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A non-toxic Hsp90 inhibitor protects neurons from Aβ-induced toxicity

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Abstract—The molecular chaperones have been implicated in numerous neurodegenerative disorders in which the defining pathology is misfolded proteins and the accumulation of protein aggregates. In Alzheimer's disease, hyperphosphorylation of tau protein results in its dissociation from microtubules and the formation of pathogenic aggregates. An inverse relationship was demonstrated between Hsp90/Hsp70 levels and aggregated tau, suggesting that Hsp90 inhibitors that upregulate these chaperones could provide neuroprotection. We recently identified a small molecule novobiocin analogue, **A4** that induces Hsp90 overexpression at low nanomolar concentrations and sought to test its neuroprotective properties. **A4** protected neurons against Aβ-induced toxicity at low nanomolar concentrations that paralleled its ability to upregulate Hsp70 expression. **A4** exhibited no cytotoxicity in neuronal cells at the highest concentration tested, 10 μM, thus providing a large therapeutic window for neuroprotection. In addition, **A4** was transported across BMECs in vitro, suggesting the compound may permeate the blood–brain barrier in vivo. Taken together, these data establish **A4**, a C-terminal inhibitor of Hsp90, as a potent lead for the development of a novel class of compounds to treat Alzheimer's disease.

The accumulation of aggregated, fibrillar proteins is a unifying characteristic of chronic, late-onset neurodegenerative diseases, leading to intense research on the protein quality control systems within cells. The accumulation of protein aggregates within or outside neurons is a characteristic of the two most common age-related neurodegenerative diseases, Alzheimer's (AD) and Parkinson's disease (PD). AD is characterized by two distinct cytopathologies: β-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs).[1] Normally, the microtubule-associated protein tau is expressed in the neuronal cytoplasm where it serves to stabilize the microtubule network in axons. In AD, however, tau becomes hyperphosphorylated and results in misfolded proteins that dissociate from microtubules and form filamentous aggregates that polymerize into NFTs.[2] PD is characterized by the accumulation of Lewy bodies composed primarily of fibrillar α-synuclein.[3] Other neurodegenerative diseases such as Huntington's disease, amyotrophic lateral sclerosis, prion diseases, and the taupathies are also characterized by similar protein aggregates.

Molecular chaperones are responsible for the conformational maturation of nascent polypeptides, refolding denatured and aggregated proteins, and directing the ubiquitination and degradation of proteins that cannot be repaired. One family of molecular chaperones, the heat shock proteins (Hsps), has a profound effect on critical cellular processes such as cell cycle regulation and apoptosis due to its diverse biological activities.[4,5]

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The major Hsp, Hsp90, exerts its effects through a wide-array of co-chaperones, partner proteins, and immunophilins, forming the major chaperone system responsible for protecting cells against toxicity resulting from the accumulation of protein aggregates. Based on these properties, it was proposed that increased chaperone activity may refold aggregating proteins and have therapeutic applications for the treatment of neurodegenerative diseases. Recently, Greengard and coworkers demonstrated that increased Hsp70 and Hsp90 levels resulted in decreased tau aggregates and provided neuroprotection in cells expressing mutant tau that becomes hyperphosphorylated as in AD brain.[6] Consequently, regulation of Hsp70 and Hsp90 expression levels has been pursued as a novel approach toward the treatment of diseases caused by protein aggregates.

Cellular stresses such as elevated temperature, abnormal pH, oxidative stress, and malignancy result in the denaturation of native proteins as well as the overexpression of molecular chaperones to refold these structures or target them for degradation via the ubiquitin-proteasome pathway.[7–9] Upon exposure to these stresses, Hsp90 and Hsp70 levels are increased to assist in the renaturation process. The expression of these heat shock proteins is tightly regulated by the transcription factor heat shock factor 1 (HSF1). Under normal conditions, Hsp90 forms a stable complex with HSF1 and prevents the transcriptional activation of the heat shock response.[10] Cellular stressors result in destabilization of Hsp90/HSF1, the subsequent trimerization and phosphorylation of HSF1, and its translocation to the nucleus, where it induces Hsp expression.[11,12] Another key protein in the Hsp90 heteroprotein complex is the co-chaperone CHIP (carboxyl terminus of the Hsc70-interacting protein).[13] CHIP binds Hsp70 through its tetratricopeptide repeat (TPR) domain and also possesses intrinsic ubiquitin ligase activity, suggesting a direct link between the chaperone and the ubiquitin-proteasome pathway which may modulate the cellular equilibrium of protein folding and degradation.[14]

Previously identified inhibitors of Hsp90 include geldanamycin (GA, Fig. 1) and its derivatives, which bind to the N-terminal ATP-binding pocket. These N-terminal inhibitors manifest their activity by competitive inhibition of ATP, which serves as the requisite source of energy for the Hsp90-mediated protein folding process. The concentration of N-terminal inhibitors required to induce degradation of Hsp90-dependent client proteins is approximately equal to that needed to increase Hsp70 and Hsp90 levels. Unfortunately, there is a small therapeutic window for the treatment of neurodegenerative diseases with these molecules because cytotoxicity generally occurs at these concentrations. To circumvent these issues, further development of Hsp90 inhibitors for the treatment of neurodegenerative diseases requires the identification of non-toxic inhibitors that provide a large therapeutic window, but stimulate the dissociation of HSF1 from Hsp90 at low concentrations and penetrate the blood–brain barrier

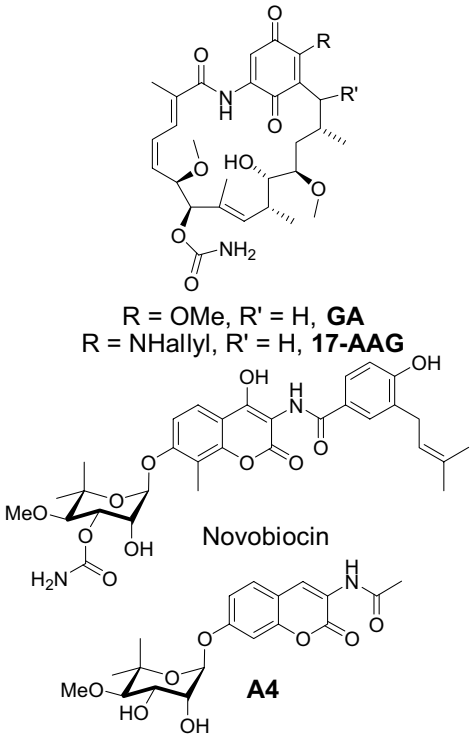
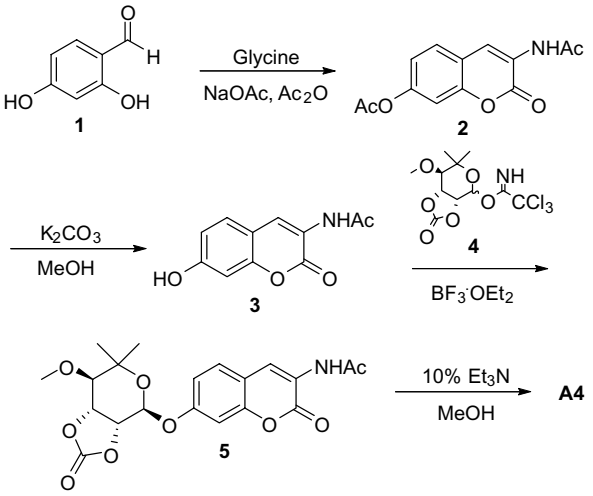


Figure 1. Structures of Hsp90 inhibitors. Structures of the N-terminal Hsp90 inhibitors, GA and 17-AAG, and the C-terminal inhibitors, novobiocin and **A4**.

(BBB). A compound with such attributes could potentially regulate the refolding of protein aggregates, including tau, or initiate their degradation through the ubiquitin-proteasome pathway and provide an alternative approach toward the development of drugs for these diseases.

Recently, an additional ATP-binding site on the C-terminus of Hsp90 was elucidated and novobiocin (Fig. 1) was identified as a competitive inhibitor of ATP with low affinity (~700 μM).[15] Elucidation of this new inhibitor and nucleotide-binding domain provided a novel opportunity to regulate the Hsp90-mediated protein folding machinery with small molecules. In an effort to prepare more efficacious inhibitors, a small library of novobiocin analogues was prepared and evaluated.[16] **A4** induced Hsp90 expression at concentrations significantly lower than those needed for client protein degradation. Because of this finding, we proposed that **A4** possessed unique properties that could prove useful for the treatment of neurodegenerative diseases. In this article, we detail the neuroprotective effects of **A4** and describe its potential promise as a therapeutic lead compound for the treatment of Alzheimer's disease.

In order to prepare sufficient quantities of **A4** for biological testing, it was necessary to devise an efficient synthetic route. As shown in Scheme 1, the coumarin ring (**2**) was constructed by the condensation of commercially available benzaldehyde **1** with glycine in the presence of acetic anhydride.[17] After selective



Scheme 1. Synthesis of **A4**.

deprotection, the free phenol was coupled with the trichloroacetimidate of noviose carbonate (**4**)[18] in the presence of catalytic boron trifluoride etherate.[19] **A4** was furnished in excellent yield by treatment of the cyclic carbonate **5** with triethylamine in methanol, resulting in solvolysis of the carbonate to afford the desired product.

Treatment of embryonic primary neurons and neuronal cell cultures with $A\beta_{25-35}$ produces distinct morphological changes and eventual cell death.[20] Pretreatment with neuroprotective agents can reduce or abolish these effects. The neuroprotective effects of **A4** were determined in primary neurons derived from embryonic rat brain exposed to Aβ (10 μM) in the presence or absence of drug for 48 h. In the majority of experiments, the toxic $A\beta_{25-35}$ was used to induce cell death. However, a smaller number of cultures were treated with the $A\beta_{1-42}$ formed in excess in AD. In all cases we found that $A\beta_{1-42}$ produced effects virtually identical to those of $A\beta_{25-35}$, and the toxicity of both was dramatically inhibited in the presence of the drug. The percentage of surviving neurons was determined by labeling with the fluorescent dyes calcein-AM and propidium iodide as previously described.[21,22] The numbers of calcein-labeled live cells and propidium iodide-labeled dead neurons in several fields were visualized via fluorescence microscopy and counted as described. In our studies, treatment of primary cortical neurons with Aβ alone (10 μM) represented the basal level for neuronal survival. Pretreatment of neuronal cells with **A4** prevented Aβ-induced toxicity in a dose-dependent fashion, with an EC_{50} value of ~6 nM (Fig. 2). While there were minor neuroprotective effects associated with **A4** concentrations as low as 0.5 nM, significant protection was not demonstrated until 5 nM. Treatment of neuronal cells with **A4** alone at 20× the EC_{50} value (100 nM) did not result in any observed neurotoxicity.

Inhibition of Hsp90 induces overexpression of both Hsp90 and Hsp70 through dissociation of Hsp90-

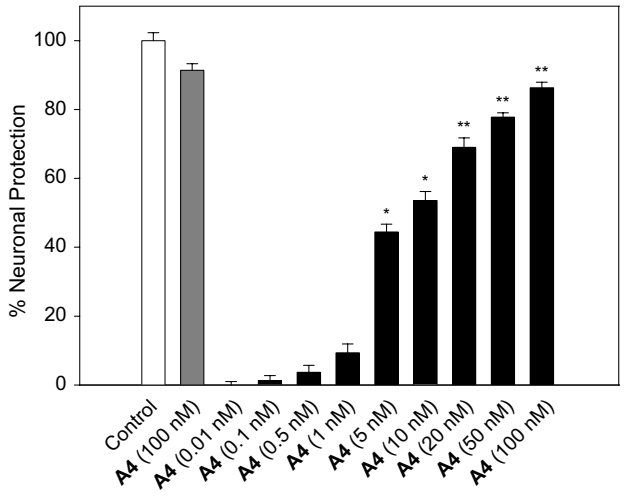


Figure 2. Dose-dependent **A4** protection against $A\beta_{25-35}$ toxicity. Neuronal cells were treated with vehicle only (clear), **A4** (100 nM, gray), or **A4** + Aβ (10 μM, black). The indicated concentrations of **A4** were added 2 h before Aβ. Cell viability was determined 48 h later.[24–27] $^{*}p < 0.05$ and $^{**}p < 0.01$, for Aβ alone versus **A4** + Aβ. Data represent mean survival ± SE for three separate experiments with ~1500 cells per treatment condition. Aβ (10 μM) alone was used as 0% survival and DMSO control was used as 100% protection.

HSF1 complexes and subsequent translocation of HSF1 to the nucleus.[23] Induction of both Hsp90 and Hsp70 by GA in cultured cells was reported to result in decreased levels of aggregated tau and increased levels of soluble tau, indicating that Hsp90 inhibitors can reduce toxic tau aggregates.[6] In our neuronal cultures, **A4** significantly increased Hsp70 levels at the concentration of 0.2 μM (Figs. 3a and b). However, concentrations of **A4** as low as 1 nM also led to increases in Hsp70 levels, compared to DMSO controls, after 48-h incubation. Hsp70 induction at 0.2 μM was comparable to that seen upon

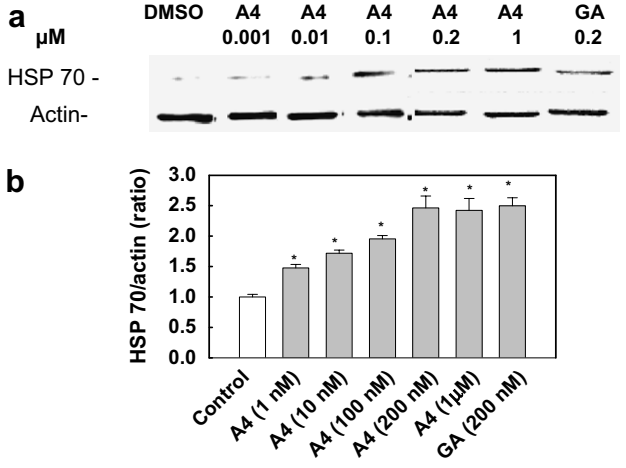


Figure 3. P upregulates Hsp70 in neuronal cells. (a) Primary cortical neurons were incubated with GA or **A4** for 48 h and probed for Hsp70 and actin (control). (b) The ratio of Hsp70 to actin was determined for each treatment as described.[28,29] $^{*}p < 0.005$ compared to DMSO control. Each bar represents the average of four separate experiments.

treatment with GA at the same concentration, suggesting **A4** could potentially attenuate tau aggregation in a manner similar to that reported for GA. In addition, incubation with higher concentrations of **A4** (1 and 10 μM) did not significantly increase Hsp70 levels, suggesting that the maximal neuroprotective effects of **A4** can be elicited at concentrations significantly lower than any potential cytotoxic effects.

To determine whether **A4** exhibited its neuroprotective effects via Hsp90 inhibition, **A4** was incubated with the well-studied mutated androgen receptor (AR)-dependent prostate cancer cell line, LNCaP. As reported previously, **A4** induced Hsp90 at the lowest concentrations tested (10 nM). In contrast, degradation of the Hsp90 client proteins AR and AKT did not occur until 1 μM, providing a therapeutic window of ⩾200-fold, suggesting increased levels of Hsps do not correlate directly with client protein degradation for inhibitors of the C-terminal ATP-binding pocket.[16]

For most Hsp90 inhibitors, efficacy in degrading client proteins, such as Her2 and AKT, in two distinct cancer cell lines, SkBr3 and MCF-7, correlates well with their anti-proliferative effects.[30] The anti-proliferative effect of GA treatment in these cell lines is well established, and we confirmed almost complete cytotoxicity with GA at 1 μM. GA elicited dose-dependent anti-proliferation in both cell lines, and the IC_{50} values were comparable to those reported previously (18 and 133 nM, respectively). In contrast, **A4** demonstrated no anti-proliferative effects in either cell line up to 100 μM (Figs. 4a and b), a concentration well above that necessary for complete neuroprotection, suggesting that C-terminal inhibitors possess a mechanism of action distinct from GA and other inhibitors of the N-terminus. When the effects of GA and **A4** alone were examined in neuronal cells, GA induced significant cytotoxicity at 10 μM after 24 h (Fig. 4c). Lower concentrations of GA led to substantial cytotoxicity after 72 h of incubation (data not shown). In contrast, 10 μM of **A4** caused no toxic effects even after incubation for 72 h, clearly indicating a novel utility for C-terminal inhibitors.

The BBB expresses high levels of P-glycoprotein (P-gp), an efflux pump responsible for the extrusion of numerous drugs and other xenobiotics from cells.[31] The rhodamine 123 assay is often used to predict whether a compound is a potential substrate for P-gp. In this assay, rhodamine 123 is used as a surrogate P-gp substrate. If **A4** is a substrate for P-gp, then its addition will increase rhodamine 123 uptake relative to the negative control determined by monitoring intracellular fluorescence. Taxol, a microtubule stabilizing agent that exhibits neuroprotective effects both in vitro and in vivo, is hampered as a CNS therapeutic because it is a known P-gp substrate.[32] Used as a positive control, Taxol significantly increased rhodamine 123 uptake in bovine brain microvessel endothelial cells (BMECs), while addition of **A4** had no effect on uptake even up to 50 μM, suggesting **A4** is not likely to be a substrate for P-gp (Fig. 5a).

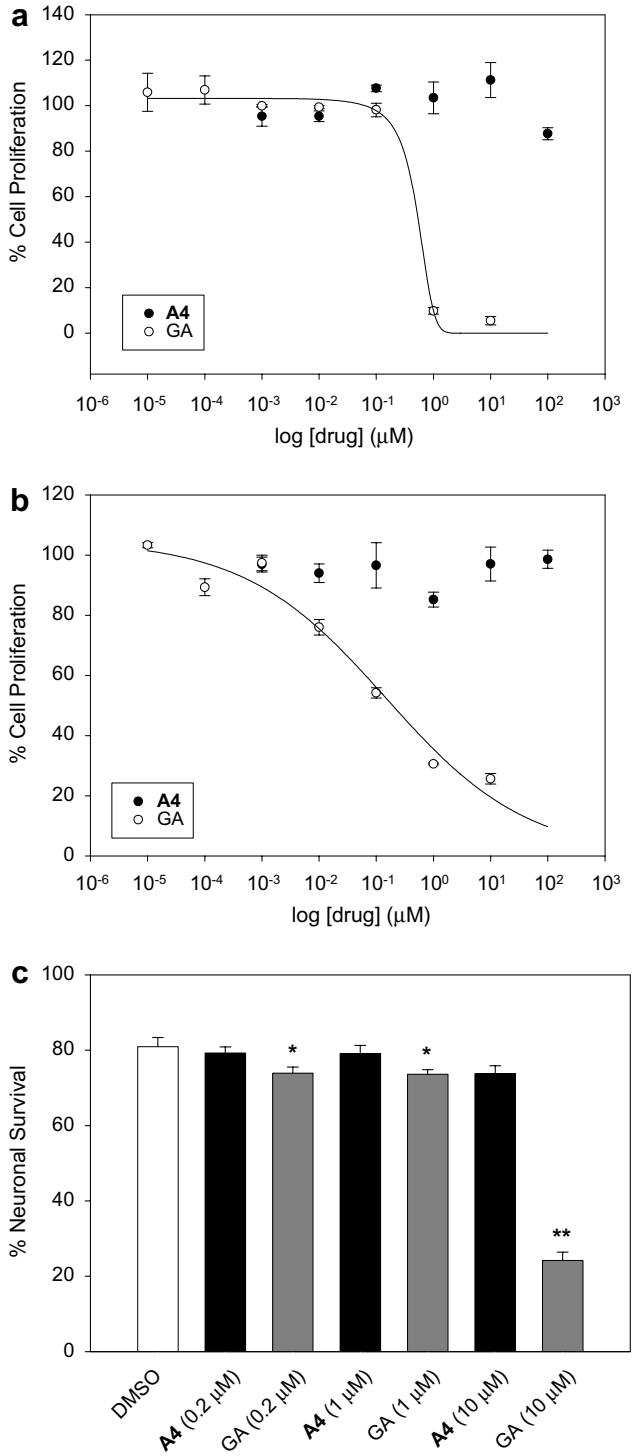


Figure 4. Anti-proliferative and toxic effects of **A4** and GA. MCF-7 (a) or SkBr3 (b) cells were incubated with **A4** (closed circles) or GA (open circles) at varying concentrations. Viable cells were quantitated using the MTS/PMS assay. Values represent means ± SE for one representative experiment performed in triplicate. Assays were replicated three times and the IC_{50} of GA correlated well with previously published values (MCF-7 = 133 ± 2 and SkBr3 = 18 ± 5 nM). In (c), neuronal cells were treated with DMSO (open bar), **A4**, or GA at the indicated concentrations, and cell viability was determined 24 h later. The data represent mean percentage ± SE of surviving neurons for three separate experiments. $^{*}p < 0.05$ for control versus GA, and $^{**}p < 0.001$ for control versus GA.

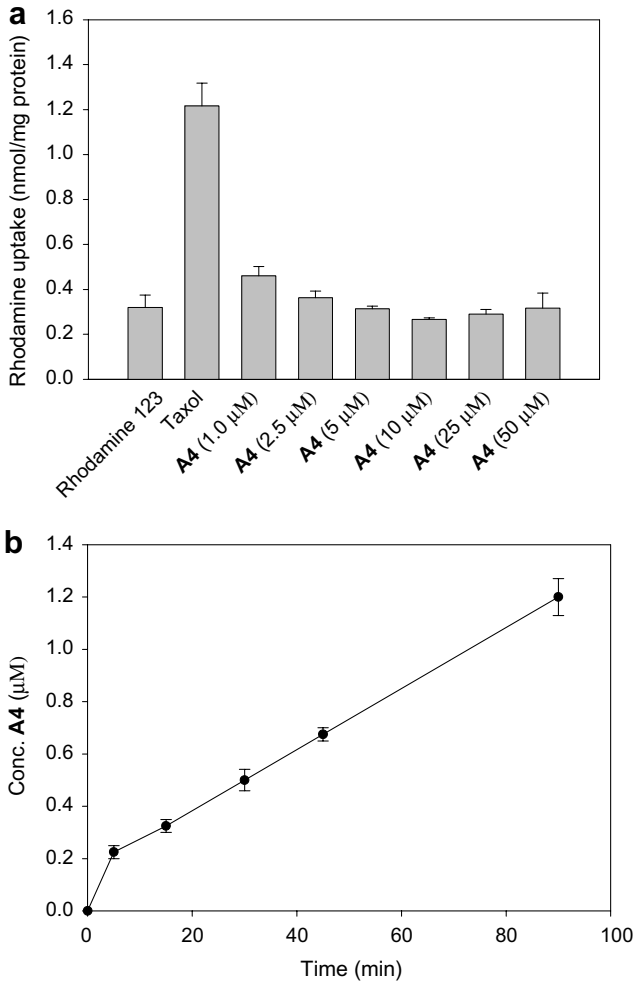


Figure 5. Efflux and transport of **A4** across BMECs. (a) BMECs were grown to confluency and incubated with Rhodamine 123 (5 μM) alone or **A4** (indicated concentrations) + rhodamine 123. Taxol (10 μM) was used as a positive P-gp substrate. (b) BMECs were grown to confluency on polycarbonate membranes and **A4** (10 μM) was added to the donor chamber. Aliquots from the receiver chamber were taken at the noted time points and analyzed by RP-HPLC for **A4** permeation across the cell layer.

The ability to partition across the blood–brain barrier is an essential property of drugs that are designed to produce beneficial effects on neuronal cells of the central nervous system (CNS). Transport across primary cultures of BMECs in vitro often correlates well with the BBB permeability of a compound in vivo.[33] In the side-by-side diffusion chamber, **A4** exhibited time-dependent linear transport across BMECs for up to 90 min (Fig. 5b). The concentration of **A4** at 90 min in the receiving chamber (1.2 μM) was 200-fold greater than the concentration necessary (5 nM) for 50% neuroprotection from Aβ-induced toxicity. These data offer preliminary indication that pharmacologically active amounts of **A4** might penetrate the BBB and result in the presence of the drug in brain tissue.

Intracellular protein aggregation is a defining pathology of numerous neurodegenerative disorders, including Huntington's disease, PD, and AD.[1] In AD, tau aggregation results from its hyperphosphorylation and subsequent disassociation from microtubules to form filamentous aggregates that polymerize into NFTs. It has been proposed that increased Aβ levels lead to alterations in intracellular kinase and phosphatase activity, which ultimately results in pathogenic hyperphosphorylation. Therapeutic strategies directed at numerous individual pharmacological targets in this pathway have been tested as potential treatments for AD. Strategies designed to reduce the levels of Aβ peptides in the brain include inhibition of proteolytic enzymes that form Aβ, prevention of Aβ oligomerization through metal chelators, and upregulation of proteases that normally degrade Aβ.[34] Previous attempts to prevent tau aggregation have generally focused on the identification and inhibition of specific kinases involved in its abnormal phosphorylation. Microtubule-stabilizing agents such as Taxol may prevent loss of the microtubule-stabilizing activity of tau and such agents have also exhibited neuroprotective activity against a variety of toxic insults.[21]

The inducible nature of Hsps makes Hsp90 inhibition a unique and exciting strategy for the treatment of AD and other neurodegenerative disorders in which protein aggregation is a major pathology. The direct mechanism responsible for the neuroprotective effects of Hsp90 inhibitors appears to be the upregulation of chaperones that can resolubilize these toxic aggregates. Dou et al. suggest that Hsps can directly associate with tau proteins and prevent their misfolding and subsequent aggregation.[6] The independent identification of a series of Hsp90 inhibitors that lower intracellular tau levels through induction of Hsps supports this hypothesis.[35] Recently, tau was identified as a substrate for CHIP, and it was suggested that increased levels of Hsp70 can shift the equilibrium toward formation of the CHIP/Hsp70/tau complex, resulting in rapid clearance of tau aggregates via the ubiquitin-proteasome pathway.[36] Therefore, induction of Hsp70 appears to play a major role in the removal of tau aggregates. More recently, a high-throughput assay designed to identify compounds that block JNK-dependent apoptosis identified AEG3482 as an Hsp90 inhibitor with potential neuroprotective properties.[37] The data suggest that JNK inhibition is a downstream result of Hsp90 inhibition and subsequent Hsp70 induction. Hsp70 is known to bind JNK and disrupt substrate interactions, thereby, inhibiting its kinase activity. Taken together, this evidence strongly suggests that increased Hsp70 levels are a key factor in the neuroprotective effects of **A4** and other Hsp90 inhibitors.

The development of Hsp90 inhibitors as chemotherapeutics has focused almost entirely on their use as anticancer agents. Numerous Hsp90 client proteins are essential for the growth and proliferation of cancer cells, resulting in cytotoxic effects. This detrimental property has been a key reason behind their lack of development as neuroprotective agents. In contrast to GA and other N-terminal inhibitors, **A4** is the first compound reported that induces Hsp70 and provides

significant protection against Aβ-induced toxicity at non-cytotoxic concentrations. In fact, no toxicity was observed in our assays even at 20,000× the EC_{50} (100 μM) in non-neuronal cells, a concentration at which GA is severely toxic. In addition, **A4** increases Hsp90 levels at concentrations ~200-fold less than those required for client protein degradation. This provides a large therapeutic window for treatment of several disorders in which chaperones provide a protective effect. These attributes make **A4** an ideal lead compound for development as a novel chemotherapeutic that might slow progression of neurodegeneration in AD. Current studies are underway to evaluate this new lead compound in animal models as well as in cells transfected with mutant tau to elicit the effects of p-tau aggregation.

A major obstacle for any drug whose site of action is within the CNS is the ability to penetrate the BBB. In general, only small molecules with high lipid solubility and relatively low molecular weight (~400–500 Da) are able to cross the BBB.[38] To date, few brain disorders have responded to small molecule therapies and many, including AD, PD, and Huntington's disease, have not been successfully targeted.[39,40] The time-dependent linear transport of **A4** across BMECs in vitro suggests that significant concentrations of the drug will be available to the CNS in vivo. Within 15 min, the concentration of **A4** present in the abluminal chamber was enough to provide full neuroprotective effects as demonstrated in vitro, suggesting that in vivo neuroprotection could be fast-acting.

In summary, we have further characterized a novel C-terminal Hsp90 inhibitor, **A4**, as a potent neuroprotective agent for the treatment of AD. The protection against Aβ-induced toxicity exhibited by **A4** at concentrations that are non-cytotoxic provides a large therapeutic window and establishes it as the first Hsp90 inhibitor to manifest this unique property. In addition, the biological activities of **A4** suggest that C-terminal inhibitors of Hsp90 work through a different mechanism than the known N-terminal inhibitors and may exhibit other unique pharmacological profiles that will be useful for the treatment of other neurodegenerative disorders.

Acknowledgments

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