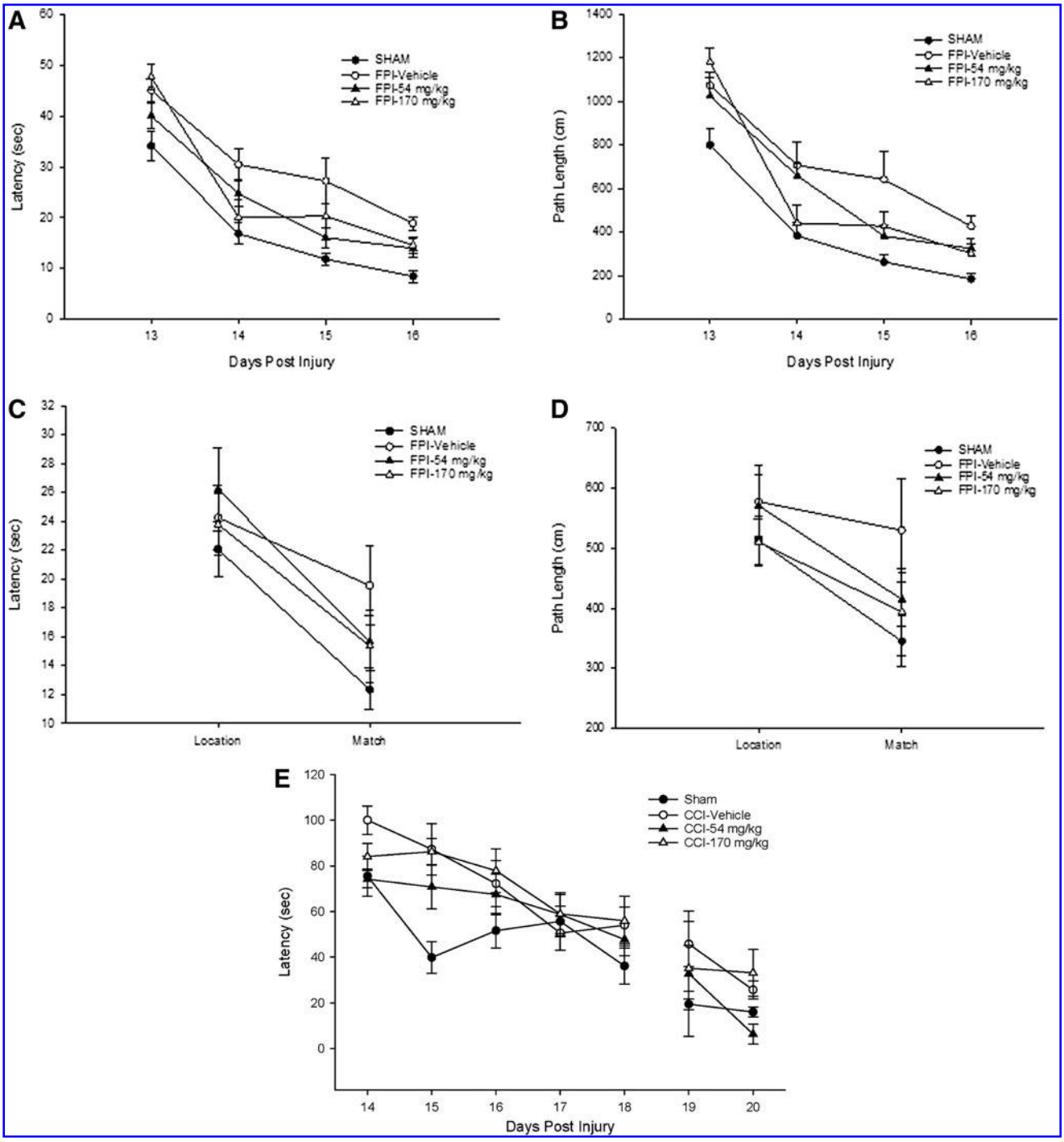

FIG. 2. Cognitive outcome. Fluid percussion injury (FPI) model (**A-D**): Graphs show spatial learning performance in the Morris water maze (MWM) task based on (A) latency and (B) path length to locate the hidden platform over 4 days of MWM testing. Working memory performance is represented by graphs showing the difference in (C) mean latency and (D) mean distance taken to reach the hidden platform between the ''location to match'' trials. Controlled cortical impact (CCI) model (**E**): Line graph shows the (E) latency to the hidden platform over 5 days of MWM testing and mean swim latencies to the ''visible'' platform on post-injury days 19 and 20. Penetrating ballistic-like brain injury (PBBI) model (**F,G**): Graphs show (F) mean latency to the hidden platform and (G) percent time spent circling the outer perimeter of the maze (thigmotaxic response) over 5 days of MWM testing. Pooled comparisons (**H, I**): Graphs show (H) the mean overall spatial learning performance (latency to locate the hidden platform) and (I) the percent time searching the target zone during the probe (missing platform) trial. In FPI, for MWM latency, sham, low dose, and high dose levetiracetam (LEV) treatment groups all performed better than vehicle ($p < 0.05$). Similarly, both doses of LEV displayed improved MWM path length. In FPI, there was no significant benefit of LEV on working memory. In CCI, there was a significant increase in latency versus sham after injury in both the vehicle and high dose LEV groups ($p < 0.05$), but not in the low dose LEV group. In PBBI there were robust injury effects on both MWM latency and thigmotaxis, but no LEV treatment effect. Pooled comparisons confirmed both the benefit on latency for LEV versus vehicle at both doses in the FPI model ($*p < 0.05$), and the blunting of a difference between injury and sham for the low dose group in CCI ($**p < 0.05$ vs. sham). Pooled analysis also showed that low dose LEV improved probe trial performance versus vehicle ($*p < 0.05$) and that although TBI vehicle differed from sham ($**p < 0.05$), high dose LEV did not. In CCI and PBBI, there were no LEV effects on probe trial. See text for details. Data represent group means ± standard error of the mean. $*p < 0.05$ vs. vehicle, $**p < 0.05$ vs. sham.

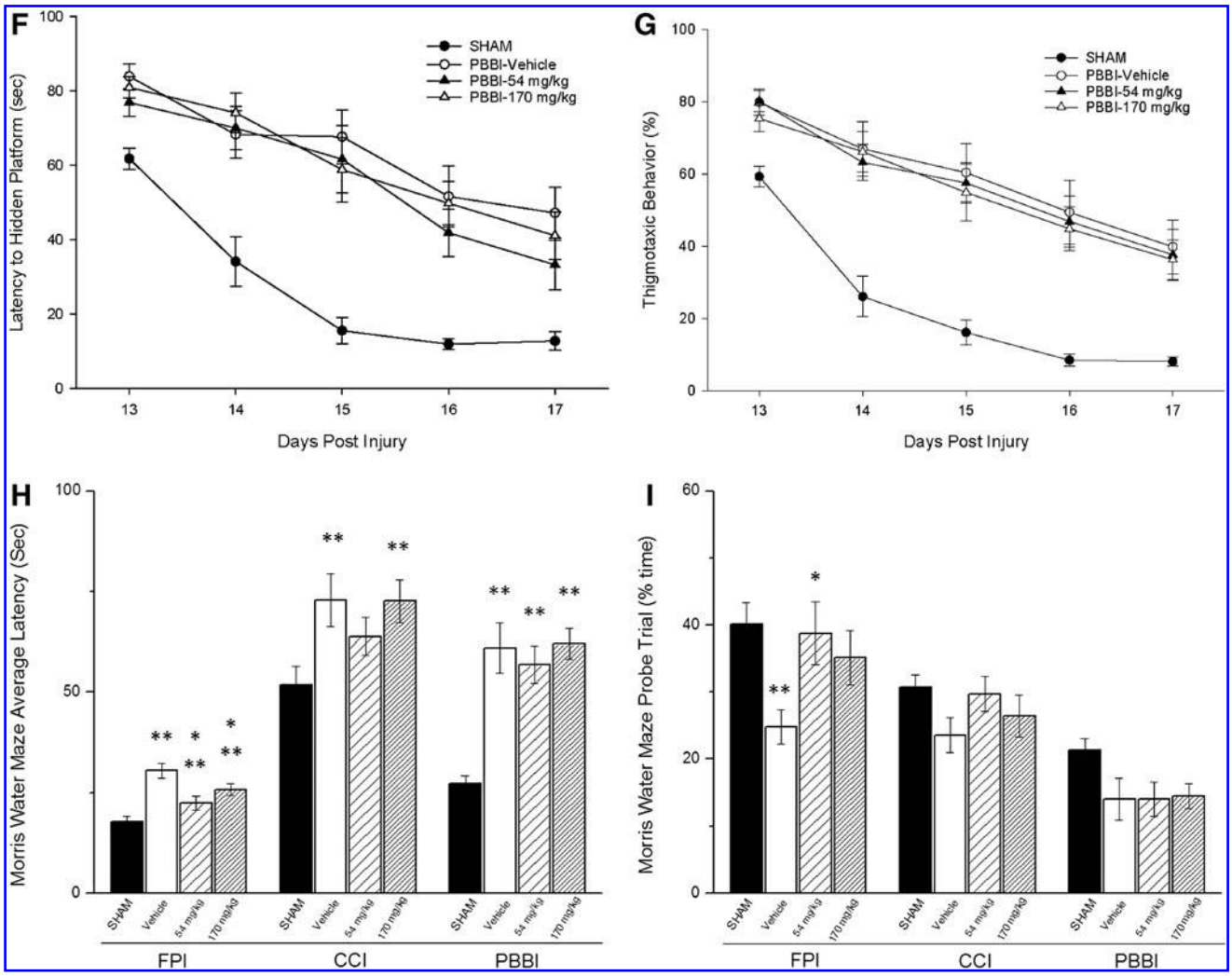

FIG. 2. *(Continued)*

dose (Fig. 5A). No significant between-group effects for any TBI group versus sham were seen for post-injury levels of UCH-L1 at 4 h or 24 h (Fig. 4A). Delta 24–4 h UCH-L1 levels also showed no treatment effect (Fig. 5A).

CCI model. A group effect on GFAP levels was detected at 4 h ($p < 0.05$), with all injured groups showing significant increases in GFAP versus sham (Fig. 4B). Although GFAP levels were lower in both TBI treatment groups, no treatment effect was found. At 24 h, both CCI-vehicle and low dose LEV groups showed significant increases in GFAP versus sham, while there was no significant difference between the high dose LEV group and sham group. In addition, levels of GFAP were significantly lower in the high dose LEV group versus CCI-vehicle group (Fig. 4B). Thus, a full positive point (+1) for high dose LEV was generated for the OBTT scoring matrix on this parameter. No significant group differences on delta 24–4 h GFAP levels were observed (Fig. 5B). Unlike GFAP, there were no significant group differences on either post-injury levels of UCH-L1 at 4 h, 24 h, or delta 24–4 h UCH-L1 levels (Fig. 4B and 5B).

PBBI model. Overall analysis revealed a significant main effect on GFAP levels at 4 h post-injury ($p < 0.05$), with all injured groups showing significant increases in GFAP versus sham. Significant between-group effects on post-injury levels of GFAP were also detected at 24 h ($p < 0.05$), but only PBBI-vehicle and high dose LEV group showed significant increases versus sham. GFAP in the low dose LEV group did not differ significantly from shams (Fig. 4C). This produced a half point (+0.5) value for this parameter for low dose LEV in this model for the OBTT scoring matrix. No significant between-group effects on delta 24–4 h GFAP levels were found (Fig. 5C). All injured groups exhibited significant increases in UCH-L1 at 4 h versus sham ($p < 0.05$) (Fig. 4C). No group effects on levels of UCH-L1 at 24 h (Fig. 4C) as well as delta 24–4 h UCH-L1 levels were seen (Fig. 5C).

OBTT outcome scoring matrix

The overall scoring matrix is shown in Table 3 for the effect of LEV across all models. Overall low dose LEV was beneficial in FPI and CCI, receiving 9.5 points in those two models as a result of cognitive benefit in FPI and motor and cognitive benefit in CCI. Low dose LEV also produced a beneficial effect on 24 h GFAP levels in PBB, providing an additional +0.5 point, for a total of 10 points. High dose LEV produced benefits in both FPI and CCI, with

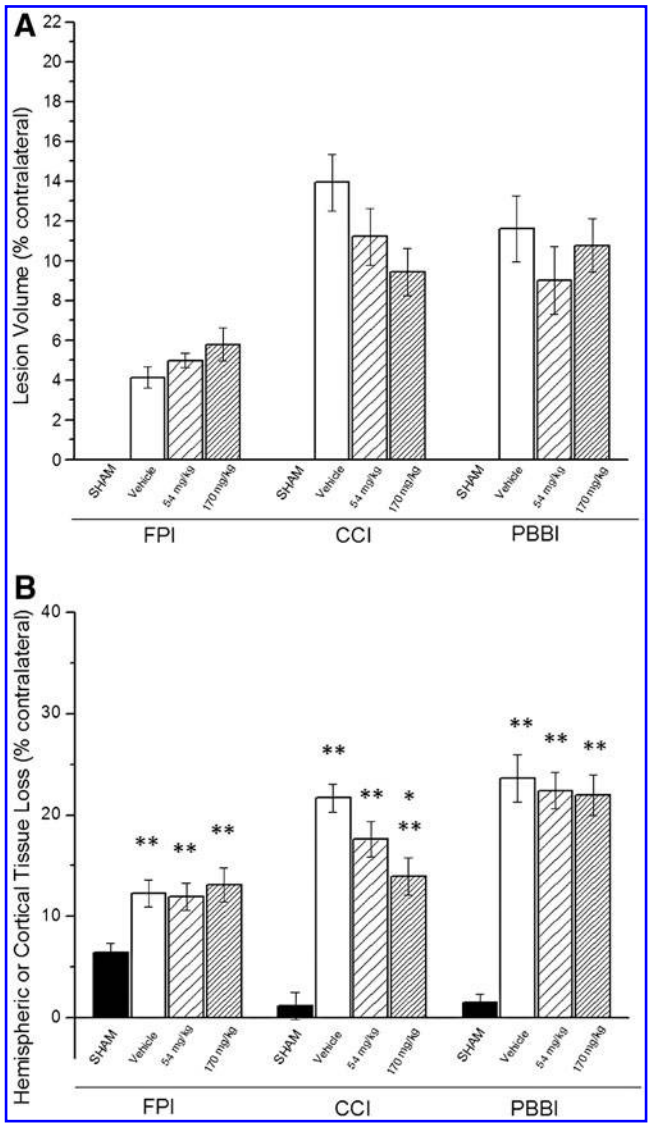

FIG. 3. Histopathology. Bar graphs showing cross-model pooled comparisons of (**A**) lesion volume as a percent of the contralateral cortex in fluid percussion injury (FPI) and hemisphere in controlled cortical impact (CCI) and penetrating ballistic-like brain injury (PBBI), and (**B**) tissue loss; cortical tissue loss in FPI (as a percent of contralateral cortex) and hemispheric tissue loss in CCI and PBBI (as a percent of contralateral hemisphere). Overall, there was no drug effect on lesion volume, although there was a trend toward a dose response reduction by levetiracetam (LEV) treatment in CCI. Consistent with this finding, high dose LEV significantly reduced hemispheric tissue loss versus vehicle in CCI ($*p < 0.05$) with a trend toward reduced hemispheric tissue loss at low dose. There were no treatment effects on hemispheric tissue loss in either FPI or PBBI. See text for details. Data represent group means ± standard error of the mean; $*p < 0.05$ vs. vehicle, $**p < 0.05$ vs. sham.

cognitive benefit in FPI, and motor and histological benefit in CCI. High dose LEV also produced a beneficial effect on 24 h GFAP levels in CCI, resulting in a total of 10 points as well. Aside from the partial point awarded for the GFAP result, there were no other benefits seen for LEV in PBBI. No negative points were generated by LEV treatment in the OBTT scoring matrix.

Morbidity and Mortality

No treatment adverse effects or apparent acute physiological problems were observed in the FPI model, and no notable mortality and morbidity were appreciated in FPI, CCI, or PBBI.

Discussion

Since its approval by the Food and Drug Administration as adjunctive therapy for partial onset seizures, clinical use of LEV has expanded dramatically. Pre-clinical studies have examined its various antiepileptic applications, neuroprotective properties, and potential use as an anti-hyperalgesic and anti-inflammatory agent.[6,23–25] Despite remarkably little pre-clinical data in TBI, various centers have begun to use LEV for post-traumatic seizure prophylaxis in adults with severe TBI.[12,26,27] The most recent TBI guidelines, however, still identify phenytoin as the prophylactic anticonvulsant of choice with level II evidence in adults and level III in pediatrics.[28] A small number of pre-clinical studies suggest benefit of LEV in TBI, including benefit versus phenytoin.[9] Given the varied use in clinical practice combined with sparse but encouraging pre-clinical TBI studies, and a favorable safety profile, we selected LEV as the fifth agent to be tested in OBTT.

A literature search performed when LEV was being considered by OBTT revealed only a single study in a pre-clinical TBI model. Wang and associates[9] showed efficacy in a mouse model of closed head injury. We chose to mimic that study and test single IV dose administration in the acute post-injury period. We selected the dose (54 mg/kg) that produced maximal benefit in that study, which we identified as our ''low dose'' group. The rationale for testing a ''high dose'' group arose from the general design of OBTT, which includes assessment of a dose response, when possible, and 170 mg/kg (high dose) was selected based on work by Klitgaard and colleagues[7] in multiple rodent models of epilepsy. They reported that extremely high doses of LEV were well tolerated in rats; detrimental effects on behavior were not appreciated until doses of 1700 mg/kg were used.[7]

In OBTT, the most encouraging results were seen in FPI and CCI. LEV, at both doses, significantly improved cognitive outcomes in rats after FPI, and depending on the dose, produced favorable effects on motor, cognitive, and/or histological outcomes in CCI. In addition, we were likely underpowered for the motor testing performed in FPI. The biomarker data revealed reductions in GFAP 24 h levels with high dose LEV in CCI and with low dose LEV in PBBI; however, this was the only positive result produced by LEV in PBBI.

The mechanisms underlying the benefit of LEV in FPI and CCI remain undefined, given that the goal of OBTT is screening therapies rather than studying mechanism. Published reports, however, suggest benefit via effects on post-traumatic seizures and/or subclinical status epilepticus, glutamate signaling, excitotoxicity, neuroinflammation, and/or neuromodulation.[6,12,23] The contribution of post-traumatic seizures to secondary injury has not been clearly defined in any of the three pre-clinical rat models used by OBTT, although post-traumatic seizures are seen in these models.[29,30]

While there is limited pre-clinical work examining LEV in TBI and none, to our knowledge, addressing the effects of LEV on post-traumatic seizures in rodents, there are intriguing results in pre-clinical stroke and hypoxic-ischemic brain injury. Cuomo and coworkers[31] found that one dose of LEV 100 mg/kg given before middle cerebral artery occlusion in rats reduced seizure activity, lesion volume, and neurologic deficits. A recent study[32] examined the effects of LEV on neonatal rat pups after hypoxic ischemic

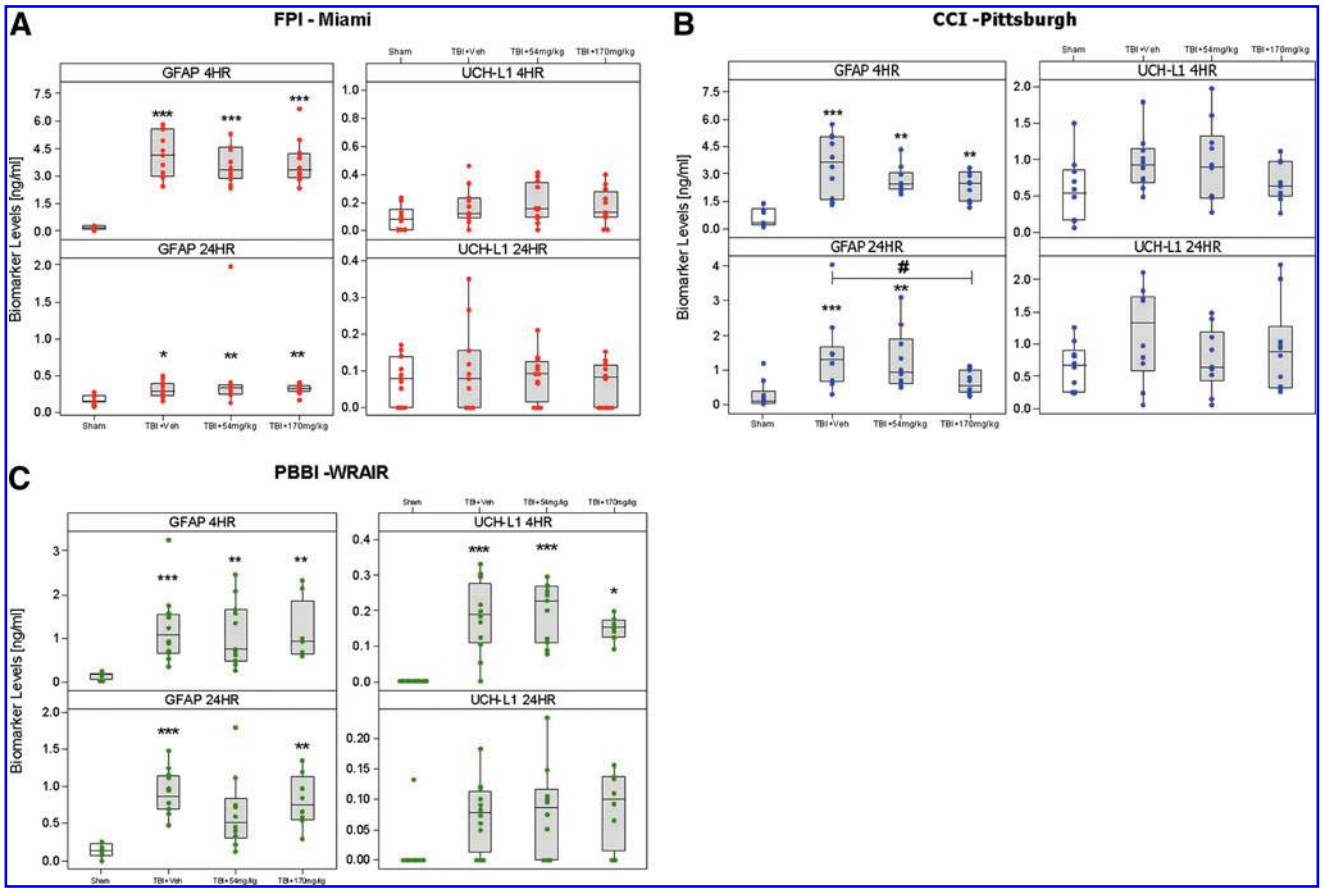

FIG. 4. Box plots illustrating serum glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L) concentrations in blood. GFAP and UCH-L1 concentrations in blood at 4 and 24 h post-injury in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. There were significant increases in GFAP in the vehicle groups at both 4 and 24 h versus sham in all three models. In addition, in CCI, high dose levetiracetam (LEV) significantly reduced GFAP levels at 24 h after injury (#$p < 0.05$ vs. vehicle). In PBBI, GFAP levels in vehicle and high dose groups were significantly increased versus sham, but low dose LEV was not. UCH-L1 levels were increased versus sham only in the PBBI model, and there were no treatment effects. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. sham; #$p < 0.05$ vs. vehicle. TBI, traumatic brain injury.

brain injury. A single dose of LEV (200 mg/kg) decreased apoptotic neurons and improved MWM outcomes possibly from reduced oxidative stress and seizure activity.

LEV may also ameliorate the initial glutamate surge after TBI or alter glutamate signaling and it modulates GABA-ergic signaling leading to calcium channel inhibition.[33,34] These pathways may converge to diminish post-synaptic depolarization, calcium accumulation, excitotoxicity, cell death, and inflammation. While the exact mechanisms remain unanswered, the ability of LEV to produce benefit in multiple models after only a single early dose indicates a potent effect—particularly given the fact that a number of other therapies have produced limited benefit tested in the rigors of OBTT.

Treatment was restricted to a single dose at 15 min after injury, suggesting benefit early after TBI. Loscher and colleagues,[8] however, used a chronic rat seizure kindling model and reported that 54 mg/kg of LEV IP blunted kindling for weeks after treatment despite a half-life of 2–3 h in rats. Thus, sustained effects on post-traumatic seizures cannot be ruled out with our approach. Delayed or sustained use of LEV in patients, however, has the potential to cause behavior and mood disturbances—some so severe that treatment must be discontinued.[35] We wish to emphasize that benefit was seen in OBTT using single IV dose administration early after TBI.

Surprisingly, LEV is the only therapy that has been shown thus far to have beneficial effects on cognitive outcome in any of the models used in OBTT. It has been reported to improve cognition, especially in patients with existing cognitive weaknesses.[36] Given that treatment was restricted to the early post-injury period, however, it suggests an enduring benefit from an acute post-TBI effect rather than delayed direct effects on cognitive function.

Another promising finding was the reduction in hemispheric tissue loss with high dose LEV in CCI, and the suggestion of a dose response on hemispheric tissue loss and lesion volume in CCI. Histological protection by LEV was restricted to CCI, however, and the benefit on cognitive outcome in FPI was independent of an effect on lesion volume or hemispheric tissue loss. This highlights the complexities encountered with trying to develop a therapy that crosses models and injury severities. We cannot, however, rule out histological benefit in FPI—because we did not assess outcomes such as neuron counting in cortex or hippocampus or axonal injury. Further study of additional targets with LEV treatment is ongoing in the FPI model in micropigs.

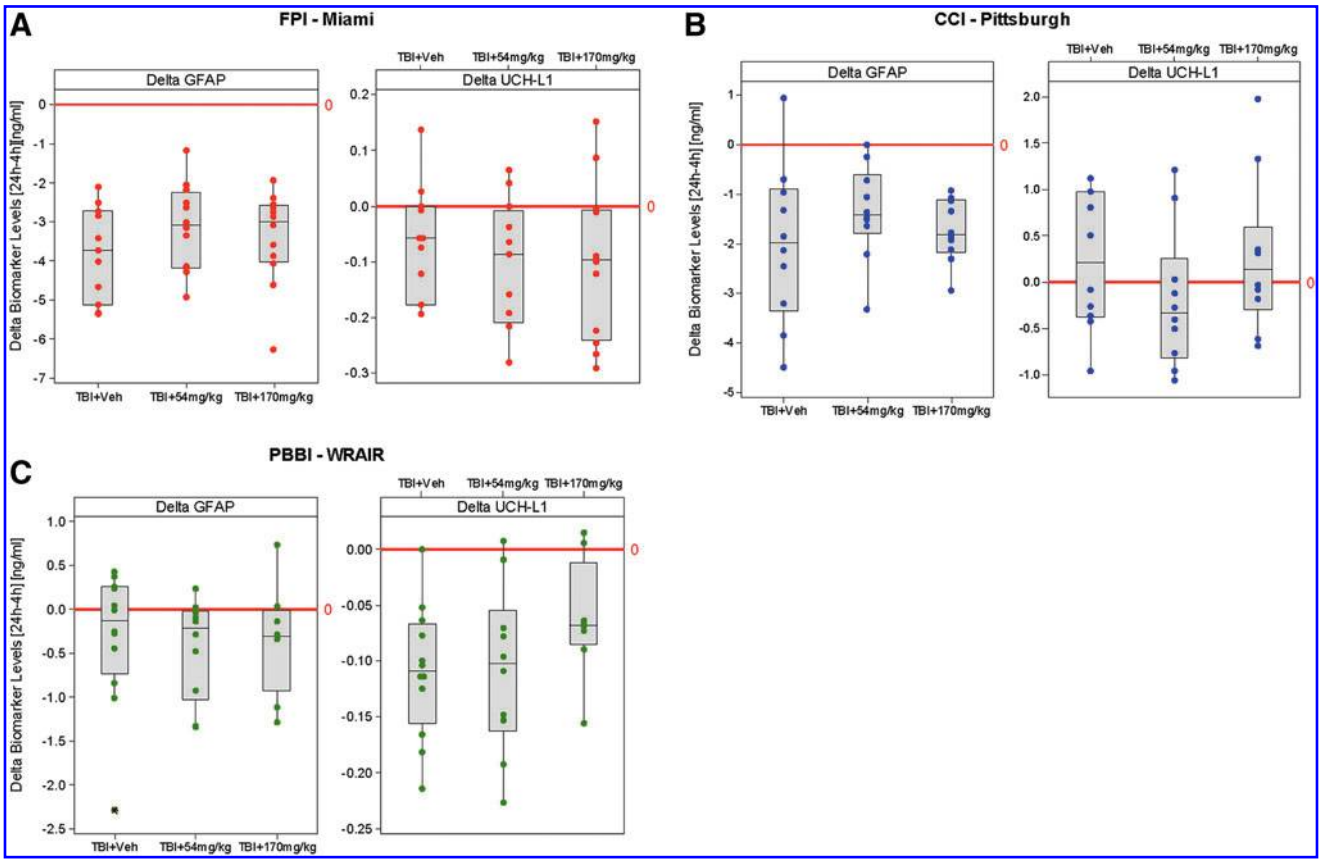

FIG. 5. Box plots illustrating delta 24–4 h glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) levels in blood. Delta 24–4 h GFAP and UCH-L1 levels in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. Overall, there were no significant changes in delta 24–4 UCH-L1 levels in any of the models, indicating no effect of LEV on net clearance of either biomarkers. Please see text for details. TBI, traumatic brain injury, WRAIR, Walter Reed Army Institute of Research.

It is intriguing that fairly robust cognitive benefit was seen with single dose administration in FPI, which is the mildest insult in OBTT. To our knowledge, LEV has not been studied in models of mild or repetitive mild TBI. We believe that such studies are needed.

In PBBI, the most severe model in OBTT, LEV produced a partial benefit on 24 h GFAP levels with no other significant effects on the other primary outcomes. Subsequent unpublished observations in PBBI using a longer treatment duration and electroencephalographic (EEG) monitoring, however, suggest that benefit can be seen with more sustained therapy.[37] It is thus possible that different dosing regimens will be required depending on the model and/or injury severity level. Further studies with continuous EEG monitoring are warranted in PBBI and the other TBI models in OBTT and in other TBI models outside of OBTT. The ability to administer high doses with what appears to be a large safety margin and with sustained antiexcitotoxic effects—exceeding those expected based on its half-life—may have given LEV a considerable advantage for the screening approach taken by our consortium.

Our findings with LEV also indicate what are likely to represent important differences between the models used in OBTT, support the OBTT concept of screening across multiple TBI models, and suggest that our models represent a reasonable spectrum of insults to generate a menu of therapeutic targets that parallel the complex injury spectrum in human TBI.

Remarkably, a theranostic effect of high dose LEV was seen in the CCI model based on 24 h GFAP levels, which were significantly reduced versus TBI vehicle. This finding paralleled the benefit of high dose LEV on motor function early after injury and hemispheric tissue loss at 21 days in CCI. This is an exciting and unique finding and suggests theranostic potential for GFAP as a biomarker in pre-clinical drug screening in TBI. Whether or not this could have clinical translation remains to be explored, but recent work suggests clinical potential for GFAP in TBI.[38,39]

We did not see a theranostic effect of LEV on GFAP in FPI despite benefit on cognitive outcome. The increase in GFAP at 24 h in FPI, however, although statistically significant, was modest and did not provide a robust target for a therapeutic effect. Similarly, UCH-L1 was only significantly increased versus sham after injury in PBBI, the most severe injury model in OBTT, and thus also did not provide a robust theranostic target.

Our study design was based, to a large extent, on work by Wang and associates,[9] and our results appear to agree with their work. Reproducibility of experimental findings is a major mandate of NIH, and thus far, in OBTT, it has been challenging, given the rigor of our approach, to reproduce some of the published benefits seen using

Table 3. Scoring Matrix for Assessment of Therapeutic Efficacy Across Models in Operation Brain Trauma Therapy

Site	*Neuro exam*	*Motor*	*Cognitive*	*Neuropathology*	*Serum biomarker*	*Model and overall total*
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform path length (2) MWM probe (2) Working memory latency (2) Working memory path length (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Miami total	N/A	4	10	4	4	
Miami						
Dose 1		0,0	2,2,2,,0,0	0,0	0,0,0,0	6
Dose 2		0,0	2,2,1,0,0	0,0	0,0,0,0	5
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh						
Dose 1		1,0	2.5,0	0,0	0,0,0,0	3.5
Dose 2		2,0	0,0	0,2	1,0,0,0	5
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
WRAIR total	1	3	10	4	4	
WRAIR						
Dose 1	0	0	0,0,0	0,0	0.5,0,0,0	0.5
Dose 2	0	0	0,0,0	0,0	0,0,0,0	0
Grand total						
Dose 1	0	1	8.5	0	0.5	10
Dose 2	0	2	5	2	1	10

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; WRAIR, Walter Reed Army Institute of Research.

( ) = point value for each outcome within each model.

Drug: Levetiracetam; Dose 1 = 54 mg/kg; Dose 2 = 170 mg/kg.

various drugs in identical and/or other TBI models. The reproducibility seen with LEV in this study—comparing both mouse and rat and in both FPI and CCI—is encouraging. It was also encouraging that LEV was similarly effective at both doses, that we encountered no deleterious side effects with our treatment regimen, and that no negative points were produced in the OBTT scoring matrix. The only hint of negativity was in the PBBI model on day 10 rotarod performance.

It may also be important that unlike the previous agents tested by OBTT (nicotinamide, erythropoietin, cyclosporine A, and simvastatin), LEV is a drug specifically developed as a neurotherapeutic. Blood–brain barrier penetration is excellent, and anticonvulsant properties could represent a primary or adjunctive benefit to neuroprotection.

Our findings provide an exciting platform on which to expand the study of LEV as a potential therapy in TBI. Since testing on LEV began in OBTT, two additional studies by Zou and associates[6,40] have emerged examining the effects of LEV on rats after CCI. In an initial study, they found that 50 mg/kg of IP LEV given daily for 20 days produced benefit on histological, molecular, and behavioral elements after TBI.[6] Treatment was not initiated until 24 h after injury. A follow-up study examined an abbreviated treatment regimen early after TBI. They gave three 50 mg/kg IP doses of LEV over the first 24 h after CCI—an immediate post-injury dose followed by doses at 12 and 24 h. Unfortunately, no benefit was seen.[40]

Our results differ from that report. One potential explanation may stem from the fact that we administered LEV IV rather than IP—which could be important to blunting excitotoxicity rapidly after TBI. It is also intriguing to consider the combined effects of acute plus prolonged treatment, perhaps targeting the initial glutamate surge and chronic inflammation.[39,40] As previously discussed, however, our work in OBTT can only speak to early, post-TBI administration with a single dose.

Conclusion

LEV is the most promising agent tested to date by OBTT. Although benefit was not seen across all three models, positive effects in both FPI and CCI across multiple outcomes, including motor, cognitive, and/or histology, with single early post-TBI dosing suggest the need for OBTT to study LEV further. This includes studies of dose response, therapeutic window, mechanism, and testing in our large animal FPI model in micropigs. Given its track record for safety early after severe TBI, it would also be reasonable to consider a randomized controlled trial examining early administration in patients with severe TBI. Finally, we observed unique and exciting theranostic potential for blood levels of GFAP as a TBI biomarker in the CCI model.

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Author Disclosure Statement

Dr. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Dr. Hayes is an employee and receives salary and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and as such all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

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Synthesis of Findings, Current Investigations, and Future Directions: Operation Brain Trauma Therapy

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Abstract

Operation Brain Trauma Therapy (OBTT) is a fully operational, rigorous, and productive multicenter, pre-clinical drug and circulating biomarker screening consortium for the field of traumatic brain injury (TBI). In this article, we synthesize the findings from the first five therapies tested by OBTT and discuss both the current work that is ongoing and potential future directions. Based on the results generated from the first five therapies tested within the exacting approach used by OBTT, four (nicotinamide, erythropoietin, cyclosporine A, and simvastatin) performed below or well below what was expected based on the published literature. OBTT has identified, however, the early post-TBI administration of levetiracetam as a promising agent and has advanced it to a gyrencephalic large animal model—fluid percussion injury in micropigs. The sixth and seventh therapies have just completed testing (glibenclamide and Kollidon VA 64), and an eighth drug (AER 271) is in testing. Incorporation of circulating brain injury biomarker assessments into these pre-clinical studies suggests considerable potential for diagnostic and theranostic utility of glial fibrillary acidic protein in pre-clinical studies. Given the failures in clinical translation of therapies in TBI, rigorous multicenter, pre-clinical approaches to therapeutic screening such as OBTT may be important for the ultimate translation of therapies to the human condition.

Key words: biomarker; controlled cortical impact; drug; fluid percussion; micropig; penetrating ballistic-like brain injury; pre-clinical modeling; rat; reproducibility; therapy; traumatic brain injury

Introduction

In this series of articles,[1–7] we have reported on the design, establishment, and implementation of the Operation Brain Trauma Therapy (OBTT) pre-clinical therapy and biomarker screening consortium. We have presented the findings of the first five therapies that were evaluated—namely, nicotinamide, erythropoietin (EPO), cyclosporine A (CsA), simvastatin, and levetiracetam[2–6]—and reported on the performance of two biomarkers of brain injury, Ubiquitin carboxyl-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) across the three rodent traumatic brain injury (TBI) models used in therapeutic screening.

As described in the individual articles, the design of the OBTT screening consortium featured three different TBI rat models (parasagittal fluid percussion injury [FPI], controlled cortical impact [CCI], and penetrating ballistic-like brain injury [PBBI]), a battery of established and conventional functional and histological outcomes, a careful and comprehensive approach to therapy selection, a literature-based approach to treatment protocol development that was implemented in an identical fashion across sites,

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and a highly rigorous approach to therapy and biomarker assessments.

In this concluding article, we synthesize the key findings from this initial work by the consortium, provide insight into the ongoing investigations, and formulate potential avenues for future directions.

Summary and Synthesis of Findings

Strategy

Crafting and establishing a multicenter consortium approach to pre-clinical therapy development represented a novel initiative for the field of TBI; however, we were fortunate that there was some precedent on which to base our overall plan for therapy testing—namely, the Multicenter Animal Spinal Cord Injury Study (MASCIS) from the late 1990s.[8,9] As discussed in the introductory article in this issue of the journal,[1] in MASCIS, there was an attempt to standardize the approach to therapy testing by using a single model of spinal cord injury across all sites. Regrettably, that led to major challenges in reproducibility and differential complications across the sites, and ultimately a large number of therapies were not evaluated. The failure to reproduce the purported efficacy of the bellwether agent methylprednisolone by the MASCIS consortium also blunted momentum.

Building on that valuable knowledge, in OBTT we chose to use a range of models that were already established and being actively used for therapy testing at each site, and instead, rigorously standardized the approach to treatment across sites. We believe that that decision contributed critically to the success of rapidly launching OBTT and facilitated the prompt and ongoing screening of multiple therapies, as presented in this issue. Similarly, we chose to use outcomes that were already established at each site—rather than attempting to mandate use of identical outcomes across sites—outcomes that might have needed to be established at a given site. Thus, as was evident from the reports on each therapy, the motor tasks used, for example, differed across sites.

This approach, however, allowed investigators to use established injury levels at each site and immediately begin therapy screening, rather than try to define an injury level that produced usable deficits on outcomes that may have neither been established nor routinely used in their model and/or center. We were fortunate that the Morris water maze (MWM) was already established and routinely used at each site, and thus a key cognitive outcome tool was able to be readily incorporated into the OBTT scoring matrix. We were also able to establish an approach to histopathological screening that was readily applicable with either no or only minimal modifications at each site. We believe that those initial decisions were essential to the ability of OBTT to launch and produce meaningful data in a prompt and successful manner.

We also chose to weigh cognitive outcome as having the greatest impact on perceived therapy success in our scoring matrix. That decision was in some ways arbitrary—although the importance of cognitive outcome as a therapeutic target is certainly not questioned in the setting of severe TBI.[10]

Although we believe that the scoring matrix that was developed is reasonable, we recognize, however, that the exact weighting of the various outcomes across the sites was defined simply by the consensus of our collective investigative team and might not be optimal in some applications. For example, a therapy targeting brain edema might be important to preventing herniation and/or the need for decompressive craniectomy in humans with severe TBI, but that might not be readily reflected in improved cognitive outcome in our models—each of which includes a craniotomy as part of its design. As with any pre-clinical data, these and other related factors should be carefully vetted when considering any therapy for clinical translation.

Models

Another goal of OBTT was to use a broad menu of TBI models in rats in primary screening in an attempt to specifically address the well-recognized issue of heterogeneity of TBI as a roadblock to successful therapy development.[11,12] It became clear rapidly that the three models that we selected achieved that goal and provided both a wide range of injury severity, behavioral deficits, neuropathological alterations, biomarker levels, and responses to therapy.

We also believe that OBTT provided a heretofore unexplored direct comparison between three rodent models commonly used in the field of pre-clinical TBI research—providing unique insight for the field. For example, visual comparison of the TBI vehicle groups in the pooled outcomes figures in any of the individual treatment articles in this special issue[2–6] immediately reveals the marked differences in outcomes between models. Specifically, examination of the pooled analysis graphs in each of the aforementioned manuscripts comparing lesion volume at 21 days after injury across models demonstrates the relatively small focal lesion in the parasagittal FPI model in rats compared with either CCI or PBBI, and the similarities between CCI and PBBI for this outcome with, in general, slightly greater lesion volume in PBBI versus CCI.

Careful consideration of the methods also indicates that the difference in the size of the focal lesion in FPI versus either CCI or PBBI is even greater than suggested by those figures, given the fact that lesion volume is normalized to cortical volume in FPI, while it is normalized to hemispheric volume (a much larger denominator) in both CCI and PBBI. These types of comparisons are, we believe, unique in our field and also help in explaining the differences between models in the observed behavioral outcomes such as average latency to find the hidden platform in the MWM paradigm–which in general were much shorter in the FPI model versus either CCI or PBBI. Surprisingly, despite what would amount to apparently much smaller therapeutic targets in FPI, in general, the greatest number of therapeutic benefits were shown in FPI and CCI.

Issues such as severity or manipulability of the insult, other facets of the type of injury produced, and the ability of a chosen therapy to target a given injury substrate may be paramount to being able to demonstrate therapeutic efficacy. This issue is discussed in greater detail later in this article. In any case, given that the same rigor was applied across sites, we believe that OBTT provides special insight for model comparisons in this regard. Given the primary goal of OBTT to identify the most promising therapies for clinical evaluation in severe TBI, we have only scratched the surface related to data analysis on cross model comparisons from the myriad results of OBTT in this regard.

Biomarkers

We would be remiss to not mention the fact that in its original form, OBTT was proposed purely as a drug screening consortium. The concept of incorporating circulating biomarker assessments into the program came at the request of the reviewers of our original OBTT grant submission to the U.S. Department of Defense. We are grateful to those reviewers for that suggestion and believe that the incorporation of circulating biomarkers of brain injury into the work of our consortium has generated some remarkable results and has added considerable richness to our findings. In addition, by using biomarkers that are currently in clinical trials, we believe that the

results generated thus far by OBTT could provide insight on biomarker use and interpretation germane to clinical investigations—where the exact nature of the injury is often unclear or complex.

A number of very interesting findings were generated by OBTT based on our biomarker results—we will highlight three of them in this summary article. First, our data strongly suggest circulating levels of GFAP represent an excellent biomarker of brain injury for pre-clinical investigations, and that this is likely to be the case in the clinical arena. As clearly demonstrated in the article in this issue by Mondello and associates,[7] GFAP levels were not only reproducibly increased at 4 and 24 h after injury across models comparing the TBI vehicle and sham groups, from study to study, the 24 h levels were correlated strongly with histological outcomes and in some cases, with behavioral outcomes.

These correlations were seen across models, and were truly exceptional in the CCI model for the relationships between 24 h GFAP levels and 21 d lesion volume and hemispheric tissue loss. UCH-L1 did not perform as well, although it might merit evaluation at earlier time points after injury, given its short half-life and rapid appearance in serum after TBI in humans.[13] The ability to use time points, however, with broad clinical relevance such as 4 h and 24 h, as shown with GFAP, is attractive for a biomarker.

Second, the biomarker data revealed some very surprising cross-model findings that may provide special insight into pre-clinical and clinical data interpretation. One of the most interesting in this regard was the fact that despite the modest lesion size in FPI, serum GFAP levels were higher at 4 h after injury than in the PBBI model, as assessed when comparing TBI vehicle groups. By 24 h, this finding had reversed—although serum GFAP levels were still only modestly greater in PBBI than in FPI despite hugely different amounts of tissue loss.

A number of factors could be involved. For example, cerebral blood flow is likely much more well preserved in the FPI model and thus a larger volume of injured tissue may still be well perfused in FPI compared with PBBI, where a large area of brain is rapidly and severely damaged in the experimental ballistic tract by the PBBI mechanism that mimics what is seen in the clinical setting of a ballistic tract after a penetrating brain injury. Other factors could be involved such as lesion location and differences in blood–brain barrier permeability (in regions that remain perfused).

One other emerging area of biomarker research relates to the role of the glymphatic system on movement of parenchymal biomarkers to the circulation,[14] and differences between models on the impact of injury on that pathway could also be involved. In those studies, the impact of disruption of the glymphatic system was shown at 18 h after TBI. Differences between models that we observed as rapid as 4 h after injury, however, may suggest more direct transfer of biomarkers into the circulation. Further study is needed in this regard.

Third, we were very pleased that circulating GFAP levels at 24 h after injury in the CCI model predicted a benefit of most efficacious therapy tested to date in OBTT (levetiracetam) on ultimate hemispheric tissue loss at 21 days after injury. The benefit of levetiracetam in pre-clinical TBI was suggested in the initial work of Wang and colleagues,[15] and thus OBTT was able to replicate, in many ways, that positive effect.

It was also interesting to see that the increase in lesion volume seen with simvastatin treatment in the FPI model was also reflected in an increase in serum GFAP levels at 24 h—although this was not as clearly delineated as in the case with levetiracetam, because both the low and high dose treatments with simvastatin increased serum GFAP, while only the high dose exacerbated tissue loss. In any case, the potential theranostic utility of GFAP is exciting, particularly given that 24 h circulating levels are predicting long-term histology at 21 d.

One could also argue that although not statistically significant, a similar trend of reduced GFAP levels at 24 h after injury predicting a reduction in hemispheric tissue loss was seen in the CCI model with the only other drug that significantly affected this histological outcome parameter in OBTT—namely high dose nicotinamide.[7] Histological benefit was suggested in the CCI model by the work of Hoane and coworkers,[16] which served as the basis of the treatment protocol used for nicotinamide by OBTT. A larger sample size would be needed to appropriately test the utility of GFAP to predict tissue loss in the CCI model with nicotinamide—but it is clear that this is worthy of additional exploration.

Although both levetiracetam and nicotinamide reduced hemispheric tissue loss in CCI, neither drug produced a statistically significant reduction in contusion volume in the CCI model. Hemispheric tissue loss in CCI comprises both the contusional volume loss and an additional volume of tissue lost outside of the contusion in the impacted hemisphere—an amount that often is similar in magnitude to the tissue volume in the contusion proper. It might be that this ''occult'' or ''silent'' volume loss is more therapeutically manipulable than the parenchyma directly impacted by primary injury located in the contusion proper. It will be interesting to follow this parameter in OBTT to determine whether other drugs can successfully reduce contusion volume versus hemispheric tissue loss in CCI and the other models being used.

Finally, as previously discussed,[17] hemispheric tissue loss often better correlates with MWM latency than lesion volume in CCI.

Therapies

In general, within the exacting approach used by OBTT, most of the therapies performed below or well below what was expected based on the published literature. One of the major goals of OBTT is to define a therapy that is highly effective across all three models, in an attempt to address the heterogeneity of TBI that has been suggested to be vital to successful pre-clinical drug development to mimic the clinical condition.[11,18]

None of the first five therapies proved beneficial across all three screening models, although levetiracetam showed beneficial effects on multiple outcomes in both the parasagittal FPI model and CCI (Fig. 1). In addition, surprisingly, it was the only therapy to show beneficial effects on cognitive outcome in any of the models. Remarkably, its tissue sparing effect in CCI seen at 21 days after injury was predicted theranostically by 24 h blood GFAP levels—an exciting finding in the field of TBI biomarker research.

Modest and relatively sporadic beneficial effects were seen for nicotinamide (at the highest dose) and simvastatin (on motor function). A complete lack of benefit in any model was also quite surprising to see with EPO—which had >20 articles supporting its use specifically in pre-clinical TBI and many other supportive studies across other brain injury models.[3] Supporting our findings, however, EPO failed to demonstrate benefit in a recent high-quality single center trial in TBI[19] and similarly demonstrated a suggestion toward a detrimental effect in clinical stroke.[20] Effects of CsA in OBTT were complex and highly model dependent—with some modest benefits in the FPI model—the mildest injury used for therapy screening in OBTT, but lack of benefit in CCI (and some toxicity) and deleterious effects in PBBI, the most severe injury model.

Some thoughts on why only levetiracetam met the performance standard suggested in the literature whereas these other seemingly promising therapies failed to show benefit in OBTT are provided in

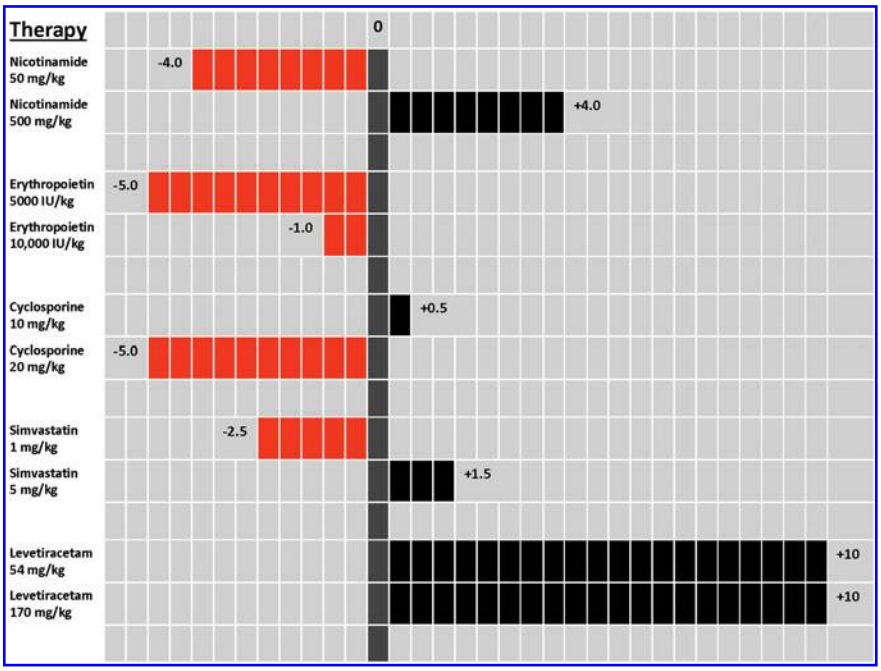

FIG. 1. Graphic representation of the overall scores from the Operation Brain Trauma Therapy (OBTT) scoring matrices generated from testing of each of the first five therapies evaluated across three rat models (parasagittal fluid percussion injury, controlled cortical impact, and penetrating ballistic-like brain injury). Note that for each drug, two doses were tested. Specifics of the dosing are provided in each of the treatment articles in this issue of the journal.[2–6] Scores depicted in red indicate negative effects, while those in black indicate an overall positive effect. In general, most of the therapies underperformed relative to the published literature. Levetiracetam, however, was the most promising drug tested, was the only drug that showed strong effects on cognitive outcome in any model, and had no deleterious effects that generated negative points in any of the models. Levetiracetam is currently being evaluated in a micropig model within OBTT.

the sections below addressing (1) limitations of OBTT versus failure of reproducibility, (2) seeking a therapy that crosses models versus model specific therapy, and (3) translation of brain specific versus broader mechanism-targeting therapies.

Limitations of OBTT versus failure of reproducibility. One possible reason for the limited therapeutic success in OBTT is that there are many limitations to the approaches taken by OBTT. It is, thus, not OBTT's position to serve as a bully pulpit with regard to formulating impressions on the rigor of previous pre-clinical research or to forcefully shape the translational potential of the pre-clinical TBI arena and/or dismiss other results.

Some of the limitations of OBTT are obvious. OBTT does not study mechanism and thus did not demonstrate that the drugs and/or doses used affected the desired mechanistic target or targets. OBTT also screened only two doses of each therapy and performed studies in only a single injury level in each model. Although OBTT based its treatment protocols on the published literature as much as possible, given our goal of having a protocol that could have relevance to combat casualty care and be readily translated to a clinical trial, we often chose the intravenous rather than intraperitoneal route for drug administration. That likely changed drug kinetics in several cases and may have impacted efficacy and/or toxicity, as discussed later.

Dosing regimens different from those used thus might produce different results. This could be particularly true in OBTT given the fact that, depending on the drug, the optimal dose could vary across models. For example, this might in part be reflected in the highly variable effects of CsA in OBTT. CsA is a drug with a narrow therapeutic index that has complicated distribution and elimination kinetics that are altered after brain injury. It also has limited blood–brain barrier permeability, and brain levels that are achieved likely vary greatly with model severity—and could produce a spectrum of benefit versus harm.[4] Supporting this concept, levetiracetam—a drug with simple pharmacokinetics, excellent blood–brain barrier penetration, and a very large margin of safety—was the most successful.

Within OBTT for CsA, we observed some modest benefit in the mildest model (FPI) but substantial toxicity in the most severe insult (PBBI). To select the best possible doses, we have sought in many cases the specific input from our team members in the University of Pittsburgh School of Pharmacy (SMP and PEE), and in some cases have measured serum levels when there is limited and/or equivocal literature support. To optimize clinical translation of the effective therapies, future studies should aim at not only evaluating the dose mediating improved outcomes but also assess the concentration required in the brain to mediate that observed effect.

As mentioned previously, there is also another possible issue related to drug administration and dosing that could impact reproducibility of treatment effects when compared with the existing pre-clinical literature by OBTT. In general, OBTT sought to use the intravenous route for drug administration, given the likelihood of the need for that route in the setting of combat casualty care and/or severe TBI in the civilian setting. Often the majority of studies in the published literature in rodent models, however, involved intraperitoneal drug administration where systemic drug bioavailability may be variable or reduced. Injury can also influence drug metabolism, further complicating dosing.

Finally, as discussed previously, the injury levels in each of the models in OBTT did not always produce an optimal therapeutic

target for each therapy and a certain amount of ''wobble'' in the models was observed given the desire to produce behavioral deficits that were potentially manipulable by the treatments that were being screened. This may have underpowered some studies, particularly in FPI. Consistent with this hypothesis, the only model that produced a robust MWM latency target in every study was PBBI, yet that was the model that demonstrated the fewest therapeutic successes. This fact epitomizes the challenges faced when screening drugs across multiple models.

Thus, we appreciate the fact that despite the strong and carefully constructed methodologic rigor of OBTT on many fronts, pharmacologic rigor may have fallen short, particularly with respect to replicating specific previous literature reports. Other limitations inherent to the approach taken by OBTT are possible and some were discussed in the other articles in this special issue of the journal.

(1). Rigor and reproducibility. One alternative possible explanation for the performance of therapies in OBTT at a level below or well below what was expected based on the published literature is that the literature is inflated. Recently, inadequate rigor in pre-clinical research has been identified as an important potential contributor to the failure of clinical trials. This was brought to light in a landmark article by Begley and Ellis[21] and Begley and Ioannidis [22] that called into question pre-clinical research in the field of cancer therapy development when the Hematology and Oncology Department at the biotechnology firm Amgen tried to confirm published findings from 53 articles that were published in prestigious high impact basic science journals.

By the investigator's criteria, only 11% of the studies were able to be reproduced. This reproducibility assessment was performed because, mirroring TBI, randomized controlled clinical trials in the field of oncology were stated to have extremely high failure rates.

It is interesting that in several cases in OBTT, we based our selection on as many as 10–20 supportive published articles in pre-clinical TBI models, and in some cases we used the same or a similar dosing regimen suggested in some of the reports within a given body of work. This did not even take into account reports in other pre-clinical models such as stroke. Ironically, the most successful therapy in OBTT (levetiracetam) had only a single supportive paper in pre-clinical TBI at the time it was selected for screening, but it had other favorable characteristics including a purported brain-specific mechanism of action, a chemical structure with proven blood–brain barrier permeability with an ability to achieve therapeutically relevant concentrations in the brain, sporadic clinical use in TBI that was already ongoing, and a robust safety record supporting its selection. The rationale behind the selection of levetiracetam versus other potential therapies is discussed in greater detail later.

The role of the aforementioned failure of reproducibility on the findings of OBTT remains unclear, but reproducibility of published pre-clinical studies was not the stated goal of OBTT. Rather, we sought to use the published pre-clinical literature to aid in (1) selecting potential therapies, (2) formulating a pharmacological approach that was as clinically relevant as possible given the available literature, (3) screening those therapies at two doses across three very different rat models of TBI, and (4) seeking breakthrough effects in either one or multiple models across the consortium. This approach involved difficult compromises often related to issues relevant to pharmacology.

Another factor to consider is that unlike *in vitro* basic research, where it might be relatively straightforward to reproduce findings, pre-clinical TBI has always placed high value on behavioral outcomes and neuropathology at relatively long-term outcome time points. Clearly, reproducing those types of studies is demanding and expensive, and quite a high bar. This suggests that approaches such as OBTT (or others with a multicenter strategy with a high level of rigor) could be quite valuable to help direct the field toward the strongest possible candidates—using the established literature more as a repository of ''clues'' rather than as a stronger verdict on a given therapy.

Ramping up the rigor in all of our individual laboratories is also logical as suggested in recent publications in the fields of stroke, spinal cord injury, and central nervous system (CNS) injury[23–25] and in the recent article on common data elements in pre-clinical TBI.[26] Specific recommendations include carefully addressing issues such as group randomization, study blinding, sample size analysis, appropriate statistical approach, and transparency with regard to conflict of interest, among other issues, along with the important recommendation to reproduce the work before publication. With regard to increasing pharmacologic rigor, directly measuring dose-concentration relationships on mechanism and outcomes could be helpful.

Finally, in our current state of knowledge, it is very difficult if not impossible to define a treatment effect on either MWM or histology in a pre-clinical study that is linked to a known clinical outcome success.

(2) A therapy that crosses models versus model specific therapy. As discussed previously, one factor believed to be important to the failed translation of therapies from the pre-clinical to the clinical arena is the fact that clinical TBI is extremely heterogeneous. Thus, it is believed that the chances for successful translation might be increased if a drug was shown to have benefits when tested across multiple models of TBI. That possibility was one of the premises on which the design of OBTT was based.

To date, no therapy tested by OBTT has shown robust benefit across all three primary screening models—particularly on long-term cognitive outcome. To date, only levetiracetam has shown fairly robust benefit, but that is limited to two of the three models and does not include robust benefit on cognitive outcome in both models. An alternative conclusion of the findings of OBTT to date may thus be that a personalized or precision medicine (model specific) approach to the treatment of TBI is needed.

This is certainly a real possibility and might be even more likely than suspected from the data provided thus far in this issue given the fact that in OBTT, we limited our modeling to clinical analogs of severe TBI or possibly (given the injury level seen in parasagittal FPI) moderate or moderate to severe TBI. Broadening the clinical target to include the full spectrum of injury severity suggests that the need for a personalized medicine approach for clinical translation is even more likely. This would suggest that clinical investigators should consider testing therapies in patients that mimic the models that demonstrate the greatest beneficial effects of a given therapy. Those studies should also measure drug exposure and theranostic markers if available. OBTT can thus contribute special insight into such an approach given that the three models that are being used are quite different.

We did not, however, include a diffuse closed head injury model, a blast TBI model, or a TBI plus polytrauma model (among others) in our OBTT screening approach, and there is substantial evidence that various aspects of the secondary injury mechanisms involved in the exacerbation of damage are unique.[27–33] An even broader model representation for OBTT might thus be desirable for future

investigations. Given that screening of only five drugs is reported, however, and that only two additional drugs have completed studies (along with an eighth drug currently in testing), it would be, in our opinion, incorrect to come to premature closure on the ability to identify a highly robust therapy that crosses all three models.

It is also possible that for any of the drugs tested, the optimal dose to show benefit would differ between models tested in OBTT. For therapies targeting acute neuroprotection, however, clinical dosing has, to our knowledge, not been titrated to severity of injury in randomized controlled trials (RCTs)—so from a clinical translation standpoint, we believe that the goal of OBTT to demonstrate efficacy of a therapy across the three models (despite their great differences in severity) at a given dose is justified. Even in the meticulously executed recent trial of progesterone by Wright and colleagues[34] where patients with both moderate and severe TBI were randomized, for example, all patients received the same dose.

Clinical trials invariably test the therapy in an RCT at one or possibly two doses as used in our OBTT study design. Thus, in an attempt to maximize clinical translation, we may in fact have underestimated the potential efficacy of individual drugs in individual models using the screening strategy taken by our consortium. Nevertheless, we believe that this represents a strength rather than a liability for OBTT. If a therapy indeed is robust across all models at the same dose, it would greatly strengthen the chances of successful clinical translation.

Thus, there are both strengths and weaknesses to the approach used by OBTT. We remain optimistic that a more potent and robust therapy that crosses models will be identified by our approach.

(3) Brain specific versus broader mechanism-targeting therapies. Another intriguing finding based on the results of the first five therapies screened by OBTT is the fact that the drug that has demonstrated the most benefit (levetiracetam) is the only one that was drug specifically designed/developed to treat a pathophysiological process in the brain—namely, seizures. Given the fact that there is an empiric use of a number of therapies in neurocritical care in the treatment of patients with severe TBI—such as anticonvulsants, analgesics, and sedatives, and hyperosmolar agents, among others—there has, in general, been a focus in the pre-clinical literature on unique therapies that target broader secondary injury mechanisms (that operate both within and outside of the CNS) such as apoptosis, mitochondrial failure, oxidative stress, proteolysis, autophagy, and/or other pathways.

The findings of OBTT presented, however, suggest that such an approach, although tantalizing for identifying a unique breakthrough therapy, may actually have a lower chance of success than exploring more highly brain specific mechanisms. Bullock and associates[35] and Tolias and Bullock[36] have long suggested that a major limitation of TBI research in clinical translation has been in the area of the clinical assessment of brain pharmacokinetics and pharmacodynamics of therapies. Issues such as robust blood–brain barrier permeability and lack of neurotoxicity, for example, may be paramount to success and dwarf other mechanistic factors—which are often highlighted in pre-clinical reports.

There are many brain specific targets in the secondary injury cascade such as excitotoxicity, spreading depression, axonal injury, glial alterations, and loss of trophic support, and these may represent important targets using drugs specifically designed as CNS targeting therapies. One could argue, alternatively, that demonstrating benefit from a drug such as levetiracetam by OBTT has limited value. Rats, unlike patients, do not routinely receive anticonvulsants in the acute phase after severe TBI, and thus it might remain difficult to demonstrate a clinical benefit of levetiracetam in an RCT. Studies by Darrah and coworkers[37] suggest, however, that unlike levetiracetam, phenytoin demonstrates deleterious effects in the CCI model in rats, and given the fact that both phenytoin and levetiracetam are used clinically, an advantage might be able to be shown in a clinical RCT.

In addition, one of the most interesting findings in our studies with levetiracetam is that it improved multiple long-term outcomes despite the fact that it was administered as a single bolus at 15 min after injury. As discussed earlier in this special issue,[6] that may suggest beneficial effects on mechanisms other than seizures. In any case, we believe that the findings to date in OBTT suggest that additional drugs that were specifically designed for use in the CNS should be explored.

Current Investigations

Investigations are ongoing in OBTT. Given the success of levetiracetam, it has been advanced to testing in a gyrencephalic animal model—namely, FPI in micropigs. It is noteworthy that the outcomes in that model include assessments of axonal injury and cerebrovascular responsivity, along with serum and tissue biomarkers (GFAP, UCH-L1, and ionized calcium-binding adapter molecule 1 [IBA-1]). This will allow both a direct comparison of rodent and large animal response to a promising therapy, but it will also provide some unique therapeutic targets that are not part of therapeutic screening in the rodent models in OBTT. This cross-species investigation within OBTT is exciting.

With regard to therapeutic screening in the rat model, currently studies have been completed and data analysis ongoing on two additional drugs—namely, glyburide and Kollidon VA64. In addition, with research support provided by the U.S. Department of Defense based on performance of the OBTT consortium and on the desire to test additional therapies that may be somewhat earlier in development and/or proprietary, a grant titled OBTT-Extended Studies (OBTT-ES) is currently supporting assessment by our consortium of the aquaporin 4 antagonist (AER-271); the OBTT-ES program just launched.

In addition, exploratory dosing studies and protocol planning are under way for minocycline and amantadine, which will likely be tested by this year by OBTT, along with other agents. These agents are specifically targeting mechanisms such as cerebral edema,[38] neuroinflammation,[39,40] and cognitive enhancement,[41,42] which have not been explored to a significant extent thus far by our consortium and are logical candidate mechanisms. We are particularly interested in testing amantadine given the fact that it has shown success in a RCT in the setting of severe TBI in humans[43]—and that work was based on the seminal study by Dixon and colleagues[41] in the CCI model using that therapy.

Future Directions

Therapy

One of the key questions in the search for new therapies for TBI that future studies by OBTT and/or other similar initiatives should address is whether the most fruitful path lies in using therapies to prevent the evolution of secondary damage or manipulate the remaining circuitry.[44] It is certainly logical to pursue both strategies, with the ultimate goal of new therapy development on both fronts. Nevertheless, it is not clear which approach is most likely to lead to major improvements in outcome.

Another question that is often raised at presentations of the work of OBTT is whether or not the consortium is considering

combination therapy. It is likely that combination therapy will ultimately be needed to maximize outcomes in pre-clinical models and patients with severe TBI; however, we believe that we are at an early point in the evolution of the consortium approach to pre-clinical therapy development and have sought to first carefully evaluate individual therapies, generating a body of individual comparisons of therapies that can—at the least—serve as a future road map. Indeed, key basic elements such as drug levels and dose response deserve to be more carefully and thoroughly evaluated, even in studies of individual therapies.

Thus far in OBTT, we have focused on assessment of drugs, but we recognize that approaches such as cellular therapies[45,46] or other nonpharmacological therapies[47] should be considered. Issues such as prevention or resilience are also of potential importance particularly when considering military relevance, and thus a pre-treatment approach might also be worthy of consideration for certain therapies. Given the goal of identifying a robust therapy for a RCT in the setting of severe TBI in civilians (which would be necessary for ultimate translation), however, we have logically focused on post-TBI drug administration.

Finally, we have focused most of our efforts on acute administration of therapies, given the premise that delay in the onset of treatment for most mechanisms reduces therapeutic efficacy. Given the role of mechanisms such as subacute neuroinflammatory cascades,[48] however, additional consideration might be given to the prolonged administration of a given therapy. Thus far, prolonged therapy was tested for only one agent by OBTT—namely, simvastatin. Unfortunately, we did not see robust benefit in any of the models using simvastatin despite prolonged treatment. Other agents potentially targeting neuroinflammation and/or neurodegeneration related pathways might be more successful and deserve exploration.

Biomarkers

(1) Use and optimization of current biomarkers. As discussed previously, using a protocol that included blood sampling at 4 h, 24 h, and 21 days after TBI, GFAP outperformed UCH-L1 both as a diagnostic and theranostic in the initial five therapeutic screening studies in OBTT. Given its short half-life,[13] it is possible that the performance of UCH-L1 could be improved with sampling earlier after TBI. We have recently added a 1 h sampling point to the protocol and are currently reexamining how UCH-L1 performs across our models. In addition, assays for GFAP and UCH-L1 are currently in development, and testing for work in the micropig model and serial blood sampling is being performed in that model along with an assessment of correlation with brain tissue levels of both markers as assessed by immunohistochemistry.

(2) Additional biomarkers in development. Using an assay developed at the University of Florida, we are beginning to explore the potential utility of serum levels of IBA-1 as a TBI biomarker. These studies have been initiated in the micropig model, and if successful, there is a plan to add this biomarker to the rat panel. This is a very logical biomarker to pursue given the robust and sustained microglial response seen across our models after TBI including both rat and micropig. Several other circulating biomarkers are being considered for assessment.

Modeling

Given the fact that we have shown feasibility of the OBTT consortium concept, a number of potential avenues for expansion and/or modification can be raised. With the emergence of the importance of mild TBI and mild repetitive TBI, inclusion of a representative model of these insults would seem to be an additional and valuable opportunity. This was not considered to be feasible when OBTT was planned and launched, because at that time, there were few established pre-clinical models of mild TBI. One concern with regard to mild TBI and the OBTT concept is the fact that although new models are emerging, only a limited number of therapies have been tested in pre-clinical models of mild TBI; thus the basis for therapeutic testing would likely still rest on experience in pre-clinical models of severe TBI.

OBTT includes an important model of TBI that is highly relevant to combat casualty care—namely, PBBI. In future work, however, some consideration might be given to inclusion of a blast TBI model, where some therapy screening has been performed.[49] At the least, the most promising agents identified by our screening approach taken in OBTT—in our opinion—should be tested in blast TBI models using the treatment protocols identified as successful by our consortium.

A progressive encephalopathic process (characterized by progressive tissue loss and prolonged behavioral deficits) over as long as 1 year has been demonstrated in pre-clinical models of TBI,[50–52] including studies in some of the specific models in use in OBTT. Consideration thus might be warranted for the use of an OBTT-like approach to the assessment of the impact of acute and/or chronic treatment on longer-term outcomes. The emerging importance of the link between TBI and various neurodegenerative diseases suggests that such an approach could be quite important. Given the limited experience with this approach even within individual laboratories, the labor intensive nature of these studies, and their high cost, however, careful planning would be essential. In that regard, the lessons learned from the past and ongoing OBTT studies by OBTT would be important to guiding that work.

Conclusions

OBTT is an established, fully operational, rigorous, and highly productive multicenter, pre-clinical drug and circulating biomarker screening consortium. Based on the results generated from the first five therapies evaluated, within the exacting approach used by OBTT, four (nicotinamide, erythropoietin, cyclosporine A, and simvastatin) of the five therapies performed below or well below what was expected based on the published literature. OBTT, however, has identified the early post-TBI administration of levetiracetam as a promising agent and has advanced it to a FPI model in micropigs. Two additional therapies (the sixth and seventh) have just completed testing (glibenclamide and Kollidon VA 64) with results on those agents beginning to emerge, and an eighth drug (AER 271) is currently in testing.

Incorporation of circulating biomarker assessments into these pre-clinical studies has suggested potential for diagnostic and theranostic utility of GFAP—which could potentially simplify and/or aid in initial screening of TBI therapies in pre-clinical models. Additional validation of the use of GFAP as a theranostic tool in pre-clinical work is needed, however, both in future studies in OBTT and outside of the OBTT consortium. Given the concerns related to what has been described as a reproducibility crisis in basic and pre-clinical science across disciplines, and the many failures in clinical translation of therapies specifically in TBI, rigorous multicenter pre-clinical approaches to therapeutic screening as carried out by OBTT may be important for the ultimate translation of therapies to the human condition.

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Author Disclosure Statement

Drs. Hayes and Mr. Richieri own stock and are both officers of Banyan Biomarkers Inc. Drs. Hayes and Catania, Mr. Richieri, and Ms. Glushakova are employees and receive salaries and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and, as such, all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, No competing financial interests exist

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