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Combination Therapies for Traumatic Brain Injury: Retrospective Considerations

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Abstract

Patients enrolled in clinical trials for traumatic brain injury (TBI) may present with heterogeneous features over a range of injury severity, such as diffuse axonal injury, ischemia, edema, hemorrhage, oxidative damage, mitochondrial and metabolic dysfunction, excitotoxicity, inflammation, and other pathophysiological processes. To determine whether combination therapies might be more effective than monotherapy at attenuating moderate TBI or promoting recovery, the National Institutes of Health funded six preclinical studies in adult and immature male rats to evaluate promising acute treatments alone and in combination. Each of the studies had a solid rationale for its approach based on previous research, but only one reported significant improvements in long-term outcomes across a battery of behavioral tests. Four studies had equivocal results because of a lack of sensitivity of the outcome assessments. One study demonstrated worse results with the combination in comparison with monotherapies. While specific research findings are reported elsewhere, this article provides an overview of the study designs, insights, and recommendations for future research aimed at therapy development for TBI.

Key words: pharmacological interventions; preclinical therapeutic development; treatments

Introduction

THE LACK OF PROVEN EFFECTIVE THERAPIES for traumatic brain injury (TBI) is both a huge unmet global health need and a challenging endeavor. The challenge associated with the development of therapies for TBI is related to the range of injury severity, the complexity of approaching a disease that affects multiple types of tissues and cells, and the rapid onset of pathophysiology[1,2] and typical presentation with co-morbidities and other environmental and developmental factors.

All of the previous phase III clinical trials for TBI have failed to demonstrate greater effectiveness at improving outcomes of monotherapies for TBI.[3] Most recently, two multicenter phase III clinical trials for progesterone (PROTECT III and SYNAPSE) were halted for lack of significant treatment effect, despite compelling preliminary data in humans and rodents, and efforts to treat rapidly and include moderate as well as severe brain injury.[4,5] Intuitively, a combination of treatments seems more likely to be effective than a monotherapy; however, testing and validating multiple treatments presents additional challenges.

To explore these challenges, as well as opportunities for combination therapies, the National Institute of Neurological Disorders and Stroke (NINDS), with support from the National Institute of Child Health and Development (NICHD), the National Heart,

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Lung, and Blood Institute, and the Department of Veterans Affairs, convened a workshop in February 2008. The major recommendations were to: (1) select therapies that in combination target multiple, complementary mechanisms rather than hitting a single target at multiple times; (2) set high standards and rigorous tests for comprehensively and systematically evaluating the effects of the treatments on brain injury and repair processes for both pre-clinical and clinical studies; (3) use standard protocols and data elements to enable data sharing and meta-analysis across studies; (4) design efficient studies—e.g., include multiple treatment arms and/or interim analyses to test for futility; (5) be aware of the Food and Drug Administration regulations to ensure that the study design meets their requirements.[6]

In April 2008, NICHD and NINDS published a funding opportunity based on the workshop recommendations that was entitled ''Multi-drug combinations to promote neurological recovery in traumatic brain injury.'' Six pre-clinical studies were funded—three that aimed to develop treatments for adult TBI and three for pediatric TBI (Table 1)—all using male rats as subjects, and all with a focus on TBI associated with overt neuropathology, but requiring no medical intervention for survival. Clinically, these injuries would be classified as ''moderate severity,'' while acknowledging that the terms mild, moderate, and severe TBI are being challenged as insufficient to represent the heterogeneity of TBI.[7]

To learn from the experiences of the six studies, a symposium ''Preclinical TBI Trials for Combination Therapies'' was held as part of the National Neurotrauma Society Meeting in 2013. Each of the funded principal investigators was asked to describe their institution's scientific rationale for combining therapies and their selection, how they designed and modified their study to address anticipated and unanticipated challenges, and to provide recommendations regarding approaches for future TBI therapeutic trials.

This article is not intended as a full report of their research experiments, and some of the studies have been published separately with more detailed information about the methods, results, and interpretation of their studies. Given the challenge of publishing negative or equivocal results, however, not all of the studies were published. The goal of this article is to share their collective experiences and insights from all of their combination therapy preclinical studies.

Concurrent pleiotropic therapy combination: progesterone and vitamin D hormone

Rationale. TBI is heterogeneous, triggering multiple response cascades, and depending on the severity of the injury and other factors, can result in long-term neurodegenerative processes that last for months or even years.[8,9] To address the heterogeneity of the pathophysiology, we used a concurrent pleiotropic approach by simultaneously using two broad-spectrum agents to stem the unfolding cascade of destructive events and provide trophic support for neuroregeneration. Progesterone (PROG) was selected as one of the combination therapy agents because, at the onset of the study, it was well established as a neuroprotective and pleiotropic hormone treatment in 22 different models of injury using four different animal species[10]; however, more recent PROG phase III clinical trials failed to show benefit of the monotherapy for TBI.[4,5] Vitamin D hormone (VDH) is a neuroprotective secosteroid hormone that has also been shown to be neuroprotective in a variety of injury models.[11]

The simultaneous combination of VDH with PROG was attractive for their redundant and complementary actions and for their synergy. First, like PROG, VDH is pleiotropic and not only shares some mechanisms of neuroprotection with PROG, but also acts on different and protective signaling pathways that are missing in the PROG signaling cascade.[12] Second, VDH deficiency/insufficiency is a serious disorder that can impair several physiological processes associated with healthy CNS functions.[11] Third, we have shown

Table 1. Summary Table of Effectiveness of Combination Therapies

Therapies	*Target*	*Developmental stage**	*Injury model*	*Behavioral tests***	*Timing post-TBI*	*Effect versus mono-therapy*
PROG & VDH	Multiple	Adult	CCI	*MWM* Adhesive removal test Open field activity	20 days	No SD
GPE & Minocycline	Multiple	Immature	Closed head impact injury	Not evaluated	No data	No behavioral data
SiAQP4 & D-JNKI-1	AQP4 JNK	Immature	CCI	*MWM* Foot fault test Beam balance Rotarod Open field activity Zero maze	2 months	No SD
PROB & NAC	GSH	Immature	CCI	*MWM* Beam balance Inclined plane	14 days	Modest increase
Creatine & choline	Multiple	Adult	CCI	*MWM*	14 days	Decreased
NAM & PROG	Multiple	Adult	CCI	*Adhesive removal test* *Placing test* *Forelimb asymmetry test*	28 days	Increased

TBI, traumatic brain injury; PROG, progesterone; VDH, vitamin D hormone; SD, significant differences compared to monotherapy; CCI, controlled cortical impact; MWM, Morris water maze; siAQP4, siRNA aquaporin-4; D-JNKI-1, c-Jun N-terminal kinase-1 inhibitor; PROB, probenecid; NAC, N-acetylcysteine; GSH, glutathione; NAM, nicotinamide; PROG, progesterone.

*Male rats were used for all of the studies.

**Behavioral tests in italic are the ones selected as primary outcomes to evaluate effectiveness.

that VDH deficiency can reduce the beneficial effects of PROG treatment after TBI, especially in older subjects.[11]

Approximately 30–35% of the general American population has VDH deficiency,[13] and we designed our study to investigate this potential co-morbidity. We hypothesize that VDH supplementation could improve the recovery process and enhance the neuroprotective effects of PROG, with and without VDH deficiency before TBI.

Experimental design and findings. We investigated whether combination treatment with PROG + VDH beginning 1 h after focal cortical contusion would enhance the efficacy of PROG alone on functional recovery (Morris water maze [MWM], somatosensory neglect, open field activity), and neuropathology after TBI in young adult male VDH-sufficient rats. Functional testing was performed on multiple days, up to 21 days post-TBI. In addition, we investigated whether the neuroprotective effect of PROG is diminished in VDH-deficient senescent animals.

Our results indicate first that in young adult VDH-sufficient rats, low-dose administration of VDH (1 μg/kg) is optimal for combination therapy with PROG and shows better functional recovery than PROG alone.[14] Second, we found that systemic VDH deficiency increases baseline brain inflammation in uninjured animals and also reduces benefits of PROG treatment after TBI in aged animals. Moreover, a single bolus dose of VDH (5 μg/kg) combined with PROG can suppress post-TBI inflammation and attenuate early behavioral deficits better than PROG monotherapy.[11] Taken together, our findings demonstrate that combination treatment with PROG + VDH produces better functional outcomes than PROG alone.

In support of PROG + VDH as a simultaneous pleiotropic combination therapy for patients with severe TBI, a recent Iranian phase I/II clinical trial showed that patients who received PROG + VDH had a significantly higher recovery rate than PROG alone compared with patients given standard of care plus placebo.[15] In addition to its TBI therapeutic potential, PROG + VDH treatment for ischemic stroke also results in better functional recovery and smaller infarction volume than PROG alone.[16]

Challenges. We found that producing long-term improvements with a combination therapy was more complex than we had anticipated. Unexpectedly, we observed first that VDH deficiency alone for 3 weeks in middle-aged male rats did not significantly reduce baseline behavioral functions or aggravate impaired cognitive outcomes during long-term functional assessment.[17] We speculate that a much longer period of chronic VDH deficiency in older animals might be needed to produce significant impairments in the recovery process as measured over long-term assessment and that species differences could also play a role.

Second, unlike our previous study with multiple treatments over 8 days,[14] a single dose of VDH (5 μg/kg) combined with PROG was insufficient to enhance PROG's efficacy during long-term functional assessment. Importantly, we concluded that for VDH supplementation, the severity of the VDH deficiency, dosing, duration of treatment, and appropriate drug/dose combinations may not be linear, and this idea will require a more thorough study.[17] Extrapolating these pre-clinical data to patients will clearly be more complex because dietary factors, combined morbidities, sex differences, exercise, and climate, among others, may have to be taken into consideration in the planning of clinical trials with agents whose mechanistic and functional actions could be affected by any of these factors.

Another critical challenge in the face of the negative TBI clinical trial results with PROG,[4,5] despite the compelling pre-clinical data supporting PROG use at lower doses,[18–21] is that it may be more difficult to find support to test whether other doses of PROG might have been more effective[18,19,22] or to evaluate combination therapies using PROG that might provide better salutary effects over PROG monotherapy.

Recommendations. Drug interactions are not always linear; thus, a ''combination dose-duration-response'' efficacy should be optimized for the combination of the agents rather than selecting and combining the most effective monotherapy doses of each agent. Second, because our study was limited to males, future pre-clinical studies of PROG + VDH treatment should be conducted in older female animals (pre- and post-cessation of normal cycles) to determine whether the interactions between sex and age are important in outcomes.

Third, female animals may be more or less sensitive to the PROG/VDH treatment at different stages of their estrous cycle than male animals. Accordingly, a separate combination evaluating dose-duration-response efficacy needs to be optimized in female animals rather than testing the best combination dose selected from male animals. Also, females may need a longer/shorter period of VDH deficiency than male animals to show long-term functional deficits. Finally, for clinical testing we recommend a type of ''informed combination therapy'' approach for VDH and PROG whereby the dose of VDH is tailored to degree of deficiency as determined by a simple serum assay in patients with TBI.

Concurrent targeted therapy combination: AQP4 and JNK inhibition

Rationale. Using commercially available agents with demonstrated safety in humans, we silenced expression of the water channel aquaporin-4 AQP4 water channel by siRNA (siAQP4)[23] and inhibited MAP-Kinase c-Jun N-terminal kinase (JNK) with the application of D-JNKI-1[24] to attenuate edema and excitotoxicity, two major components of the secondary injury cascade in pediatric TBI.[25–32]

Because previous studies have demonstrated that intracortical injection of siAQP4 specifically decreases AQP4 expression and water movement in the brain[23] and siRNA treatment has already been tested in Phase I clinical trials for peripheral tumors (for references, see review [33]), we included siAQP4 in our candidate combination therapy study. To inhibit JNK, we used D-JNKI-1, which is a protease-resistant competitive JNK-inhibiting peptide[34] that strongly inhibits all three JNK isoforms,[24,35] and systemic intravenous and intraperitoneal (IP) administrations of D-JNKI-1 provides neuroprotection in several stroke models, including in juvenile animals.[24,35,36]

Experimental design and findings. Behavioral, imaging, and histological outcomes after administration of siAQP4 or D-JNKI-1 after focal TBI were compared with those for the combination therapy. Within 10 min after unilateral controlled cortical impact (CCI), immature (P17) male rats were injected intracortically with siAQP4 adjacent to the site of the impact.[37,38] A second siRNA injection was repeated 2 days later, to maintain the decrease of AQP4 expression.[23] Based on previous monotherapy studies with D-JNKI-1 in juvenile animals,[35] 11 mg/kg was administered IP at 3 h post-injury.[39] For the combination treatment, siAQP4 and D-JNKI-1 were administrated with the same route and doses applied for the monotherapy arms of the study.

A unique longitudinal combination of vertically integrated outcomes (from behavioral end-points to cellular responses) was used to evaluate the benefits of the single and combination treatments at several time points: a battery of behavioral tests, neuroimaging (magnetic resonance imaging [MRI] with T2-weighted imaging and diffusion imaging (for details, see [37,40]), and histology analysis. At 1, 3, and 7 days post-injury, foot-fault, beam balance, and rotarod treadmill tests were performed to evaluate motor functions, as well as MRI to evaluate edema.[38] At 1 and 2 months post-TBI, MRIs were repeated, and open field, zero maze, and water maze tests were added to the test battery to evaluate, respectively, general motor activity, anxiety, and spatial learning and memory. Animals were sacrificed at either 3 days (at the peak of the edema formation) or 2 months after TBI to perform classical immunohistochemistry for neuronal cell death, astrogliosis, and microglia.[37,40]

Analysis of the behavioral tests to assess the benefits of the combination treatment has been completed, and the histology and edema analysis is in progress. Individual administration of siAQP4 or D-JNKI-1 improved motor and cognitive performance, and decreased edema and neuronal cell death in comparison with untreated animals.[37,39] The combination of siAQP4 and D-JNKI-1 improved spatial memory, compared with nontreated juvenile TBI rats at 2 months post-injury. Improvement plateaued, however, such that the combination treatment had no improved benefit over monotherapies and was statistically indistinguishable from siAQP4 or D-JNKI-1 alone for the behavioral outcomes (unpublished results).

Challenges. The absence of synergistic effects of the combination treatment with siAQP4 and D-JNKI-1 could be because of several dynamics. First, because the battery of tests was repeated with a 1-month interval, practice effects may have contributed to the overall improved performance between 1 and 2 months after TBI,[41] minimizing the opportunity to observe a therapeutic benefit from any treatment. The combination of the training and monotherapy efficacy may have created a ceiling effect, limiting our ability to discriminate improvement with the combined therapy. A second challenge was the siRNA injection to the cortex, which may have created a local injury,[34] as well as the potential for off-target gene silencing with deleterious consequences[34] that overshadowed the added therapeutic benefits of D-JNKI-1 in the combination treatment.

A third challenge to this approach was the potential contribution of competitive inhibition between siAQP4 and D-JNKI-1. In another injury model (intracranial hemorrhage induction), investigators observed increased expression of AQP4 around the lesion 2 days after its use in combination with D-JNK-1.[28] We postulate that D-JNKI-1 treatment may have stimulated AQP4 expression and overwhelmed the effects of siAQP4 to inhibit expression.

The behavioral tests have been evaluated to assess the functional benefits of the combination treatment. The MRI and histology analysis in progress may help us to understand the difficulties encountered in this new multi-drug treatment.

Recommendations. First, we recommend a behavior and cognition test battery that includes both repeated tests and new or more challenging tests at later time points to increase the sensitivity in discrimination between the experimental groups. Second, we recommend avoiding siRNA injection via an intracortical route because of its limited clinical relevance and potential to cause additional injury. Instead, we suggest future studies focus on the intranasal route of drug delivery, already a promising, efficient route to target the CNS.[34,37]

Third, to minimize potential competitive effects between the siAQP4 and D-JNKI-1, the timing of the treatments should be revised. Indeed, timing is critical, because there is evidence to suggest that induction of AQP4 expression at 3 days post-TBI may actually be beneficial in the resolution of the edema.[37] Finally, we recommend using multiple outcomes to assess treatment effectiveness, using a hierarchical scale approach, from the cellular and molecular level to behavior at the organismal level, and that acute and long-term (from 2–6 months post-injury) treatment benefits should be evaluated. Because multi behavioral tests may also be providing exercise and enriched environment, which could have possibly enhanced the treatment response, it is important to include injured vehicle controls for comparison.

Concurrent targeted therapy combination: glypromate and minocycline

Rationale. Because clinical and animal studies have demonstrated that the pathogenic mechanisms in the acute and chronic post-traumatic periods are different between the immature and adult brain,[42–48] we selected two successful interventions that target separate pathologies (i.e., neurodegeneration and axonal injury) in the adult brain to test their effectiveness in the immature brain as monotherapies and in combination. Using our clinically relevant closed head impact injury model in the immature rat that results in acute and chronic cognitive deficits, neuronal and axonal degeneration, caspase-3 activation, and glial reactivity in multiple brain regions,[49,50] the chosen reagents were the antibiotic minocycline and glypromate (GPE), the N-terminal tripeptide of insulin-like growth factor-1.

As a monotherapy for TBI, minocycline has anti-inflammatory and anti-apoptotic properties and has been tested in adult models of TBI and neonatal models of hypoxic-ischemia with some success.[51–53] Similarly, systemic or intracerebroventricular administration of GPE was neuroprotective as a monotherapy when administered after hypoxic-ischemic injury in adult and pediatric rats.[54,55]

GPE enters the brain when the blood–brain barrier (BBB) is compromised, targeting it to sites of injury[54,56] and preferentially binds to glial cells after hypoxia-ischemia[55]; neuroprotection after ischemia was associated with suppression of microglial proliferation and attenuation of caspase-3 dependent neuronal apoptosis.[54] An analog of GPE, NNZ-2566, reduced microglial activity, proinflammatory cytokine expression, and apoptosis while attenuating motor behavior deficits after adult TBI in rats.[57,58] We hypothesized that administration of GPE would be neuroprotective by attenuating microglial reactivity and neuronal apoptosis after diffuse brain trauma to the immature rat.

Experimental design and findings. First we measured monotherapy dose effectiveness in the immature brain, guided by adult and pediatric dosing levels. Using our closed head impact injury model of diffuse brain trauma in the immature rat,[49] structural deficits were observed in the white matter tracts where intra-axonal dephosphorylation of neurofilament and intra-axonal accumulation of APP (amyloid precursor protein, a marker of impaired axonal transport) led to axonal degeneration. This was accompanied by functional deficits in the white matter tracts with compound action potential (CAP) deficits of both myelinated and unmyelinated axons.[59]

To confirm efficacy as a monotherapy in the immature brain, immediately after injury, minocycline was administered as a

monotherapy at 45 mg/kg/injection IP every 12 h for 3 days, based on adult and neonate CNS injury models.[60,61] Minocycline monotherapy reduced impaired axonal transport at 3 days post-injury, which was associated with reduced microglial reactivity and axonal degeneration. While reduced microglial reactivity persisted up to 7 days post-injury, there was no further reduction of axonal degeneration (unpublished observations).

Immediately after injury, we administered continuous subcutaneous (SC) infusion (osmotic mini-pump) of GPE as a monotherapy at doses of 6 or 12 mg/kg/day for 3 or 7 days post-injury, using the dosing paradigm based on adult and neonatal animal models of hypoxic-ischemia and the short half-life of GPE.[54,55] Post-traumatic administration of GPE at 6 mg/kg/day for 7 days, but not for 3 days, attenuated the extent of microglial reactivity that was associated with a reduction in caspase-3 activation and neurodegeneration in the gray matter region (cortex, hippocampus, and thalamus) but had no effect on the white matter tracts. Using the higher dose of 12 mg/kg/day of GPE as a monotherapy produced a similar beneficial effect on the gray matter region by 3 days post-injury but no effect in the white matter tracts (unpublished observations).

Further studies need to address whether higher doses and longer duration of administration may be associated with white matter benefits. Functional assessments (CAP and spatial learning and memory) with GPE as a monotherapy are currently being performed.

In a combination therapy, we administered GPE (12 mg/kg/day for 3 days) and minocycline (for 3 days) to the brain-injured neonate animal. Our preliminary observations indicate that the combination synergistically decreased microglial reactivity and impaired axonal transport, which were associated with decreasing axonal and neuronal degeneration and caspase-3 activation in the white matter tracts and in the thalamus. Although the combination of drugs synergistically decreased microglial reactivity and caspase-3 activation in the cortex, neurodegeneration was not affected (unpublished observations). Collectively, these data suggest that while combination therapy may have an overall benefit in limiting the acute inflammatory and apoptotic response, there may be regional selective effects that could impact reversal of functional deficits in the traumatically injured pediatric brain.

Challenges. Our major challenge was that our original proposed combination therapy included FK506, rather than minocycline. We had based our study design on previous *in vitro* and *in vivo* preclinical studies in adults.[62–65] In our initial age-appropriate monotherapy conformational studies with FK506, however, we found that when FK506 was administered IP as a monotherapy immediately and 6 h after TBI at doses of 10 or 25 mg/kg, the signature dephosphorylation of neurofilaments and axonal degeneration[66] was significantly reduced, but benefits were achieved only at doses that were 2–5 times greater than in the adult[62–64,67]; there was no amelioration of impaired axonal transport or CAP deficits.[59]

Ultimately, because FK506 failed to improve functional outcome associated with persistent CAP deficits in the white matter tracts after diffuse trauma to the immature brain, we abandoned the idea of using this medication for our combination therapy; instead, we evaluated minocycline and GPE. Based on this combination therapy challenge, we concluded that treatment strategies that reduce white matter damage in the traumatized adult brain might not always be effective in the traumatized pediatric brain because the mechanisms of damage may be different. Further, we emphasize the importance of age-appropriate dosing.

Recommendations. Our data reveal important age-at-injury and region-dependent responses, which should be considered in future pre-clinical trial designs. We recommend first that before large-scale pre-clinical trials, pilot efficacy data should be obtained from age-appropriate cohorts. Second, we tested therapeutic effectiveness with therapy administration immediately after injury. For future studies, we recommend testing a delayed administration paradigm, because this would be more clinically practical. Third, different doses of combination therapy should also be studied; the optimal dose for monotherapy may not be the best dose for combination therapy because of drug interactions. Further, while multiple outcomes may be evaluated, we believe that assessing functional or behavioral outcome is most important during the acute and chronic post-traumatic period.

Sequential pleiotropic therapy combination: pre-injury creatine and post-injury choline

Rationale. We hypothesized that by using the sequential combination of two agents with proven benefits for TBI as monotherapies, pre-injury administration of creatine and post-injury choline supplementation would provide enhanced benefit compared with either agent alone. Creatine is a guanidine compound that helps maintain adenosine triphosphate (ATP) levels in tissues experiencing a high level of energy fluctuation and demand, such as skeletal muscle, the heart, and the brain. Although a small amount of *de novo* synthesis of creatine occurs in the human liver and pancreas, diets high in fish and meat products provide a more substantial quantity of creatine.[68,69]

Previous studies have shown that creatine, phosphocreatine, and the enzyme creatine kinase comprise a cellular alternative energy network that can be used by cells in times of high-energy demands, such as after brain injury. Impairments in mitochondrial ATP biosynthesis seen after TBI can be partially offset by providing a diet high in creatine as a pre-treatment.[68,70] Indeed, dietary creatine supplementation as a mono-agent pre-injury treatment has been shown to afford significant neuroprotection in animal models of TBI[71–73] as well as other conditions such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and cerebral ischemia. Increased phosphocreatine availability and ATP synthesis, in conjunction with reduced mitochondrial impairment and improved calcium dynamics, are thought to contribute to the neuroprotective actions of creatine observed in these models.

Choline is a multifunctional molecule that serves several important physiological functions including serving as a precursor for phospholipid and other signaling molecule biosynthesis, a source of methyl groups used in methionine and protein synthesis, a precursor for acetylcholine (ACh) synthesis, as well as a direct acting agonist at alpha 7 neuronal nicotinic receptors. Although the human body synthesizes some choline *de novo*, dietary sources are more common, and include, eggs, nuts, liver, and other meats. In 1998, choline was classified by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Sciences as an essential nutrient.[74]

Choline as well as its precursor, cytidine 5′-diphosphocholine have been investigated as potential therapeutic compounds for treatment of patients with diseases that involve significant membrane perturbations (cerebrovascular disease, TBI) as well as disorders where a loss of acetylcholine biosynthesis is thought to contribute to pathology (Alzheimer's disease). In a rat model of focal TBI (CCI), we showed that dietary supplementation with 2% choline before and after experimental TBI results in significant

behavioral improvement (Morris Water task), decreased cortical tissue damage, and less brain inflammation.[75] In our Sequential Combination Therapy study, we combined dietary creatine pre-treatment before injury with dietary choline supplementation after injury, based on the rationale of possible additive effects because both agents have pleiotropic mechanisms of action and were demonstrated to be efficacious as monotherapies. Results of the combination therapy were compared with monotherapy treatment with each supplement alone.

Experimental design and findings. In a two-by-two design, we compared monotherapy using two agents with proven benefits for TBI with a combined therapy administered in a sequential combination approach. Specifically, we evaluated administration of creatine supplementation for 14 days before injury, choline supplementation for 14 days after TBI, and their combination, and compared them with untreated injured adult rats (60 days old at start of study). Details are provided elsewhere.[75]

Pre-injury rats were fed either a standard diet containing ''sufficient'' choline (0.2% choline; TD 03118; Harlan Teklad, Madison, WI) or the identical diet, supplemented with 1% creatine (TD 04250; Harlan Teklad). Diets were matched with respect to all of the nutritional ingredients, besides choline and creatine. Post-injury or surgery (sham), animals were maintained on either a standard diet (no creatine, 0.2% choline) or a choline-supplemented diet (no creatine, 2.0% choline).

Spatial memory was assessed using a standard MWM paradigm between the 8th and 14th day post-TBI, and analyzed using a three-way (diet, surgery, and day of training) repeated measure analysis of variance. After completion of the MWM testing (14 days after CCI surgery), animals were euthanized, and brain inflammation was evaluated using [^{3}H]-PK11195 autoradiography to identify the translocator protein 18kDA (TSPO), which is thought to be located on the outer mitochondrial membrane of activated microglia. A cortical tissue sparing analysis was also performed as described previously.[75]

Results comparing monotherapies and combination therapies varied by assessment metric (e.g., behavior, inflammation, tissue sparing), but no metric demonstrated superior performance of the combination therapy. In fact, counter to our predictions, animals supplemented with creatine before the injury and 2% choline after injury performed the worst overall. Only the group fed standard diet before injury and 2% choline supplementation after CCI (i.e., choline monotherapy) resulted in significant improvement in the acquisition phase of the MWM test.

Creatine pre-treatment did not result in any significant functional improvement compared with animals given the standard diet before and after injury. Animals that started 2% choline supplementation after injury also had significantly reduced brain inflammation in the dentate gyrus of the hippocampus compared with all of the other treatment groups. The group of animals that received only choline supplementation after injury (choline monotherapy) was the only group with a significant decrease in inflammation; all other groups has increased inflammation after injury. In contrast, creatine pre-treatment was the only strategy that had a significantly beneficial cortical tissue sparing compared with injured animals on a standard diet, which duplicated our previously published monotherapy findings.[71,73] Further, the combination therapy did not improve tissue sparing over creatine monotherapy.

In summary, there was no outcome measure that demonstrated improved outcome with our Sequential Pleiotropic Therapy Combination strategy, compared with monotherapy approaches with the same doses and timing relative to injury. In addition, no one monotherapy showed consistent benefit across all metrics compared with a standard diet. Interestingly, histological outcomes did not correlate with behavioral outcomes, which was another informative result from this study.

Challenges. Our first challenge was that the determination of the best therapy in our studies varied depending on which metric one examined. For example, the improved cortical tissue sparing was not accompanied by improved performance in the MWM or reduced brain inflammation. Supplementation with 2% choline after injury only was associated with some benefits including reduced brain inflammation and improved performance in the MWM (but not significant tissue sparing).

Our second challenge was that we found no evidence of any synergistic or additive benefits of two dietary supplements that show more impressive results when administered alone for any measure. Choline supplementation after the injury seems to obfuscate the tissue sparing effect of creatine pre-treatment, and beneficial effects observed with choline alone after injury are absent if creatine pre-treatment was used. Because there are no obvious pharmacodynamic or pharmacokinetic (PK) interactions between choline and creatine, we were very surprised that we got no evidence of any additive or synergistic actions of these drugs. Because both creatine and choline require transporters to cross the BBB, we speculate that there is some competition or compensatory changes that could have led to reduced brain choline levels when animals were switched from the creatine supplemented diet to the choline supplemented diet.

In examining potential mechanisms to explain this unexpected outcome, food consumption was measured across the duration of the experiments, and there were no significant differences in food intake when animals were consuming diets with supplements, and there were similarly no significant changes in feeding parameters when animals were switched form one diet to another. Another important observation of these studies is that dietary choline supplementation has much better efficacy for neuroprotection when a pre-treatment strategy is included, because our previous studies with choline supplementation before and after injury showed better results in each area of assessment. Choline supplementation as a post-TBI strategy has also recently been disappointing in a National Institutes of Health sponsored clinical trial. The Citicoline (CDP-choline) Brain Injury Treatment Trial (COBRIT), a double-blinded randomized phase III clinical trial, failed to show any functional or cognitive improvements in patients given CDP-choline supplementation after TBI.[76]

Recommendations. Future research should focus on understanding mechanisms responsible for the lack of efficacy of this sequential combination drug therapy and on finding additional combinatorial approaches that might have better efficacy. In summary, because creatine pre-treatment and choline supplementation each provides some beneficial actions in our experiments but the combined effects were less than either individual treatment alone, our recommendation is to examine the potential antagonistic effects of even two seemingly independent agents under consideration for a combined therapy. As with VDH therapy, dosing strategies may be informed by biomarkers of nutritional status.

Concurrent targeted therapy combination: probenecid and N-acetylcysteine (NAC)

Rationale. Brain bioavailability of systemically administered drugs is limited not only by penetration across the BBB, but also by

energy-dependent transporters that export drugs back across blood–brain and blood–cerebrospinal fluid barriers. This may contribute to some of the failures of previously tested pharmacological treatments in clinical TBI trials.[3]

One such pharmacotherapy is N-acetylcysteine (NAC), which has notoriously poor BBB penetration and brain bioavailability.[77] NAC is the antidote for preventing hepatic necrosis in patients with an acetaminophen overdose,[78] functioning as a cysteine donor to replete the endogenous antioxidant glutathione (GSH). Systemic administration of NAC alone restores GSH levels and reduces mitochondrial dysfunction,[79] and NAC in combination with minocycline attenuates inflammation and cognitive deficits, after TBI in rats.[80,81] Moreover, a recent clinical trial suggests that NAC improves neurological outcome after blast-induced mild TBI.[82]

These studies suggest that normal BBB penetration may be less of a factor after TBI, where some degree of BBB disruption is part of its pathophysiology. We hypothesize that an adjunctive combinational strategy to improve brain bioavailability of NAC would theoretically enhance NAC dose effectiveness.

As an adjunctive agent to synergize NAC's therapeutic potential, we selected probenecid, which has been used as an antibiotic adjuvant to achieve higher concentrations of therapeutics since the 1940s when it was synthesized in response to shortages of penicillin needed to treat wounded soldiers during World War II.[83] Further, probenecid inhibits active efflux of biological compounds similar to NAC (including GSH) through ABC drug transporters and/or organic anion transporters.[84] We therefore posited that probenecid would also serve to increase brain bioavailability of NAC when used in combination.

Probenecid on its own has other effects that may be protective after TBI, including maintenance of endogenous GSH levels,[85] inhibiting activation of the inflammasome,[86] and preventing spreading depression.[87] Thus, probenecid has the capacity to increase brain bioavailability of NAC, thereby enabling synthesis of GSH, while also preventing depletion of endogenous GSH. Probenecid would also inhibit renal excretion of NAC, thereby maintaining serum concentrations. As such, the combination of NAC and probenecid has the potential to act synergistically above and beyond a role as ''drug and drug-adjuvant.''

Experimental design and findings. For proof of concept, we first tested the effect of probenecid and NAC alone, and probenecid + NAC in combination on brain total antioxidant reserves (AOR) after CCI in mice. Anesthetized, adult male mice were subjected to moderate CCI (1.2 mm depth, 6 m/sec velocity), then randomized to receive normal saline (NS) vehicle, NAC (163 mg/kg), probenecid (150 mg/kg), or a combination of NAC and probenecid IP 10 min after CCI. The combination strategy group received probenecid 10 min after CCI, then NAC 10 min after that.

Total AOR were determined by quantifying the capacity for protein homogenates to quench AAPH-derived peroxyl radicals in ipsilateral cortex and hippocampus harvested 6 h after CCI. Total AOR in injured brain were reduced by 24% versus control. Post-treatment with systemic NAC had no effect on total AOR, compared with vehicle 6 h after CCI. In contrast, post-treatment with probenecid prevented this reduction in total AOR levels, and combined treatment with NAC and probenecid had a synergistic effect, with repletion of total AOR in the injured brain to a level that was 35% higher than in naïve mice ($p < 0.05$ vs. vehicle).

Given that, to date, carrier-mediated transport of NAC has not been reported,[77] we then performed PK studies to determine if probenecid inhibited transport of NAC from plasma and brain tissue. This is important because, as mentioned above, both NAC and probenecid may preserve AOR independently after TBI. For PK studies, we used postnatal day (PND) 17 rats to facilitate placement of vascular catheters for frequent blood sampling, and NAC and probenecid measured using a validated liquid chromatography-mass spectrometry/mass spectrometry assay. The same doses of NAC (163 mg/kg) and probenecid (150 mg/kg) were administered IP, and blood was sampled at intervals from 30 min to 8 h.

In naïve PND 17 rats, NAC was undetectable in brain homogenates at 6 h when administered alone. In contrast, NAC levels were >50 ng/g tissue when administered with probenecid. We found that both plasma and brain NAC concentrations were increased by co-administration of probenecid, and that probenecid increased brain NAC to a degree beyond that explained by increased plasma levels alone. These findings suggested that NAC is a transporter substrate, and that probenecid inhibits NAC plasma clearance and brain efflux. Similar PK studies performed in PND 17 rats after CCI also showed significant increases in plasma and brain NAC levels with probenecid co-administration, although after CCI, brain NAC levels were detectable when NAC was administered alone, reflecting BBB disruption after injury.

Pre-clinical outcome studies performed used a post-treatment multi-dosing paradigm informed by the PK studies. Rats were randomized to one of four groups: vehicle 1 + vehicle 2, NAC (163 mg/kg) + vehicle 2, vehicle 1 + probenecid (150 mg/kg), and NAC (163 mg/kg) + probenecid (150 mg/kg). All doses were administered IP starting 10 min after CCI, then every 12 h for six total doses. Afterward, rats underwent beam balance, inclined plane, and MWM testing over a 14-day period. The combination NAC + probenecid group performed better in the probe trial MWM test ($p < 0.05$ vs. vehicle groups); however, the overall treatment effect was modest and not seen in other functional outcome paradigms evaluated.

Challenges. The first hurdle to overcome was simply related to the compatibility of multiple drugs. NAC and probenecid precipitate in aqueous solution without pH adjustment, and this pH adjustment necessitates addition of a substantial amount of sodium hydroxide. Thus, one must account for both pH of the final solution and matching of the amount and concentration of sodium delivered in the vehicle(s). An additional challenge with combinational strategies is that off-target consequences are increased.

Relevant to our strategy, NAC is known to result in nausea in a dose-dependent manner,[88] and we observed weight loss predominately in the NAC + probenecid treated rats, necessitating administration of parenteral fluids for future pre-clinical trials. Further, probenecid has been found to ''prolong and enhance'' fever produced by direct injection of pyrogenic substances,[89] and we found that rats treated with probenecid were more susceptible to hyperthermia, but only after CCI and only if placed in a high-efficiency incubator (and not on a simple Plexiglas container on a warming blanket). Both of these undesirable consequences were discovered because of vigilant physiological monitoring, will need to be prevented in future pre-clinical trials, and will mandate equally vigilant monitoring in clinical trials of these drugs individually and in combination.

Recommendations and future studies. Based on this experience, we recommend designing pre-clinical combinational drug studies with more ''clinical trial rigor.'' Specifically, drug combinations should be tested for compatibility, and placebos should be carefully matched with vehicles.[6] Dosing strategies

should be informed by PK studies of the drugs/interventions in combination, because they may have significant interactions, either intentional as with NAC and probenecid, or unintentional (e.g., phenytoin and hypothermia[90]). Finally, rigorous physiological monitoring is recommended at least during the development phase of combinational strategies, because drugs/interventions may also have additive physiological consequences, both desirable and undesirable.

Screening concurrent therapy combinations: a PK, behavioral, and mechanistic approach to polytherapy treatment design

Rationale. The primary objective of our research was to screen potential multi-drug therapies for complementary effects on neurological recovery after experimental TBI using behavioral, histological, and gene expression markers of neuroprotection. Drugs were selected by evaluating commercially available candidates with preclinical evidence of efficacy in three general pharmacology mechanistic categories: antioxidant, anti-inflammatory, and excitotoxicity inhibitors, with an emphasis on agents that target multiple biological pathways associated with secondary injury after TBI.

Anakinra,[91] erythropoietin,[91] memantine, minocycline,[92] nicotinamide,[93,94] progesterone,[18,94] simvastatin,[92] and topiramate were evaluated. Because anti-epileptic drugs (AEDs) are frequently used post-TBI, levetiracetam was included to determine whether the AED alters the efficacy of a polytherapy.

Experimental design and findings. Studies were conducted in two phases. First, studies were performed in healthy adult rats to determine the optimal dosage regimen to target serum concentrations used for non-TBI clinical indications. The initial dose of drugs with prolonged absorption in rodents (peak concentrations >1 h post-dose) or rapid absorption (peak concentrations ≤1 h) were administered either 2 h or 4 h post-injury, respectively, to attain targeted concentrations by 4 h post-injury.

Clinically, in patients with TBI, administration of an intravenous loading dose results in rapid peak concentrations, compared with the delay in peak found with some of our drugs in the CCI model when administered IP, SC, or oral gavage. Drugs with short elimination half-lives (nicotinamide, levetiracetam, minocycline) were administered by SC infusion. The duration of treatment was based on the proposed mechanism of action of the selected drug: 48 h for excitotoxicity inhibitors, 72 h for drugs with anti-inflammatory and/or antioxidant activity, and 7 days for an antiepileptic drug. After the dose finding studies in healthy rats, blood draws at specific time-points during treatment after CCI were used to rule out possible differences in the PKs of healthy and injured rats, as well as any drug interactions in the combination therapy studies.

In the second phase, the effects of the treatments (using optimal doses determined in healthy male adult rats) were compared with vehicle control treatments after moderate to severe CCI or sham injury.[94] Outcomes assessed were PKs, histology, gene expression in brain and liver, and a battery of motor, sensorimotor, and cognitive tests for 4 weeks (placing test, Rotor-rod, adhesive removal, forelimb asymmetry, and the MWM for reference and working memory). We deliberately included some tests that show some level of spontaneous recovery.

Of the drugs evaluated, only nicotinamide and progesterone demonstrated sufficient neuroprotection to propose for possible polytherapy.[94] The gene expression data used gene ontology and Ingenuity pathway analyses to identify molecular mechanistic pathways and to suggest rational combinations of drugs based on complementary biological pathways. In our gene expression studies, progesterone[18] and nicotinamide[93] were identified as a promising pair, because nicotinamide mitigated the gene expression changes causes by TBI and progesterone treatment affected genes that were not altered by TBI.

We subsequently confirmed the efficacy of the nicotinamide-progesterone (NAM-Prog) combination therapy in comparison with vehicle or either therapy alone, and details are published elsewhere.[19] Briefly, male adult (3.5 month old) rats were designated as surgical shams or experienced unilateral CCI TBI. Injured animals received Prog or NAM monotherapy, the NAM-Prog combination therapy, or only vehicle, for a total of four injured groups, plus shams. Group sizes were N = 9 for behavioral outcomes and N = 6 for histopathology analysis.

Those receiving NAM or Prog alone received a vehicle dose of the other therapeutic at the same timing and route of the combination therapy cohort. Prog (10 mg/kg) was administered via an IP injection at 4 h post-CCI, and every 12 h until sacrifice. In contrast, NAM was administered with osmotic pumps to provide a continuous IP infusion (12 mg/kg/h; 240 μL/day) after a 75 mg/kg IP loading dose at 4 h post-CCI. To control for effects following injections and/or pump implantation, all animals were injected, anesthetized, and implanted with osmotic pumps. Three behavioral metrics were evaluated at 4, 6, 8, 11, 17, 24, and 28 days post-injury: a bilateral tactile adhesive removal task as a sensory assessment, and both a forelimb asymmetry task and foot placing task as motor assessments.

At 24 h and 29 days post-TBI, rats were euthanized, brains fixed for histopathology, and lesion volume and glial fibrillary acidic protein (GFAP) activity (only at 24 h) were assessed. In all three behavioral assessments, mono- and combination therapies significantly improved outcomes compared with vehicle-treated injured animals, with the combination therapy improving outcomes significantly more than both monotherapies in two behavior assessments, and improving outcomes significantly more than one monotherapy for the third metric. Lesion volume was significantly reduced relative to the vehicle-treated injured group at 24 h only for the combination therapy; by 29 days post-CCI, all treatments were equivalent in reducing lesion volume compared with untreated injured animals. Finally, while all treated groups had significantly fewer GFAP positive cells 24 h post-CCI, the NAM-Prog combination demonstrated superior reduction over monotherapies.

Challenges. The pre-clinical studies were complex, involving combination and comparative studies that necessitated multiple routes of administration and multiple controls. We conducted dose-finding studies to identify doses associated with target blood concentrations; however, concentration targeted dosing required us to develop a wide range of assays using multiple analytical methodologies including enzyme-linked immunosorbent assay, high pressure liquid chromatograph with ultraviolet and mass spectrometry detection. The targeted concentrations provide information regarding the maximum concentration (dose) for drugs with low therapeutic ranges (i.e., erythropoietin, Anakinra). More importantly, concentration target dosing based on non-TBI approved indications assumes that the neuroprotective effect of the drug occurs at the same concentrations attained in the non-TBI indication.

Thus, one should use caution basing concentration targets on non-TBI approved indications. For example, simvastatin is a prodrug with active metabolites; therefore traditional concentration target dosing was not possible, and HMG-reductase inhibition activity in plasma was used as a surrogate marker. We found minimal

efficacy in the CCI model using HMG-reductase inhibition paired with a 10-fold higher dose of simvastatin,[92] compared with a lower efficacious dose reported by other investigators.[95,96] Therefore, for drugs with possible U-shaped dosing curves, which demonstrate decreased efficacy at higher doses, the most effective dose may not be identified by concentration targeted dosing, unless multiple doses are evaluated. Others[20,21] and we[94] have found U-shaped neuroprotective effects with progesterone in experimental models of TBI.

Conducting an appropriate pre-clinical screening study requires the use of multiple behavioral measures. In addition, these tests should be conducted over multiple time points spanning a moderately lengthy assessment period (i.e., 30 days minimum).

Recommendations. First, it is important to use multiple assessments and time windows to determine a general therapeutic effectiveness, because acute and long-term results could differ across the specific assessments. While an emphasis on long-term improvement may be desired, success may also be defined as a more rapid rate of recovery. Second, as a next step, future studies should evaluate the optimal treatment duration and windows for NAM-Prog combination therapy. Additional studies could target isoboles, or pairs of NAM-Prog doses that result in comparable improvement in outcomes. Finally, it would be important to evaluate at lower doses, to establish a lower dosing bound, with the goal of reducing the risk of unintended consequences of treatment. These optimization studies can often add considerable cost to the pre-clinical phase, but may be important in laying the foundation for successful clinical trials.

Discussion

Three major challenges were encountered in this first major attempt to use pre-clinical animal models of TBI to develop an effective combination therapy for TBI (see Table for summary of outcomes). One challenge was to define ''success.'' Many of the studies demonstrated significant improvements in histology or other biomarkers but not in behavioral outcomes; different outcomes were used as primary end point assessments (italic, in Table); improvements were observed in some but not all of the behavioral outcomes; or early improvements in behavioral outcomes were not sustained. Most of the investigators suggested that a ''long-term'' battery of behavioral outcomes is probably the best metric, but consensus about the recommended battery is currently lacking.

Pre-clinical common data elements to promote standardization of protocols and definitions for animal studies are currently under development, but additional work to reach consensus on a ''gold standard'' behavioral battery for rat and mouse models of TBI is needed. Finally, the criterion used for ''success'' was a statistically significant difference from untreated injured animals; future studies may include establishing success metrics with an effect size that is also clinically meaningful.

A second challenge that impacted three of the studies was a lack of sensitivity of their ''long-term'' outcome measurements to detect differences between the monotherapies and the combination therapies because of a ''ceiling effect.'' The one study that did detect significant improvements used a different battery than the other studies; and this test battery was also different from the original articles demonstrating effectiveness of the monotherapies.[81,84] We speculate whether the lack of significant improvement in combination therapies we report are attributable to insensitive test batteries; it would be interesting to compare effectiveness of all therapies using that novel battery of tests. We value the one study that demonstrated worse outcomes with the combination of therapies because this unexpected finding highlights the current limitations in how to select drugs for combined use. It would be interesting to evaluate whether an integrated agent selection approach using gene expression data and Ingenuity analysis would have anticipated the negative outcomes to combination therapy. If so, *in vitro* and *in silico* studies may prove to be an important step to inform preclinical study design.

A third major challenge is the extraordinary amount of work needed to rigorously evaluate combination therapies at multiple doses and times. This phase is especially important when initial (ineffective) dosing strategy was derived from diseases or injuries other than TBI, or in a different species, sex, or stage of maturity. Because agents may have an interaction or off-target effects when combined, it is important to optimize dose-duration-response efficacy in combination doses, rather than selecting the best effective dose of each agent as independent monotherapies and then combining them.[97] Even though the preponderance of TBI occurs in young adult males,[98–100] TBI also occurs in females, children, and the elderly; pre-clinical therapeutic screening across developmental age and sex can inform clinical trial design.

Clinical trials are addressing similar challenges by developing and validating biomarkers for Phase II studies[101–103] and by also incorporating secondary efficacy analyses for these factors. To reduce sample sizes and/or enhance power of including even modestly promising therapies for more rigorous subsequent evaluations, clinical trials now incorporate innovative study designs, (e.g., adaptive, factorial, crossover), and perform interim analyses to identify and eliminate futile dosing strategies from further study.[104] Population heterogeneity in TBI injured patients[7] undermines the potential to find a statistically significant therapeutic effect size; clinical trials in other fields are now reducing variability in the group by using genetic or biomarker screening.[104] All of these innovative approaches being added to enhance the statistical power and reduce costs of clinical studies should also be exploited for pre-clinical translational therapy research.

Several limitations of the studies are important to note. Given the paucity of effective translation of monotherapy trials from rodents to human clinical trials, we emphasize that proof-of-concept in male rodents does not ensure efficacy in humans. First, we recommend performing additional pilot studies in females and in non-rodent species, including humans, to confirm translation of therapeutic promise, before conducting large-scale clinical trials.[105–107]

Second, all studies were performed in ambulatory rodents with moderate TBI with demonstrable histopathology and behavioral deficits. While this degree of severity represents a cohort of the TBI population with a good potential to respond to treatments, agent efficacy, therapeutic dosing strategies and administrative route practicality for more or less severe TBI should be established, and clinical trial designs should include assessment of injury severity by biomarkers or imaging to inform treatment options. Third, a variety of strategies were used to evaluate and select monotherapies that could be combined for even greater therapeutic benefit (distinct or overlapping mechanisms, synergistic effects, administered concurrently or sequentially); no one strategy emerged as exemplar.

Conclusion

All of the studies have increased our understanding of the challenges associated with testing combination therapies in animal models of TBI, as well as opportunities for the future. In addition, ongoing studies to more precisely classify patients with TBI based on evolving pathoanatomical and pathophysiological responses to

injury will provide additional opportunities for developing targeted, effective combination treatments.

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