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Authors
Kim, N
Kim, JY
Yenari, MA

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PHARMACOLOGICAL INDUCTION OF THE 70-kDa HEAT SHOCK PROTEIN PROTECTS AGAINST BRAIN INJURY

N. KIM, J. Y. KIM AND M. A. YENARI*

Department of Neurology, University of California, San Francisco and Veterans Affairs Medical Center, San Francisco, CA 94121, USA

Abstract—The 70-kDa heat shock protein (HSP70) is known to protect the brain from injury through multiple mechanisms. We investigated the effect of pharmacological HSP70 induction in experimental traumatic brain injury (TBI). 3-month-old male C57/B6 mice were given 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) intraperitoneally (IP, 2 mg/kg) or intracerebroventricularly (ICV, 1 μg/kg) to determine whether HSP70 could be induced in the brain. Mice were subjected to TBI via cortical controlled impact, and were treated with 17-AAG (or vehicle) IP according to one of two treatment regimens: (1) 2 mg/kg at the time of injury, (2) a total of three doses (4 mg/kg) at 2 and 1 d prior to TBI and again at the time of injury. Brains were assessed for HSP70 induction, hemorrhage volume at 3 d, and lesion size at 14 d post-injury. Immunohistochemistry showed that both IP and ICV administration of 17-AAG increased HSP70 expression primarily in microglia and in a few neurons by 24 h but not in astrocytes. 17-AAG induced HSP70 in injured brain tissue as early as 6 h, peaking at 48 h and largely subsiding by 72 h after IP injection. Both treatment groups showed decreased hemorrhage volume relative to untreated mice as well as improved neurobehavioral outcomes. These observations indicate that pharmacologic HSP70 induction may prove to be a promising treatment for TBI. Published by Elsevier Ltd. on behalf of IBRO.

Key words: animal studies, traumatic brain injury, therapeutic approaches.

INTRODUCTION

The 70-kDa class of heat shock proteins (HSP70) comprise a highly conserved family of ATP-dependent, cytosolic chaperones that function primarily in facilitating protein folding, degradation, complex assembly, and translocation, consequently preventing harmful protein aggregation (Giffard et al., 2004). They are present in nearly every type of cell in the body, and some are specifically upregulated in response to stress, such as cytotoxic and potentially pathogenetic accumulation of unfolded proteins that arises when normal cellular processes are interrupted by stress (Adachi et al., 2009; Henderson, 2010). The HSP70 family includes an inducible form also known as HSP72, HSP70i, or simply HSP70. HSP70 has also been shown to be neuroprotective in animal models of various brain insults, including neurodegenerative disorders, cerebral ischemia, and traumatic brain injury (TBI) (Yenari et al., 2005; Turturici et al., 2011).

Whether by their function as chaperone or by some other yet undetermined mechanism, HSP70 appears to play a role in cytoprotection, reducing inflammation and apoptosis in brain injury models including stroke and TBI (Giffard et al., 2004). Overexpression of HSP70 has been shown to reduce apoptosis, though the exact mechanism remains unclear (Giffard and Yenari, 2004). Thus, strategies to increase intracellular HSPs might be relevant in many neurological conditions such as TBI. Studies have shown that immune response pathways arising after acute neurological insults can exacerbate brain injury, and that suppressing inflammation can reduce cell death and improve recovery. Overexpression of HSP70 in such circumstances appears to be largely anti-inflammatory, as intracellular, innate immune responses appear to be in play (Giffard and Yenari, 2004). Previous studies have also identified a link between inducible HSP70 and matrix metalloprotease regulation in injury conditions (Lee et al., 2004). Recent findings from our lab have shown that HSP70 overexpression suppresses matrix metalloproteinase (MMP) 9, protecting the brain in experimental TBI. Selective knock-down of HSP70 led to more pronounced MMP 2 and MMP 9 activity in the brain and reversed the reduction in hemorrhage and lesion sizes corresponding with HSP70 overexpression (Kim et al., 2013). However, much of the existing research in neuroprotective HSP70 overexpression has been conducted in transgenic models or by gene transfer which may not be practical in clinical settings (Whitesell et al., 1994; Giffard et al., 2008). Pharmaceutical induction of HSP70 may prove to be a viable therapeutic approach for limiting damage due to brain injury.

Under normal, non-stressful conditions HSPs are located intracellularly and are bound to heat shock
factors (HSFs) (Kwon and Yenari, 2002). Inducible HSP70 is upregulated following a denaturing stress such as trauma or ischemia. Next, HSFs dissociate from HSPs, leaving HSPs free to bind target proteins. HSFs are then phosphorylated and form activated trimers which bind to highly conserved regulatory sequences on the heat shock gene known as heat shock elements (HSEs). Once bound to HSEs, HSFs control the generation and expression of more HSPs. Newly generated HSPs can then bind denatured proteins and act as a molecular chaperone by contributing to repair, refolding, and trafficking of damaged proteins within the cell. HSP90 can also influence HSP70, since HSP90 is bound to HSF-1. When HSP90 dissociates from HSF-1, HSF-1 leads to HSP70 induction (Kim et al., 2012). HSP90 antagonists can thus function as HSP70 inducers.

At present, there are few published findings evaluating pharmacological HSP70 induction in neurological disorders, and these have exclusively involved ansamycins in acute injury. The research to date has particularly focused on geldanamycin (GA), one of the ansamycins in acute injury. The research to date has determined that HSP70 induction by GA represses expression of pro-inflammatory markers, reduces infarct size, and downregulates apoptotic pathways in stroke and hemorrhage. Because of toxicity, GA remains a poor candidate for a pharmaceutical treatment, and investigators in the cancer biology field have developed a less toxic GA analog 17-allylamino-17-demethoxygeldanamycin (17-AAG or tanespimycin), which has been shown to be an effective HSP90 antagonist in addition to a more favorable safety profile (Chakraborty et al., 2008; Vaishampayan et al., 2010). 17-AAG has primarily been studied as a pro-apoptotic drug in several cancers, though these studies often use the HSP90 inhibitor in conjunction with other anti-survival conditions (Chatterjee et al., 2007; Tavernier et al., 2012; Zhang et al., 2012). Apart from cancer biology, 17-AAG has been shown to protect neural progenitor cells in vitro and reduce inflammation in other non-neurological disease models such as atherosclerosis, uveitis, and acute lung injury (Poulaki et al., 2007; Madrigal-Matute et al., 2010; Wang et al., 2011). There are presently no studies of 17-AAG in brain trauma. Findings from our lab are the first to suggest that 17-AAG may also confer protection in TBI, reducing hemorrhage and improving neurobehavioral outcomes. Further investigation is needed to conclusively determine a potential role in stroke or other neurological insults, although the existing data highlight 17-AAG as a promising therapeutic.

**EXPERIMENTAL PROCEDURES**

**Animals**

Male, 3-month-old C57B6 mice weighting 25–30 g each (Simonson Labs, Gilroy, CA, USA) were used for all groups. Mice were housed and handled in compliance with NIH regulations, according to protocols approved by the local Institutional Animal Care & Use Committee.

**Controlled cortical impact (CCI)**

Mice were anesthetized with isoflurane (5% for induction in a designated chamber, 2% for maintenance by nosecone) and fixed in a stereotaxic frame. CCI was induced using methods previously published by our group (Whitesell et al., 1994). A midline scalp incision was made to expose the skull. A 5-mm-diameter burr hole over the parietal lobe bregma was drilled. The dura was not disturbed during craniectomy. Impactor with 3 mm-diameter convex tip (Pinpoint Precision Cortical Impactor, Hatteras Instruments, Cary, NC, USA) was perpendicularly applied at exposed area with 1.5 m/s velocity to a depth of 2 mm and for a 100-ms dwell time. Sham operated mice were studied in parallel. Post-injury, mice were monitored daily and survived for up to 14 d. At the end of the observation periods, mice were euthanized and brains harvested for further study.

**17-AAG treatment**

For the initial immunohistochemistry panel to determine whether 17-AAG treatment induced HSP70 in the brain, uninjured mice were treated with 1 μl of 17-AAG (Sigma, St. Louis, MO, USA) at a dose of 1 μg/kg via intracerebroventricular (ICV) injection or 100 μl of the same concentration via intraperitoneal (IP) injection. In subsequent uses, sham and injured mice in the treatment groups were given 100 μl of 17-AAG at 100 μg/mL via IP-injection at indicated time points. IP dose of 4 mg/kg was determined based on previous studies and drug safety profile (Sausville et al., 2003). 17-AAG was solubilized in dimethyl sulfoxide (DMSO) and diluted in phosphate-buffered saline (PBS). Single treatment (1X) mice were administered a single injection of 17-AAG concurrent to injury. Triple treatment (3X) mice were given two initial doses, 2 d and 1 d prior to injury, followed by a final injection at the time of injury (Table 1). Untreated groups were given diluted DMSO via IP injection at time of injury as well as at matching pre-injury time points. 1X and 3X DMSO groups did not show significant variation (not shown).

**Histology**

Frozen tissue sections were prepared from brains collected at 1 d after treatment with 17-AAG or vehicle only (n = 3 per group). Mice were euthanized using (5% isoflurane, dislocation), transcardially reperfused with saline, and brains were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were then blocked with 10% sucrose in 0.1 M phosphate buffer (pH 7.4) for 1 d at 4°C and then gradually embedded in 30% sucrose in 0.1 M phosphate buffer (pH 7.4). Brains were sectioned using a Leica sliding microtome at 30 μm, mounted on glass slides, and stored in 0.01% thimerosal in 0.1 M phosphate buffer (pH 7.4) at 4°C. Sections were equilibrated 30 min in 0.01% thimerosal in 0.1 M phosphate buffer (pH 7.4) at 4°C, washed in buffer, and rinsed in 0.01% thimerosal in 0.1 M phosphate buffer (pH 7.4) at 4°C for 5 min. Sections were then rinsed in 0.01% thimerosal in 0.1 M phosphate buffer (pH 7.4) at 4°C for 5 min.

**Table 1. Treatment paradigms for the CCI experiments.** All injections were given intraperitoneally (IP); CCI = controlled cortical impact, 17-AAG = 17-allylamino-17-demethoxygeldanamycin

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>Control</td>
<td>Vehicle injection at the time of CCI</td>
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<tr>
<td>17-AAG (1X)</td>
<td>IP injection at the time of CCI</td>
</tr>
<tr>
<td>17-AAG (3X)</td>
<td>IP injections 2 d prior, 1 d prior, and at time of CCI</td>
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PBS and afterward with 4% paraformaldehyde (PFA). Samples were post-fixed by overnight incubation in 4% PFA overnight, followed by overnight incubation in a 30% sucrose solution with sodium azide. Tissues were coated in optimal cutting temperature embedding media and kept at −80°C before being sectioned into 12- or 25-μm-thick section using a cryostat. Immunohistochemical analysis was performed using anti-mouse HSP72 (1:500; Enzo Life Sciences, Farmingdale, NY, USA) and anti-rabbit microtubule-associated protein 2 (MAP-2) (1:500; Millipore, Billerica, MA, USA), glial fibrillary acidic protein (GFAP) (1:500; Cell Signaling, Danvers, MA, USA), CD11b/c (1:500; Abcam, Cambridge, MA, USA), each at 1:200. Biotinylated anti-rat immunoglobulin G (IgG) (Vector Laboratories, Burlingame, CA, USA) was used for the secondary antibody and visualized using peroxidase-conjugated avidin (ABC Elite, Vector Laboratories, Burlingame, CA) and diaminobenzidine substrate. Western blot

Brains were collected from TBI mice treated with 17-AAG or vehicle at 6, 24, 48, and 72 h after injury or 24 h after sham surgery for uninjured mice (n = 3 per group). Ipsilateral hemispheres were homogenized manually in lysis detergent with protease inhibitors (cOmplete Mini protease inhibitor cocktail tablets, Roche, NJ, USA). Protein concentrations were measured using the Bradford assay before running 40-μl aliquots in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis. Samples were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Target proteins were detected using anti-mouse HSP72 (1:500; Enzo Life Sciences, Farmingdale, NY) and horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000; Biorad, Hercules, CA, USA), then reprobed using anti-β-actin (1:2000; Sigma, St. Louis, MO). Relative intensity of proteins detected by blots was digitally quantified using ImageJ (v. 1.31).

Image-based measurement of hemorrhage and lesion size

For lesion volume analysis, tissue sections from brains collected 14 d post-injury were stained with hematoxylin and eosin (H&E, Sigma, St. Louis, MO). Samples assessed for lesion size included brains from TBI-only (n = 8), 17-AAG 1X (n = 8), and 17-AAG 3X (n = 8) groups. Three serial sections spanning the center of the injury (−2 mm to −3 mm from bregma) were averaged to approximate relative lesion volume according to a previously established protocol (Whitesell et al., 1994). Hemorrhage severity was assessed using brains that were harvested at 3 d post-injury from TBI-only (n = 8), 17-AAG 1X (n = 9), and 17-AAG 3X (n = 8) groups. Upon sacrifice, mice were transectadially perfused with ice-cold PBS. Freshly extracted tissues were submerged in ice-cold PBS and cut into 2-mm-thick sections. Sections were then serially imaged. Pictures of brain sections were cropped and contrasted (GNU Image Manipulation Program v.2.8, CA, USA) to isolate hemorrhage sites. We then digitally quantified the hemorrhage volume using the “Integrated Density” function in ImageJ (v. 1.31). These methods were in keeping with the formerly published methods to measure relative size and intensity of lesion or hemorrhage (Tang et al., 2010).

Neurobehavioral assays

The elevated body swing test was performed 1, 3, and 7 d after TBI to compare neurobehavioral deficits among sham (n = 3 all time points), DMSO-only (n = 17 at 1 and 3 d; n = 16 at 7 d), 17-AAG 1X (n = 22 at 1 and 3 d; n = 8 at 7 d), and 17-AAG 3X (n = 24 at 1 and 3 d; n = 17 at 7 d) groups. For the swing test, mice were suspended vertically by the tail with their heads lifted 3 inches above the test bench. A lateral swing to the left or right was counted each time the animal moved its head >10° away from the vertical axis (Borlongan and Sanberg, 1995). A total of 20 swings were counted in each trial, and frequency of right-biased swing was calculated for each animal.

The adhesive removal (sticky tape) test was performed 1, 3, 7, and 14 d after injury (DMSO control n = 8; 17-AAG 3X n = 9 for all time points). 50 mm² adhesives were attached to both forepaws, and mice were individually placed in an arena for a maximum of 2 min. An observer blinded to treatment groups recorded the time elapsed before each mouse was able to identify and remove both adhesives. Mice were evaluated prior to injury in lieu of sham. T-test with repeated measures showed significant difference between pre- and 1 d post-injury outcomes for both control and 3X groups. Each mouse performed three trials per testing day. Shorter removal times indicated improved neurological recovery (Komotar et al., 2007).

Statistical analysis

All data analyses were performed by investigators blinded to experimental conditions. Experiments were carried out in a randomized fashion to minimize bias. An analysis of variance (ANOVA) or Student’s T-test was performed on east set up data to determine statistical significance (Systat Software, Inc., San Jose, CA, USA). P-values < 0.05 were considered significant. Data are presented as ± SEM.

RESULTS

17-AAG induction of HSP70

A single dose of 17-AAG via IP injection was sufficient to boost HSP70 expression in the brain as seen in tissue sections stained 1 d after treatment (Fig. 1). Uninjured mice treated with vehicle alone expressed low levels of HSP70, while treatment with 17-AAG led to increased HSP70 expression. Double-immunolabeling located HSP70 mainly in microglia and some neurons. Co-labeling with HSP70 and GFAP antibodies suggested little to no HSP70 expression associated with astrocytes. Brain slices from mice treated with 17-AAG
by ICV-injection bore similar results (not shown). We administered 17-AAG by IP injection in subsequent experiments. Western blot analysis of tissue samples from injured and uninjured brain showed an overall increase in the expression of inducible HSP70 with 17-AAG treatment.
Comparatively, 17-AAG did not have as strong an effect in uninjured sham groups as in injured and treated groups. In injured mice, increased HSP70 expression was most pronounced at 48 h after injury, with a slight elevation over baseline at 72 h.

Since we observed peak induction of HSP70 at 48 h following 17-AAG injection, we designed a paradigm outlined in 'Experimental procedures' section and in the Table 1 to test the hypothesis that pharmacologic HSP70 induction might improve outcome in CCI. 1X mice received a single IP injection at the time of injury. 3X mice received a total of three doses: two injections prior to injury plus one at the time of injury. Control groups were given a vehicle injection concomitant to injury.

Improved neurobehavioral outcomes in treated mice

17-AAG treatment improved neurobehavioral deficits following experimental TBI (Fig. 3). Both single-treatment and pre-treatment mice showed reduced swing bias during elevated body-swing test by 3 d and 7 d post-injury (T-test, $P < 0.05$) compared to the untreated control group. Treatment groups were also observed to be more alert and engaged during 1-d and 3-d testing. In the sticky-tape test, 3X-treated mice showed a trend toward longer adhesive removal times relative to treated mice at all time points, with significant differences at 1 and 3 d (T-test, $P < 0.05$). Significant differences among treated and untreated groups were not detectable at 14 d.

Reduced lesion size and hemorrhage volume in treated mice

We observed decreased tissue atrophy 14 d after injury in samples from 3X-treated mice relative to untreated and 1X groups (Fig. 4). Untreated TBI mice presented the largest brain lesions, followed by 1X mice. 1X mice did not show significant changes in lesion size compared to the controls. The 3X treatment group showed significantly reduced lesion sizes compared to the untreated and 1X groups (T-test, $P < 0.05$).

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**Fig. 3.** 17-AAG-treated mice performed better in neurobehavioral assessments. (a) 17-AAG treatment both 1X with injury and 3X pre-injury led to decreased bias on the elevated body swing test at 3 and 7 d post CCI. Sham ($n = 3$ all time points), DMSO-only ($n = 17$ at 1 and 3 d; $n = 16$ at 7 d), 17-AAG 1X ($n = 22$ at 1 and 3 d; $n = 8$ at 7 d), and 17-AAG 3X ($n = 24$ at 1 and 3 d; $n = 17$ at 7 d). (b) The adhesive removal test showed that 17-AAG pre-treatment (3X only) led to shorter mean adhesive removal times compared to no treatment at 1 and 3 d after injury. DMSO control $n = 8$; 17-AAG 3X $n = 9$ for all time points ($P \leq 0.05$).

**Fig. 4.** 17-AAG treatment decreased lesion volume following CCI. Lesion sizes estimated by average cavity area in H/E stained serial sections were decreased in CCI exposed mice that had been treated with 17-AAG. The reduction was most pronounced in the pretreatment (3X) group. ($n = 8$ group, $P < 0.05$).
17-AAG treatment also attenuated brain hemorrhage in CCI mice (Fig. 5). Imaging analysis indicated greater hemorrhage volume in untreated groups relative to treated groups. Pre-treatment groups showed the smallest hemorrhage volumes in comparison with single-treatment and untreated groups. There were significant differences in hemorrhage measurements among the three groups (ANOVA, \( P < 0.05 \)).

![Fig. 5](image-url) 17-AAG treatment reduced hemorrhage following CCI. (a) Brains harvested 3 d post-injury showed more severe hemorrhage in untreated mice. Hemorrhage volume and intensity were estimated by imaging software analysis of hemorrhagic area and pigment in each section. (b) Integrated density was measured using imaging software to compute hemorrhage volume times the relative optical density (ROD) of the hemorrhage intensity. (c) Hemorrhage volumes were calculated by summing the hemorrhage area of each serial section and multiplying by thickness. TBI-only \( n = 8 \), 17-AAG 1X \( n = 9 \), and 17-AAG 3X \( n = 8 \) (\( ^* P < 0.01 \), \( ^\dagger P < 0.05 \)).

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**DISCUSSION**

TBI is a widespread condition, annually affecting 1.7 million people in the US alone, but there are yet no known effective interventions (Faul et al., 2010). The current research identifies a potential role for a pharmacological approach to regulating a previously demonstrated neuroprotective molecule, HSP70. We and others have shown HSP70 to be protective in experimental brain injury (Whitesell et al., 1994; Sharp et al., 1999; Rajdev et al., 2000; Kelly et al., 2002; Chatterjee et al., 2008). Though HSPs have been studied for over a decade for its neuroprotective effects, there have yet been no published trials of an HSP70 inducing drug with adequate safety profile that can treat brain injury. The present study evaluates the efficacy of pharmacological HSP70 induction in TBI using GA analog 17-AAG.

17-AAG induces HSP70 expression in mouse microglia and neurons, peaking around 48 h after administration. We saw similar effects whether 17-AAG was given ICV or IP. Endogenous HSP70 expression in the brain following injury showed that neurons were among the first cells to express HSP70, followed by astrocytes and finally blood vessels and microglia (Hoehn et al., 2001). Our findings suggest that HSP induction in microglia and neurons is sufficient to produce neuroprotective effects.

17-AAG’s known function is as an HSP90-antagonist. Thus, its ability to induce HSP70 is through HSP90 inhibition and subsequent liberation of HSF. Once liberated, HSF is free to bind and upregulate HSP70 gene and protein expression. 17-AAG is a promising pharmacological intervention because of its capacity to induce HSP70 expression in the brain and that it appears to elicit this affect when administered peripherally. Compared to GA, 16-AAG appears to have less toxicity when studied in humans (Sausville et al., 2003).

This study provides new evidence for 17-AAG as a viable agent for neuroprotective HSP70 induction in mouse TBI. Neurobehavioral assays indicated significant protection in 17-AAG treatment groups both at the acute as well as the subacute phases of injury. Treated mice tended to perform better than untreated mice and recovered motor skills in less time. Behavioral differences at later time points were difficult to detect using rudimentary behavior tests, although we speculate that more differences might be detected at the later time points using more sensitive assessments. Further, the lack of statistical differences were largely due to the control group improving neurological function, rather than worsening or lack of any improvement among treated groups.

Finally, our findings show that induced HSP70 overexpression corresponded to smaller hemorrhage volumes at 3 d post-injury and less cortical degeneration at longer time points. Previous work from our lab has shown that HSP70 overexpression either through viral vector mediated gene transfer (Yenari et al., 2005) or in transgenic animals (Lee et al., 2004) can suppress MMPs 2 and 9, which are proteases that, when activated, lead to blood–brain barrier (BBB) disruption and hemorrhage. Data presented here further support the consensus in the current literature that HSP70 induction protects the BBB under injury conditions, possibly by means of a previously reported mechanistic link between HSP70 expression and the regulation of MMPs (Kim et al., 2013). Taken together with prior findings, the present data indicate that pharmacologically-induced HSP70s may be able to protect the BBB. It has been thought that by reducing NFkB expression, HSP70 suppresses MMPs that are implicated in BBB damage and hemorrhage (Giffard et al., 2008). Other labs have shown that HSP70 upregulation by 17-AAG analog GA protects BBB integrity in a murine model of primary brain hemorrhage (Manaenko et al., 2013).
We also saw more pronounced neuroprotection in the 3X paradigm compared to 1X. This is not surprising since the 3X paradigm was designed to lead to maximal HSP70 induction at the time of injury with sustained induction for the first few days post insult. In contrast, the 1X paradigm consisted of a single dose at the time of injury to model a more clinically relevant scenario. However, our time course data would predict that HSP70 expression would not begin until about 24 h after injury onset. Mice given the 1X treatment paradigm did not significantly reduce lesion size compared to untreated controls, whereas the 3X group showed markedly smaller lesions, suggesting inducible HSP70 may have a limited therapeutic time window. We previously showed in a stroke model, using viral vector mediated HSP70 gene transfer, that the temporal therapeutic window for HSP70 might be about 6 h from injury onset (Hoehn et al., 2001). It will be important to firmly establish a similar time window for TBI. If the time window for TBI is similar to stroke, such a strategy of HSP70 induction with HSP90 inhibitors may be limited as a preventive measure.

CONCLUSIONS

We show here the potential of HSP70 induction for brain injury treatment. Our findings are in line with that recently shown by our group in HSP70 overexpressing transgenic mice. However, translational issues such as dose, route of administration, long-term protection, timing, and safety require further study. 17-AAG is a promising candidate, with its favorable safety profile as well as our observation presented here that peripheral administration can induce HSP70 induction (Porter et al., 2009). Our findings overall indicate that 17-AAG may prove an effective treatment for TBI. Heightened neuroprotection observed in pretreatment groups suggests 17-AAG may be useful as a prophylactic measure for individuals at high-risk.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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17-AAG has also been shown to attenuate endothelial deterioration (Chatterjee et al., 2008).


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