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Pharmacologic heat shock protein 70 induction confers cytoprotection against inflammation in glio-vascular cells

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Abstract

Inhibition of the 90 kDa heat-shock protein (HSP90) leads to upregulation of the 70 kDa inducible heat shock protein (HSP70). HSP70 has previously been shown to be neuroprotective and anti-inflammatory. Geldanamycin (GA) and other HSP90 inhibitors have emerged as promising therapeutic agents in cancer, presumably due to their ability to upregulate HSP70. However, the effects of HSP90 inhibition in brain inflammation are still unclear. We investigate the effect of a panel of HSP90 inhibitors on endotoxin-activated microglia and eventual protection from brain derived endothelial cells. Prior studies have shown that GA protects brain cells from oxidative stress. We show here that when astrocytes or microglial BV2 cells were pretreated with GA or other HSP90 inhibitors, endotoxin-induced cell death was reduced in co-cultures of BV2 microglia and brain derived endothelial cells (bEND.3). Endotoxin stimulated BV2 cells led to increased nitric oxide (NO) and inducible nitric oxide synthase (iNOS) which was prevented by treatment with all HSP90 inhibitors. HSP90 inhibitors also prevented LPS -induced BV2 cell death. We also found that HSP90 inhibition blocked nuclear translocation of NF- κ B and attenuated I κ B α degradation, and inhibited LPS-activated JAK-STAT phosphorylation. We show that pharmacologic inhibition of HSP90 with subsequent HSP70 induction protects cells that comprise the cerebral vasculature against cell death due to pro-inflammatory stimuli. This approach may have therapeutic potential in neurological conditions with an inflammatory component.

Keywords

cytoprotection; HSP70; HSP90; microglia; endothelial cells

Introduction

We and others have previously shown that the 70 kDa heat shock protein (HSP70) protects brain cells against ischemia and other insults (Stetler et al. 2010). One mechanism of this protective effect may be through its ability to prevent detrimental pro-inflammatory responses (Yenari et al. 2005). We previously showed that overexpression of HSP70 in transgenic animals protected against traumatic brain injury (TBI) by decreasing blood brain

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barrier (BBB) disruption and hemorrhage. This effect was correlated to the suppression of matrix metalloproteinases (Kim et al. 2013). Translating the beneficial effect of HSP70 to the clinical level may include the use of pharmacological inducers of HSP70, such as those compounds which inhibit the 90 kDa heat shock protein (HSP90) (Kim et al. 2012).

HSP90 is another molecular chaperone in the same family as HSP70, and is known to regulate HSP70 induction. It is normally bound to heat shock factor (HSF), but under conditions of stress, HSP90 dissociates from HSF, and HSF can bind the promoter of HSP70, heat shock element (HSE) (Sharp et al. 1999). Thus, by inhibiting HSP90, HSP70 can be pharmacologically induced. Several HSP90 inhibitors (also referred to as HSP70 inducers) have already been studied as a potential cancer treatment, both at the preclinical and clinical levels (Hwang et al. 2009) (Soga et al. 2013). Geldanamycin (GA) and its analog 17-Allylamino-17-demethoxygeldanamycin (17-AAG), represent a class of HSP90 inhibiting drugs, the benzoquinone ansamycin antibiotics, capable of inducing HSP70 through its ability to bind and disrupt the function of HSP90 (Georgakis and Younes 2005; Hwang et al. 2009; Kim et al. 2012; Miyata 2005). HSP90 inhibitors, by virtue of their ability to induce HSP70, has been shown to promote protection against tissue injury (Harrison et al. 2008; Kwon et al. 2008; Lu et al. 2002; Ouyang et al. 2005; Sonoda et al. 2010; Xu et al. 2003).

Recent findings also highlight the role of HSPs in modulating immune response and the inflammatory pathways, and the benefit of overexpression of HSP70 and anti-HSP90 therapy against inflammation during infection, immune disease, or ischemia/reperfusion induced injury such as experimental stroke (Jones et al. 2011; Kim et al. 2012; Poulaki et al. 2007; Rice et al. 2008; Tsan and Gao 2009; Yenari et al. 2005; Zheng et al. 2008). To our knowledge, there have been no previous studies examining the role of HSP90 inhibitors in brain inflammation.

We previously developed a model of inflammation-induced brain endothelial cell injury in which we can study the effects of inflammatory responses on the cerebral vasculature (Kacimi et al. 2011). In this model, application of the endotoxin lipopolysaccharide (LPS) to cocultures of brain derived endothelial cells and microglia lead to endothelial cell death. We use this model to examine a panel of HSP90 inhibitors, and examine their potential in protecting cells of the brain's vasculature.

Material and Methods

Materials

All reagents were high grade and were purchased from Sigma with the following exceptions. RPMI, DMEM, Calcein and other culture reagents were purchased from Invitrogen Inc (Grand Island, NY, USA) and the UCSF cell culture facility (UCSF, San Francisco, CA). Fetal bovine Serum Defined (FBS) was purchased from Hyclone Laboratories (Logan, UT, USA). Selective HSP90 inhibitors: Geldanamycin, 17-AAG, and radicicol, and BIIB021 were purchased from Calbiochem (San Diego, CA). Lipopolysaccharide (LPS, *Escherichia coli*, O26:B6) was purchased from Sigma (St Louis, MO). Drugs were dissolved in DMSO or ethanol and stored at -20°C and either used (final concentration of vehicle 0.1% (v/v or

dried down and resuspended in PBS/0.1% bovine serum albumin (BSA). HSP antibodies were purchased from Assay design (Enzo Life sciences), for MAP stress activated kinase anti-phospho-JNK/SAPK mAb (#4668) were from Cell Signaling Technology (Danvers, MA); anti-NF-kBp65 (# SC-8008), anti-IkB α (# SC-1643) and respective horseradish peroxidase-coupled secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA) and antibodies against mouse HSP70i (#SPA-810) from Enzo Life sciences, iNOS (# 61043), was from BD Biosciences (BD Biosciences, Lexington, KY); COX-2 antibody is from Cayman Chemical Company (Ann Arbor, MI (# 160106).

Cell culture

Glial cell preparation—Primary mouse astrocytes were isolated and prepared by proteolytic dissociation of neonatal cortical tissue and cultured using standard protocols as previously described (Yenari et al. 2006). Brains obtained from 1–3 day old pups were minced and resuspended in perfusion buffer with 10% fetal bovine serum (FBS). To remove debris, the suspension then was passed through a filter (Falcon). Cells that passed through the filter were pelleted by centrifugation at 500 $\times$ g for 5min, and the supernatant was removed. The pellet was washed twice, and resuspended in Minimal Essential Medium (MEM) containing 10% FBS supplemented with antibiotics (penicillin/streptomycin, 100U/ml). After 1 h of attachment to tissue culture plates, cells that were weakly attached or unattached (neuronal cells, endothelial cells, smooth muscle cells, and red blood cells) were rinsed free and discarded. Adherent cells either pure astrocytes or microglia enriched mixed glial cells were grown and used for 1–3 passages, as previously described (Yenari et al. 2006). Some astrocyte cultures were exposed to 5 h hypoxia followed by reoxygenation for 24 h, using an anoxia chamber as previously described (Lee et al. 2004). In astrocytes, hypoxia, unlike oxygen-glucose deprivation, does not cause significant cell stress or death. As a positive control, other astrocyte cultures were exposed to heat stress using a paradigm previously shown to induce endogenous HSP70 (Kim et al. 2002).

BV2 cells—The immortalized mouse microglia cell line, BV2 cells exhibit both the phenotypic and functional properties of reactive microglia cells and are a suitable model of inflammation as previously described (Kacimi et al. 2011; Webster et al. 2013). Briefly, cells were grown and maintained in RPMI supplemented with 10% fetal bovine serum and antibiotics (penicillin/streptomycin, 100U/ml). Under a humidified 5% CO₂/95% air atmosphere and at 37°C, cells were plated in 75cm² cell culture flask (Corning, Acton, MA, USA) and were split twice a week. For the experiments, cells were placed on 6-well dishes as previously described (Kacimi et al. 2011).

bEND.3 cells—The immortalized mouse brain microvascular endothelial cell line, bEND. 3, was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 450 mg/dl glucose, 10% fetal bovine defined, and antibiotics. For co-culture of BV2 cells and microvascular endothelial cells, bEND.3 cells were grown to confluence in DMEM with serum, thereafter BV2 cells were seeded on the top of the monolayer and set to adhere for 24 hours before each experimental design as previously described (Kacimi et al. 2011).

Experimental protocols

Cell treatment—Cells were cultured to approximately 80% confluence, *and cultures with a density of approximately 0.5×10^6 cells were used. Similar culture densities were verified prior to use.* Fresh serum-free media was added for 4–24h before LPS or inhibitors treatments. All inhibitors were applied 1h before experimental treatment and given in doses as follows unless otherwise specified: geldanamycin (100 nM), 17-AAG (500 nM), *BIIB021* (100 nM) and radicicol (10 μ M). LPS was used in a concentration of 1 μ g/ml, H₂O₂ 500 μ M, and poly (I:C) 100 μ g/ml. These concentrations were based on our prior work in this model of endothelial cells and microglia, as well as preliminary dose response assays for all the treatments used. The LPS, Poly (I:C) and H₂O₂ concentrations were based on those used in the literature as well as that found in our hands to cause approximately 60–70% cell death. For the HSP90 inhibitors, we used optimal concentrations that inhibited NO and NO signaling proteins without observable cytotoxic effect on cells as previously described (Kacimi et al. 2011).

Cell transfection &HSP70 knockdown

Standard siRNA transfection was used for transient silencing of Hsp72 in BV2 cells. Cells were incubated in 6 well plates for 48 to 72 hours with either nontargeting siRNA control or HSP70 smart pool siRNA constructs from Dharmacon/ Thermo Scientific, (Waltham, MA) in Opti-DMEM media. Lipofectamine RNAiMAX transfection reagent (Invitrogen) was used to enhance siRNA delivery according to the manufacturer's instructions. To validate our transfection assay we used Alexa Fluor® Red Fluorescent Control siRNA as a positive control. Overall efficiency of transfection using this protocol was about 60–70 % with minimal toxicity to the cells. After 2 days of transfection with either control siRNA or HSP70 siRNA for knockdown, cell culture media was changed to serum free defined media, cells were thereafter pretreated with 17-AAG for 1Hour and subsequently incubated with the TLR4 agonist LPS or the TLR3 agonist Poly (I:C). 24 h later, NO accumulation was evaluated using the Griess reagent as described in the methods below.

Fluorescence microscopy—Fluorescence immunocytochemistry was performed on cells as previously described (Kacimi et al. 2011). The wells were washed twice in PBS and then fixed with acetone/methanol (1:1) 5min at –20°C. Alternatively, cells were fixed in 4% paraformaldehyde for 30 min at room temperature. The cells were then washed twice with PBS containing 0.2% Triton X-100 for 15 min. Nonspecific binding sites were blocked in blocking buffer (2% BSA and 0.2% Triton X-100 in PBS) for 2hr. The cells were incubated with primary antibody specific marker for the vascular unit cells as indicated at 1:100 dilution in blocking buffer overnight at 4°C and then washed three times with blocking buffer, 10 min per wash. The cells were incubated with either alexa or FITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) at 1:100 dilution in blocking buffer at RT for 1 h, then washed 2 times in blocking buffer, and one time in PBS, 10 min per wash. Fluorescence was visualized with an epifluorescence microscope (Zeiss Axiovert; Carl Zeiss Inc), and images were obtained on a PC computer using Axiomatic software (Zeiss Inc).

NO measurement—Accumulation of NO in cultures media was determined by the Greiss reagent using nitrite as standard as previously described (Han et al. 2002; Kacimi et al. 2011). After 24h of incubation, serum-free media was removed and fresh media was added. LPS or vehicle was then added, and cells were returned to the incubator. After incubation for 24 or 48h, aliquots of the incubation media were removed and either stored at -80°C or used immediately for nitrite content analysis.

Immunoblotting—After each treatment period, cells plated on 6 well or 60-mm dishes were washed with cold phosphate buffered saline, and scraped into 500 μl lysis buffer. Lysates were sonicated and centrifuged for 5min. The supernatant was collected and either used immediately or frozen at -80°C . Protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL), and equal amounts of protein were loaded per lane onto 10–12% sodium dodecylsulfate–polyacrylamide gels, and were electrophoresed (SDS–PAGE) as previously described (Kacimi et al. 2000; Kacimi and Gerdes 2003; Kacimi et al. 2011). Gels were then transferred onto enhanced chemiluminescence (ECL)–nylon membranes in transfer buffer containing 48mM Tris, 150mM glycine, and 10% methanol using a Transblot apparatus (Biorad, Hercules, CA, USA) at 100V for 1hr at 4°C . The membranes were saturated in phosphate buffer (PBS) 0.1% Tween-20, and 5% non-fat dry milk for 1hr at room temperature and then probed with specific polyclonal antisera for iNOS and cyclooxygenase-2 (COX-2) in the same buffer for 1h at room temperature with gentle agitation. Anti-phospho-JNK and phospho-JAK2 mAbs were from Cell Signaling Technology (Danvers, MA). For all antibodies used, working dilution was (1:500 and 1:1000) for rabbit and mouse primary antibodies, respectively. Membranes were washed three times with PBS 0.1% Tween-20. Bound antibodies were identified after incubation with peroxidase-conjugated anti-rabbit antibodies (1:2000 dilution in saturation buffer) for 1h at room temperature. Membranes were then rewashed three times and the position of the individual proteins was detected by chemiluminescence ECL according to the manufacturer's instruction.

Assessment of I κ B- α degradation and NF- κ B nuclear translocation—I κ B α in cytoplasmic extracts was detected by Western blot and nuclear translocation of the p65 NF κ B subunit was determined by immunofluorescence using specific antibodies against I κ B α and NF- κ B p65 respectively.

Cell viability assays—Cell viability was assessed by a diazo dye (Sun et al. 2006)–2, 5-diphenyltetrazolium bromide (MTT) assay, that measures mitochondrial respiration, an index of cell viability. At the end of the observation period, media was harvested, and MTT was added. Thereafter the absorbance of each sample was read by a multiwell spectrophotometer at 570-nm wavelength. Cell viability was calculated as the percentage of absorbance in each sample versus the control. Alternatively, *trypan* blue exclusion and calcein stain was assayed for cell viability (calcein stain) as previously described (Kacimi et al. 2000; Kacimi et al. 2011).

Statistical analysis—All data were analyzed in a blinded fashion by investigators unaware of the experimental condition. Significant differences were determined by either

Student's two-tailed *t*-test for comparison of the means of two samples or analysis of variance (ANOVA) for the comparison of more than two sample means followed by Newman–Keuls post-hoc testing for multiple comparisons among sample means. The significance level was set at $P < 0.05$. Data are shown mean $\pm$ SEM.

Results

We first established that heat shock and geldanamycin (GA) treatments led to HSP70 induction (Figure 1A–B). GA dose dependently increased HSP70 in primary astrocyte cultures (Figure 1B). Similarly, other HSP90 inhibitors 17-AAG, radicicol and BIIB021 also induced HSP70 (Figure 1C). We then determined the kinetics of HSP70 induction in the setting of endotoxin treatment with and without HSP90 inhibition by 17-AAG (Figure 1D). BV2 cells were treated with lipopolysaccharide (LPS) and the time course of HSP70 induction was determined at different times with and without treatment with 17-AAG. We found that LPS itself induced HSP70 as early as 1 h post application, then continued to increase 3 and 6 h later, but was no longer detectable by 12 h. In the presence of 17-AAG, induction of HSP70 above that by LPS alone was evident by 6h and persisted at 12 h when LPS-induced HSP70 was no longer present.

We then treated astrocytes exposed to H_2O_2 with these compounds 24 h prior to injury, and found that in all cases, they protected against oxidative stress (Figure 2). The concentration chosen was based on pilot studies to determine the highest concentration of each compound that did not lead to toxicity (as evidenced by morphological changes in uninjured astrocytes or b.End.3 cells, data not shown). These agents also protected microglial BV2 cells from H_2O_2 induced injury (data not shown).

We then explored the effect of HSP70 induction and potential anti-inflammatory effects. Primary microglia enriched astrocyte cultures (mixed glial cultures prepared without removal of the microglia) were exposed to LPS, leading to activation of primarily microglia, but astrocytes as well, and leads to increased nitric oxide (NO) generation. Application of HSP90 inhibitors in concentrations previously shown to be cytoprotective, all led to the abrogation of NO production (Figure 2D).

We next turned to a co-culture model of brain derived endothelial cells and microglia, where LPS treatment leads to endothelial cell death via microglial activation (Kacimi et al. 2011). Cocultures of bEnd.3 and BV2 cells were prepared, then treated with HSP90 inhibitors followed by LPS exposure. In all cases, *HSP90 inhibitors* led to improved cell viability (Figure 3). Since LPS treatment in this model leads predominantly to bEnd.3 cell death, the protection was largely due to protection of bEnd.3 cells. However, when cultured alone, BV2 cells are also vulnerable to LPS exposure, and this vulnerability can be prevented by treatment with HSP90 inhibitors. BV2 cell death was reduced as assayed by calcein uptake and MTT (Figure 4). To determine whether these HSP70 inducers might exhibit anti-inflammatory effects, we measured both iNOS protein levels and NO accumulation by BV2 cells following LPS exposure. In all cases, *HSP90 inhibitor* treatment led to suppression of NO production and iNOS protein expression (Figure 5A–B). Prior work has shown that HSP70's immune modulating properties are due to interactions with TLR4, the receptor

upon which LPS acts. However, other work, including that from our own lab, has shown that HSP70 can also interfere with the pro-inflammatory transcription factor nuclear factor kappa B (NF- κ B) (Ran et al. 2004; Zheng et al. 2008). To determine whether the anti-inflammatory mechanism is through an interaction through TLR4 in our model or an alternate mechanism, we treated BV2 cells with the TLR3 agonist Poly (I:C). Poly (I:C) treatment also led to NO accumulation; however, treatment with the same HSP90 inhibitors also suppressed NO generation by Poly (I:C) in a manner similar to LPS, suggesting that the anti-inflammatory effect of HSP70 is not specific to TLR4 signaling (Figure 5C).

To determine whether the anti-inflammatory effect of HSP70 induction was through NF- κ B or other factors, we assayed for NF- κ B nuclear translocation, I κ B α degradation, and JAK-STAT and JNK activation. We chose these factors based on our previous study that showed that these are the main transcription factors that lead to iNOS induction by LPS in BV2 cells (Kacimi et al. 2011). Accordingly, we found that the *HSP90 inhibitors* prevented nuclear NF- κ B translocation, I κ B α degradation, and JAK2 phosphorylation following LPS exposure (Figure 6). JNK phosphorylation was affected *to lesser extent*.

To establish whether the effect of 17-AAG's anti-inflammatory effect was due to the induction of HSP70, we used siRNA to knock down HSP70 in BV2 cells. Using control siRNA containing Alexa Fluor to identify cells which took up the siRNA, we found that approximately 60% of the cells took up the reagent without any significant cytotoxicity (Fig 7A). In addition, in order to estimate the extent of HSP70 knockdown we performed Western blots of HSP70. Our data show that 17-AAG increased HSP70, but this was prevented by HSP70 siRNA knockdown (Figure 7B). Knockdown of HSP70 blunted the anti-inflammatory effect of 17-AAG against TLR3 & TLR4 agonists, as estimated by nitrite (NO) accumulation in the culture media (Fig 7C–D). The proportion of blunting was roughly similar to the extent of HSP70 knockdown. These data together indicate that HSP70 is responsible for the anti-inflammatory effect of 17-AAG. A proposed mechanism of 17-AAG's anti-inflammatory response against TLR4 and TLR3 in microglia BV2 cells is shown in Fig 8.

Discussion

We show that HSP70 can be pharmacologically induced by a panel of HSP90 inhibitors, and that this induction can protect brain derived endothelial cells and astrocytes against oxidative and inflammatory stress. Pharmacologic HSP70 induction in microglial cells also exhibited anti-inflammatory responses. *Further, we show that this is largely through the induction of HSP70, as knockdown of HSP70 led to the reduction of this anti-inflammatory effect.* These observations are in line with that previously shown by us, where we used genetic approaches to selectively overexpress HSP70 (Lee et al. 2005; Zheng et al. 2008). Since it is not possible to genetically overexpress HSP70 in humans, we show a potentially translatable pharmacological means of doing so. These data suggest a therapeutic potential for HSP90 inhibitors to protect from pro-inflammatory insults, such as that seen in stroke, brain trauma and neuroinflammation. We previously showed that HSP70 overexpression in either transgenic mice or cells treated with a viral vector encoding HSP70 inhibits the generation and activation of proteases implicated in the destruction of the brain's extracellular matrix

(Lee et al. 2004) and decreases BBB disruption and subsequent brain hemorrhage (Kim et al. 2002). Thus, pharmacological HSP70 induction has the potential to treat clinical conditions where preservation of the BBB and prevention of brain hemorrhage may be desirable.

We studied a panel of HSP90 inhibitors that have previously been studied at the clinical level in patients with cancer (Jhaveri et al. 2012). We replicated prior studies of geldanamycin (GA) which showed protection in brain cells (Xu et al. 2003), then explored whether similar protection could be observed with other inhibitors. While GA has previously been shown to protect against experimental stroke (Kwon et al. 2008; Lu et al. 2002) and related brain injury models (Ouyang et al. 2005; Xiao et al. 1999), it was poorly tolerated in humans due to liver toxicity (Supko et al. 1995). Thus, we expanded our observations to include additional HSP90 inhibitors, radicicol, 17-AAG, and BIIB021. 17-AAG was developed as a less toxic analogue of GA, and is the most widely studied HSP90 inhibitor in over 30 clinical studies including some at the phase 3 level (Porter et al. 2010). BIIB021 has also been studied in humans in phase 2 studies of cancer, and is also available in a form that can be given orally (Lundgren et al. 2009; Porter et al. 2010). Radicicol was identified as a naturally occurring HSP90 inhibitor, but was unfortunately found to degrade quickly in vivo (Porter et al. 2010). Most of these studies were carried out in cancer trials, but little work has been carried out in cell injury models, although radicicol was shown to protect renal cells from ischemia-like insults (Sonoda et al. 2010). Regardless, we found similar effects for all HSP90 *inhibitors* in our in vitro model, and would support further investigation of any or all of these compounds.

We used an in vitro model of endotoxin activated microglia which in turn led to damage to brain endothelial cells (Kacimi et al. 2011). This model could be said to model aspects of brain ischemia/trauma, neuroinflammation and sepsis where injurious or inflammatory stimuli activate immune cells and lead to BBB disruption (Alvarez et al. 1996; Hanisch. 2002; Lehnardt. 2010). BBB disruption can lead to secondary damage due to leakage of serum proteins into the brain leading to edema and cytotoxicity. With extreme BBB disruption, hemorrhage can occur. Thus, by protecting the brain's vasculature from immune attack through HSP70 induction, secondary consequences of brain injury could be ameliorated. This beneficial effect on endothelial cells was related to the suppression of immune responses in microglia.

Past studies have indicated that HSP70 can act as ligands for the Toll-like receptors (TLRs) present on immune cells including microglia (Giffard et al. 2008). In order for HSP70 to bind TLRs, it would need to be extracellular. In our model, HSP70 was generated intracellularly, and while it is possible that it could have been secreted, our data suggest that it likely acts in the intracellular compartment. Further, HSP70 binding to TLRs tends to result in pro-inflammatory signaling, whereas our data indicate that its actions are anti-inflammatory.

LPS was used to stimulate NO production and iNOS protein synthesis in microglia, and these stimulated microglia led to endothelial cell disruption. Treatment with HSP90 inhibitors suppressed LPS induced activation of microglia with parallel decreases in

endothelial cell death. Since LPS activates microglia by binding and activating the TLR4 pathway (Holm *et al.* 2012), we explored whether other TLR pathways might be similarly affected. Interestingly, geldanamycin also inhibited NO and iNOS generation by the TLR3 agonist Poly (I:C). Thus, the anti-inflammatory effect of HSP70 is likely downstream of the TLRs, and probably also acts intracellularly where it is generated through HSP90 inhibition.

Prior work relating HSP70 and its ability to suppress immune responses have also indicated that HSP70 may interfere with the activation of pro-inflammatory transcription factors such as NFkB (Ran *et al.* 2004; Zheng *et al.* 2008). We previously showed that in our model system, LPS activated microglia lead to pro-inflammatory responses through NFkB and JAK/STAT activation, *and these responses could be inhibited by their respective inhibitors* (Kacimi *et al.* 2011). Accordingly, we found HSP90 inhibitors prevented IκB degradation, nuclear NFkB translocation and JAK-STAT phosphorylation due to LPS. *Thus, the mechanism of HSP90 inhibitors on inflammation may be related to the inhibition of the pro-inflammatory factors.*

In sum, HSP90 inhibitors induce HSP70, lead to anti-inflammatory effects and protect brain derived endothelial cells from death. These inhibitors should be studied in appropriate in vivo models of brain injury and inflammation. Work from our lab indicates that at least GA and 17-AAG will penetrate into the brain following parenteral administration, and will decrease BBB disruption and brain hemorrhage (Kim *et al.* 2015). Future work should be considered in terms of bringing one or more of these compounds to the clinic for neurological disorders.

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References

- Alvarez JI, Katayama T, Prat A. Glial influence on the blood brain barrier. *Glia*. 2013; 61:1939–1958. [PubMed: 24123158]
- Georgakis GV, Younes A. Heat-shock protein 90 inhibitors in cancer therapy: 17AAG and beyond. *Future Oncol*. 2005; 1(2):273–81. [PubMed: 16555999]
- Giffard RG, Han RQ, Emery JF, Duan M, Pittet JF. Regulation of apoptotic and inflammatory cell signaling in cerebral ischemia: the complex roles of heat shock protein 70. *Anesthesiology*. 2008; 109(2):339–48. [PubMed: 18648242]
- Han HS, Qiao Y, Karabiyikoglu M, Giffard RG, Yenari MA. Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen production in experimental stroke and inflammation. *J Neurosci*. 2002; 22(10):3921–8. [PubMed: 12019311]
- Harrison EM, Sharpe E, Bellamy CO, McNally SJ, Devay L, Garden OJ, Ross JA, Wigmore SJ. Heat shock protein 90-binding agents protect renal cells from oxidative stress and reduce kidney ischemia-reperfusion injury. *Am J Physiol Renal Physiol*. 2008; 295(2):F397–405. [PubMed: 18562631]
- Hwang M, Moretti L, Lu B. HSP90 inhibitors: multi-targeted antitumor effects and novel combinatorial therapeutic approaches in cancer therapy. *Curr Med Chem*. 2009; 16(24):3081–92. [PubMed: 19689285]

- Jhaveri K, Taldone T, Modi S, Chiosis G. Advances in the clinical development of heat shock protein 90 (Hsp90) inhibitors in cancers. *Biochim Biophys Acta*. 2012; 1823(3):742–55. [PubMed: 22062686]
- Jones Q, Voegeli TS, Li G, Chen Y, Currie RW. Heat shock proteins protect against ischemia and inflammation through multiple mechanisms. *Inflamm Allergy Drug Targets*. 2011; 10(4):247–59. [PubMed: 21539516]
- Kacimi R, Chentoufi J, Honbo N, Long CS, Karliner JS. Hypoxia differentially regulates stress proteins in cultured cardiomyocytes: role of the p38 stress-activated kinase signaling cascade, and relation to cytoprotection. *Cardiovasc Res*. 2000; 46(1):139–50. [PubMed: 10727662]
- Kacimi R, Gerdes AM. Alterations in G protein and MAP kinase signaling pathways during cardiac remodeling in hypertension and heart failure. *Hypertension*. 2003; 41(4):968–77. [PubMed: 12642504]
- Kacimi R, Giffard RG, Yenari MA. Endotoxin-activated microglia injure brain derived endothelial cells via NF-kappaB, JAK-STAT and JNK stress kinase pathways. *J Inflamm (Lond)*. 2011; 8:7. [PubMed: 21385378]
- Kim JY, Kim N, Zheng Z, Lee JE, Yenari MA. The 70 kDa heat shock protein protects against experimental traumatic brain injury. *Neurobiol Dis*. 2013; 58:289–95. [PubMed: 23816752]
- Kim JY, Kim YJ, Giffard RG, Yenari MA, Lee JE. Heat treatment and hsp70-overexpression suppressed matrix metalloproteinases in the primary astrocytes after ischemia-like injury. *Neurology*. 2002; 58(7 Suppl 3):A364.
- Kim N, Kim JY, Yenari MA. Anti-inflammatory properties and pharmacological induction of Hsp70 after brain injury. *Inflammopharmacology*. 2012; 20(3):177–85. [PubMed: 22246599]
- Kim N, Kim JY, Yenari MA. Pharmacological induction of the 70-kDa heat shock protein protects against brain injury. *Neuroscience*. 2015; 284:912–9. [PubMed: 25446362]
- Kwon HM, Kim Y, Yang SI, Kim YJ, Lee SH, Yoon BW. Geldanamycin protects rat brain through overexpression of HSP70 and reducing brain edema after cerebral focal ischemia. *Neurol Res*. 2008; 30(7):740–5. [PubMed: 18534054]
- Lee JE, Kim YJ, Kim JY, Lee WT, Yenari MA, Giffard RG. The 70 kDa heat shock protein suppresses matrix metalloproteinases in astrocytes. *Neuroreport*. 2004; 15(3):499–502. [PubMed: 15094511]
- Lee JE, Yoon YJ, Moseley ME, Yenari MA. Reduction in levels of matrix metalloproteinases and increased expression of tissue inhibitor of metalloproteinase-2 in response to mild hypothermia therapy in experimental stroke. *J Neurosurg*. 2005; 103(2):289–97.
- Lu A, Ran R, Parmentier-Batteur S, Nee A, Sharp FR. Geldanamycin induces heat shock proteins in brain and protects against focal cerebral ischemia. *J Neurochem*. 2002; 81(2):355–64. [PubMed: 12064483]
- Lundgren K, Zhang H, Brekken J, Huser N, Powell RE, Timple N, Busch DJ, Neely L, Sensintaffar JL, Yang YC, et al. BIIB021, an orally available, fully synthetic small-molecule inhibitor of the heat shock protein Hsp90. *Mol Cancer Ther*. 2009; 8(4):921–9. [PubMed: 19372565]
- Miyata Y. Hsp90 inhibitor geldanamycin and its derivatives as novel cancer chemotherapeutic agents. *Curr Pharm Des*. 2005; 11(9):1131–8. [PubMed: 15853661]
- Ouyang YB, Xu L, Giffard RG. Geldanamycin treatment reduces delayed CA1 damage in mouse hippocampal organotypic cultures subjected to oxygen glucose deprivation. *Neurosci Lett*. 2005; 380(3):229–33. [PubMed: 15862891]
- Porter JR, Fritz CC, Depew KM. Discovery and development of Hsp90 inhibitors: a promising pathway for cancer therapy. *Curr Opin Chem Biol*. 2010; 14(3):412–20. [PubMed: 20409745]
- Poulaki V, Iliaki E, Mitsiades N, Mitsiades CS, Paulus YN, Bula DV, Gragoudas ES, Miller JW. Inhibition of Hsp90 attenuates inflammation in endotoxin-induced uveitis. *Faseb J*. 2007; 21(9):2113–23. [PubMed: 17400913]
- Ran R, Lu A, Zhang L, Tang Y, Zhu H, Xu H, Feng Y, Han C, Zhou G, Rigby AC, et al. Hsp70 promotes TNF-mediated apoptosis by binding IKK gamma and impairing NF-kappa B survival signaling. *Genes Dev*. 2004; 18(12):1466–81. [PubMed: 15198984]
- Rice JW, Veal JM, Fadden RP, Barabasz AF, Partridge JM, Barta TE, Dubois LG, Huang KH, Mabbett SR, Silinski MA, et al. Small molecule inhibitors of Hsp90 potentially affect inflammatory disease

- pathways and exhibit activity in models of rheumatoid arthritis. *Arthritis Rheum.* 2008; 58(12): 3765–75. [PubMed: 19035474]
- Sharp FR, Massa SM, Swanson RA. Heat-shock protein protection. *Trends Neurosci.* 1999; 22(3):97–9. [PubMed: 10199631]
- Soga S, Akinaga S, Shiotsu Y. Hsp90 inhibitors as anti-cancer agents, from basic discoveries to clinical development. *Curr Pharm Des.* 2013; 19(3):366–76. [PubMed: 22920907]
- Sonoda H, Prachasilchai W, Kondo H, Yokota-Ikeda N, Oshikawa S, Ito K, Ikeda M. The protective effect of radicicol against renal ischemia–reperfusion injury in mice. *J Pharmacol Sci.* 2010; 112(2):242–6. [PubMed: 20093793]
- Stetler RA, Gan Y, Zhang W, Liou AK, Gao Y, Cao G, Chen J. Heat shock proteins: cellular and molecular mechanisms in the central nervous system. *Prog Neurobiol.* 2010; 92(2):184–211. [PubMed: 20685377]
- Sun Y, Ouyang YB, Xu L, Chow AM, Anderson R, Hecker JG, Giffard RG. The carboxyl-terminal domain of inducible Hsp70 protects from ischemic injury in vivo and in vitro. *J Cereb Blood Flow Metab.* 2006; 26(7):937–50. [PubMed: 16292251]
- Supko JG, Hickman RL, Grever MR, Malspeis L. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother Pharmacol.* 1995; 36(4):305–15. [PubMed: 7628050]
- Tsan MF, Gao B. Heat shock proteins and immune system. *J Leukoc Biol.* 2009; 85(6):905–10. [PubMed: 19276179]
- Webster CM, Hokari M, McManus A, Tang XN, Ma H, Kacimi R, Yenari MA. Microglial P2Y12 Deficiency/Inhibition Protects against Brain Ischemia. *PLoS One.* 2013; 8(8):e70927. [PubMed: 23940669]
- Xiao N, Callaway CW, Lipinski CA, Hicks SD, DeFranco DB. Geldanamycin provides posttreatment protection against glutamate-induced oxidative toxicity in a mouse hippocampal cell line. *J Neurochem.* 1999; 72(1):95–101. [PubMed: 9886059]
- Xu L, Ouyang YB, Giffard RG. Geldanamycin reduces necrotic and apoptotic injury due to oxygen-glucose deprivation in astrocytes. *Neurol Res.* 2003; 25(7):697–700. [PubMed: 14579785]
- Yenari MA, Liu J, Zheng Z, Vexler ZS, Lee JE, Giffard RG. Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection. *Ann N Y Acad Sci.* 2005; 1053:74–83. [PubMed: 16179510]
- Yenari MA, Xu L, Tang XN, Qiao Y, Giffard RG. Microglia potentiate damage to blood-brain barrier constituents: improvement by minocycline in vivo and in vitro. *Stroke.* 2006; 37(4):1087–93. [PubMed: 16497985]
- Zheng Z, Kim JY, Ma H, Lee JE, Yenari MA. Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke. *J Cereb Blood Flow Metab.* 2008; 28(1):53–63. [PubMed: 17473852]

Main points

Activated microglia are toxic to brain derived endothelial cells. Pharmacologically inducing HSP70 by HSP90 inhibitors protects endothelial cells via an anti-inflammatory mechanism.

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Figure 1. A: Heat shock induces HSP70 in astrocytes

A Shown is a representative Western blot of primary astrocyte whole cell extracts using a mouse antibody specific for inducible HSP70 and graph to quantify these observations. HXR: 5h hypoxia/ 24 h reoxygenation, HS: Heat shock; 30min @42.5°C +1day recovery, CT: control cultures were not exposed to insults. B: Geldanamycin (GA) mimics the heat shock response by inducing HSP70 in astrocytes. A representative Western blot and graph of the data are shown. Treatment of astrocytes with GA for 24h leads to dose dependent increases of HSP70 protein, much like that seen with heat shock treatment. C: GA and

similar HSP90 inhibitors increased HSP70 in astrocytes. Treatment of astrocytes with GA and GA analog 17-AAG, radicicol or BIIB021 for 24h induces HSP70 protein expression to similar levels as GA and heat shock treatment. *D-G : Kinetics of HSP70 induction in the setting of endotoxin treatment with and without HSP90 inhibition by 17-AAG. BV2 cells were treated with lipopolysaccharide (LPS) and the time course of HSP70 induction was determined at different times (1–12 h) with and without treatment with 17-AAG. Optical densitometric values were normalized to β -actin as a housekeeping control, and are expressed as percentage of controls. Data are shown mean $\pm$ SEM, n = 3–5 independent experiments *P<0.05*

Figure 2. HSP90 inhibitors protect astrocytes against oxidative stress

A: Astrocytes were treated with geldanamycin (GA+), 17-AAG (17-AAG+), radicicol (Radicicol+) or vehicle (H₂O₂) 24 h prior to H₂O₂ exposure. Light microscopy shows astrocyte damage due to 2h H₂O₂ exposure, and this was prevented by all three HSP90 inhibitors. Control, uninjured and untreated cultures are shown for comparison (Control). B: Cell viability was quantified by MTT. n=3–5 experiments, P< 0.05 *versus control, # versus H₂O₂. C: **HSP90 inhibitors prevent LPS induced NO generation in glial cells.** Mouse primary glial mixed cells (astrocytes plus ~10% microglia) were treated either alone with

vehicle (control), LPS alone (LPS), or LPS plus individual HSP90 inhibitors (GA+, 17-AAG +, radicicol+, BIIB021+) for 24h, followed by media change, then LPS was added for another 24h. Nitrite accumulation was measured by the Griess reagent. n=5–8 experiments, *P< 0.05 versus control, # versus LPS alone.

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Figure 3. HSP90 inhibitors prevented endothelial cell death due to microglial activation
 A. While LPS did not affect bEND.3 cells alone (bEnd.3+LPS), when cultured with BV2 cells (LPS), LPS increased cell death and monolayer disruption of predominantly bEND.3 cells compared to control cocultures (control). Treatment of cocultures with LPS plus geldanamycin (GA+) prevented monolayer disruption. B. Quantitation of coculture experiments show that cell viability is decreased following LPS treatment, and this is prevented by GA (GA+), 17-AAG (17-AAG+), radicicol (radicicol+). GA: geldanamycin

(0.1–0.2 M), 17-AAG, and (0.5–1 mM); radicicol: (RDC, 10 μ M). n = 4–6 independent observations, *P < 0.05 vs. control, #P < 0.05 versus LPS.

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Figure 4. Geldanamycin prevents LPS-induced injury in microglia BV2 cells

A BV2 cells were treated with LPS or LPS plus HSP90 inhibitors for 24h. Light microscopy (blue cells) or green live cell with calcein immunofluorescence shows LPS- induced BV2 cell injury, which was prevented by all three HSP90 inhibitors. **B**. MTT assay shows that 4 different HSP90 inhibitors prevented LPS induced death of BV2 cells. $P < 0.05$ vs *control, # versus LPS.

Figure 5. HSP90 inhibitors decrease iNOS induction and NO accumulation by LPS in microglia BV2 cells were treated with LPS, vehicle, or LPS plus HSP90 inhibitors for 24hr. **A:** iNOS protein induction by LPS is prevented by all HSP90 inhibitors. **B:** Nitrite accumulation as measured by Griess reagent shows that LPS increases NO, whereas HSP90 inhibitors all decrease it. $n=5-8$ experiments, $P < 0.05$ was considered significant. *versus control, # versus LPS. **C: HSP90 inhibitors prevent TLR3 agonist induced NO in BV2 cells.** BV2 microglia cells were treated with the TLR3 agonist, Poly (I:C) (PIC), either alone with vehicle or HSP90 inhibitors for 24 h. NO levels were estimated by the amount of nitrite

accumulation using the Griess reagent. N=5–8 experiments, $P < 0.05$, *versus control, # versus LPS.

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Figure 6.

A: Immunofluorescent stains of NF-κB in BV2 cells show predominantly cytosolic staining in unstimulated control cells (control). LPS treatment led to overall NF-κB upregulation with staining within the nucleus (LPS). Treatment with 17-AAG (17-AAG+) prevented the LPS induced NF-κB upregulation and nuclear staining is decreased. *Cells were also stained with DAPI to delineate nuclei. Merging (Merge) of the two stains shows NF-κB relative to the nucleus.* **B:** Western blots of BV2 cells show that LPS decreases expression of NF-κB's endogenous inhibitor IκBα. This is consistent with NF-κB activation, since IκB is degraded,

thus permitting NF- κ B to translocate to the nucleus. This decrease is prevented by 17-AAG treatment. C & D: Similarly, LPS led to increases in phosphorylated JAK2 (p-JAK2) (C) and JNK1/2 (p-JNK1/2) (D). 17-AAG inhibited pJAK2 expression, and to a lesser extent, pJNK1/2. β -actin is shown as a housekeeping control. *Data in the graphs are expressed as the mean $\pm$ SEM of n= 5 independent experiments. $P<0.05$ *vs control, # vs LPS.*

Figure 7. HSP70 gene knockdown prevents 17-AAG anti-inflammatory response against TLR4 and TLR3 in microglia BV2

A: BV2 cells were transfected with control siRNA linked to Alexa Fluor as a marker of cell uptake. Fluorescent imaging (fluorescent siRNA Control) depicts cells which took up the siRNA as evidenced by the red stain. Phase contrast (Phase) delineates all of the cells from the same field, and these cells do not demonstrate any obvious cytotoxicity. B: BV2 cells were transfected with either control siRNA or siRNA against HSP70. 2 days after transfection, culture media was changed to serum free defined media, and cells were treated with 17-AAG or vehicle (control) for 24h. Western blot was used to determine the efficacy

*of HSP70 silencing. Cultures in which HSP70 was knocked down failed to induce HSP70 by 17-AAG (B). Data in the graphs are expressed as the mean $\pm$ SEM of n= 3 independent experiments. *P<0.05 vs control, # vs 17-AAG treatment in cells in which HSP70 was knocked down. C, D: BV2 cells were transfected with either control siRNA or HSP70 siRNA for 48 h. 2 days after transfection of the BV2 cells, culture media was changed to serum free defined media, then treated with 17-AAG for 1 h, and incubated with the TLR4 agonist LPS (C) or the TLR3 agonist Poly (I:C (PIC)) (D). Control cultures (control) were treated with siRNA but not exposed to agonists. 24 h later NO was estimated by measuring nitrite accumulation using the Griess reagent. Data in the graphs are expressed as the mean $\pm$ SEM of n= 5 independent experiments. P<0.05 * vs control, # vs LPS or PIC treatment, f vs LPS or PIC treatment in cells in which HSP70 was knocked down.*

Fig 8. Proposed mechanism of 17-AAG anti-inflammatory response against TLR4 and TLR3 in microglia BV2 cells

LPS & Poly (I:C) binds to toll-like receptor 4 (TLR4) and TLR3, respectively, on the surface of microglia leading to activation of several downstream signaling pathways: NF- κ B, JAK-STAT and JNK (JNK kinase) that lead to iNOS upregulation and the production of nitric oxide (NO). The HSP90 inhibitor 17-AAG induced HSP70, which, in turn, suppressed the inflammatory response via blockade of NF- κ B, JAK-STAT and JNK signaling pathways responsible for iNOS induction and NO generation.