

## **Hsp90 Maintains the Stability and Function of the Tau Phosphorylating Kinase GSK3 $\beta$**

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**Abstract:** Hyperphosphorylation of tau leading to aggregated tau and tangle formation is a common pathological feature of tauopathies, including Alzheimer's disease. Abnormal phosphorylation of tau by kinases, in particular GSK3 $\beta$ , has been proposed as a pathogenic mechanism in these diseases. In this study we demonstrate that the heat shock protein 90 (Hsp90) maintains the stability and function of the GSK3 $\beta$ . By using both rat primary cortical neurons and COS-7 cells, we show that Hsp90 inhibitors lead to a reduction of the protein level of GSK3 $\beta$ , and that this effect is associated with both a decrease in tau phosphorylation at putative GSK3 $\beta$  sites and an induction in heat shock protein 70 (Hsp70) levels. We further show that Hsp90 associates with the GSK3 $\beta$  regulating its stability and function and preventing its degradation by the proteasome.

**Keywords:** tauopathy, GSK3 $\beta$ , Hsp90, aberrant tau phosphorylation

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### **1. Introduction**

The heat shock proteins (Hsps) are a family of ubiquitous and abundant proteins found in all eukaryotes and prokaryotes. Their function is to mediate the proper folding of native and denatured proteins to insure that those proteins maintain their native conformation [1, 2]. In addition to their functions in co- and posttranslational folding, Hsps are also required for intracellular protein trafficking and translocation, are involved in macromolecular assembly and disassembly, and facilitate the protein degradation through the ubiquitin-proteasome pathway [3, 4]. All those functions of Hsps are well characterized and are central to maintaining cellular homeostasis and in promoting cell survival in response to stressful conditions.

During about a century of the research on neurodegenerative diseases, researchers find that a common histological feature shared by those diseases is the existence of intra-cellular and/or extra-cellular aggregations in the brain. The appearance of aggregates in diseased brain implies an underlying incapacity of molecular chaperones [5-7]. Based on this idea, the relationship between molecular chaperones, particularly heat shock proteins, and neurodegenerative disorders has been thoroughly studied. It has been demonstrated that Hsps could suppress neurodegeneration in cultured cell, fly and mouse models of Huntington's disease, Parkinson's disease and Alzheimer's disease [8-13]. While it is becoming clear that Hsps can have a profound influence on solubility and aggregation of proteins that involved in various neurodegenerative diseases, many other important roles Hsps may play remain to be examined. After all, Hsps are a group of multi-function proteins. For instance, the involvement of Hsp90 in many signal transduction pathways has been well established, Hsp90 directly associates with several important protein kinases such as Akt, ErbB2, Polo kinase and Raf1 [14-17]. Hsp90 inhibitors could induce the inactivation /degradation of those client proteins. In our previous study we have reported that inducing the expression of Hsps can reduce tau phosphorylation [13], and a subsequent study showed that Hsp90 inhibitors could cause the inactivation of ERK by facilitating the degradation of Raf [18]. In this study we report that Hsp90 could also regulate the cellular stability of GSK3 $\beta$ , an important protein kinase account for tau hyperphosphorylation. Our results show that treatment of cells with Hsp90 inhibitors results in the proteasome-dependent degradation of GSK3 $\beta$ , and subsequently cause the reduction of tau phosphorylation.

## 2. Materials and Methods

Geldanamycin (GA), ZVAD-FMK, pepstatin, MG132, leupeptin, E64 and Protein A/G plus agarose beads were purchased from Calbiochem, San Diego, CA; poly-L-lysine, novobicin are ordered from Sigma; BCA protein assay kit is ordered from Pierce; FuGENE 6 reagent is ordered from Roche Molecular Biochemicals; cDNA constructs expressing human wild type Tau (T40) and was a kind gift from Dr. Virginia Lee (University of Pennsylvania, Philadelphia, PA); PU24FCI was a kind gift from Dr. Gabriela Chiosis (Memorial Sloan-Kettering Cancer Center, New York, NY); Dulbecco's Modified Eagle Medium (DMEM), neurobasal medium, fetal bovine serum (FBS), penicillin/streptomycin and B27 supplement are ordered from Invitrogen; anti- $\beta$ -actin mouse monoclonal antibody (A1978) is ordered from Sigma, St. Louis, MO; anti-GSK3 $\beta$  (#9332) antibody is ordered from cell signaling; anti-phospho-tau (pS199/202) is ordered from Biosource; AT8, AT180, AT100, AT270 are ordered from Innogenetics; anti-tau antibody is from Santa Cruz; anti-Hsp70 (SPA-810) and anti-Hsp90 (SPA-830) are from Stressgen Bioreagents, Victoria, British Columbia, Canada.

### 2.1 COS-7 Cell Cultures and Incubation with the Hsp90 Inhibitor

COS-7 cells grown in DMEM with 10% FBS and penicillin/streptomycin (50 U/50 $\mu$ g/ml, respectively) were transiently transfected using FuGENE 6 reagent to over-express wild type hTau. At 12 h after transfection, cells were incubated for 20h with the indicated concentration of GA or a purine-based Hsp90 inhibitor PU24FCI [19]. Cells were harvested and lysed in 1 $\times$ RIPA buffer (50mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0).

The protein concentration was determined using BCA assay. Samples with equal amounts of protein were analyzed by Western blotting.

### 2.2 Primary Neuronal Cultures and Incubation with the Hsp90 Inhibitor

Primary neuronal cultures were derived from the cerebral cortices of embryonic day 17 (E17) rat embryos and maintained as previously described [20]. Dissociated neurons were plated ( $\sim 5 \times 10^6$  cells per plate) on poly-L-lysine pre-coated 6cm plates in serum-free neurobasal medium with N2 and B27 supplements and cultured for 7 days. Media were replaced every 2 days with the addition of 16.5 mg/ml uridine and 6.7 mg/ml 5-fluoro-2'-deoxyuridine to prevent proliferation of glial cells. Hsp90 inhibitor was added at day 6 of culture and cells were incubated at 37 °C as indicated. Following incubation, cells were collected and lysed. Samples were analyzed by SDS/PAGE and Western blotting. For studies involving inhibitors of protein degradation, primary cortical cultures were treated with DMSO or indicated concentration of Hsp90 inhibitor in the presence of the following protease inhibitors: 10  $\mu$ M MG132, 1 $\mu$ M pepstatin, 75 $\mu$ M leupeptin, 10  $\mu$ M E64 or 20  $\mu$ M ZVAD for 20h. Cells were collected, and the lysates analyzed by Western blotting.

### 2.3 Cycloheximide Treatments

Primary cortical cultures or COS-7 transfected cells were treated with cycloheximide (at a final concentration of 100  $\mu$ g/ml) for the indicated times. Cell lysates were run on SDS-PAGE gel and proteins transferred to PVDF membranes to be probed with anti-GSK3 $\beta$  antibody.

### 2.4 Immunoprecipitation

For isolation of the GSK3 $\beta$ /Hsp90 complexes from cultured cells, COS-7 cells were lysed in TMNSV buffer (50 mM Tris-HCl, pH7.0, 20 mM Na<sub>2</sub>MoO<sub>4</sub>, 0.09% NP-40, 150 mM NaCl, 1 mM sodium orthovanadate with added protease inhibitors mixture). Cell nuclei and debris were removed by centrifuging at 13,000  $\times$  g for 5 min. Samples were immunoprecipitated with anti-GSK3 $\beta$  antibody for 4h at 4°C. Immunoreactive materials and protein A/G agarose beads complexes were washed three times with lysis buffer and once with PBS. Immunoreactive materials were eluted from the beads by incubating with sample buffer for 5min at 95°C and subjected to SDS/PAGE followed by Western blotting.

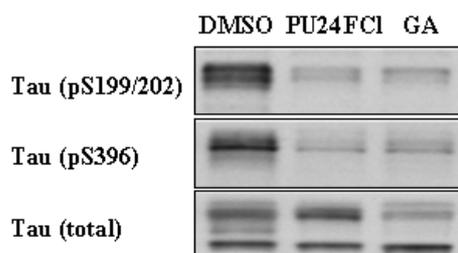
## 3. Results

### 3.1 Inhibition of Hsp90 reduces tau phosphorylation level and protein level of GSK3 $\beta$

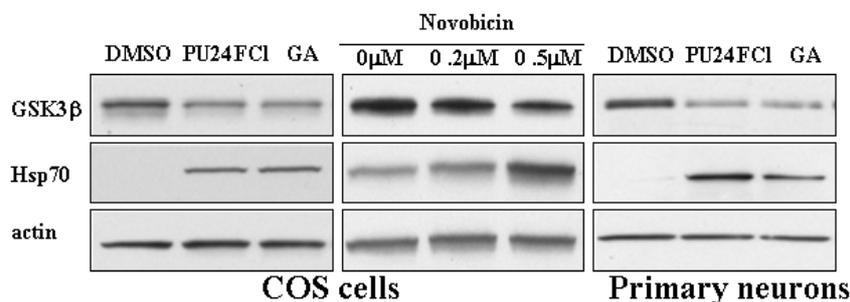
COS cells transfected with wild type hTau (the longest isoform 1-441aa) were treated with two different Hsp90 inhibitors, GA and PU24FCI. Both inhibitors bind selectively to the regulatory pocket of Hsp90 which inhibits chaperone function and alter the association of client proteins with the chaperone complex. As a consequence, these proteins do not achieve their mature functional conformation and are degraded by the proteasome. In our previous paper, we reported that the inhibition of Hsp90 resulted in a reduction of the protein level of mutant tau. While in the case of WT

tau protein, the inhibition of Hsp90 has no effect on total protein level, but to decrease the tau phosphorylation instead (Figure 1, Figure 3). Tau protein could be phosphorylated by several kinases at dozens sites, but only a few kinases have been confirmed related to the pathogenesis of AD, GSK3 $\beta$  is one of such kinases [21]. Several sites which phosphorylation level could be regulated by Hsp90 inhibitor in this study are putative GSK3 $\beta$  sites [22]. In order to study whether the reduction of tau phosphorylation is due to the inactive/degradation of GSK3 $\beta$ , the total GSK3 $\beta$  protein level has been checked in COS cells and rat primary cortical neurons. After treated with 10 $\mu$ M PU24FC1 or 400nM GA for 20h, the total GSK3 $\beta$  level in both COS cell and primary neurons are decreased dramatically (Figure 2). At the same time, the Hsp70 expression was induced because the heat shock factor 1 (HSF1) is released from Hsp90 complex as documented by Zou et al [23]. By using another Hsp90 inhibitor, novobycin, which binds to Hsp90 at c-terminus rather than the regular ATP pocket, we also observed a dose –dependent reduction of GSK3 $\beta$  (Figure 2).

The effect of Hsp90 inhibitors on kinases was well-defined and selective as the expression of casein kinase 1 (CK-1), casein kinase 2 (CK-2) and cyclic AMP-dependent protein kinase (PKA), kinases shown to prime Tau for GSK3 $\beta$  phosphorylation [24] was not affected by the Hsp90 inhibitor (data not shown).



**Figure 1.** Effects of Hsp90 inhibition on tau phosphorylation. COS-7 cells were transiently transfected to over-express human tau protein longest isoform. At 12 h after transfection, cells were incubated for 20h with 10 $\mu$ M PU24FC1 or 400nM GA, cells were collected and lysed, the resulting lysates analyzed by immunoblot.

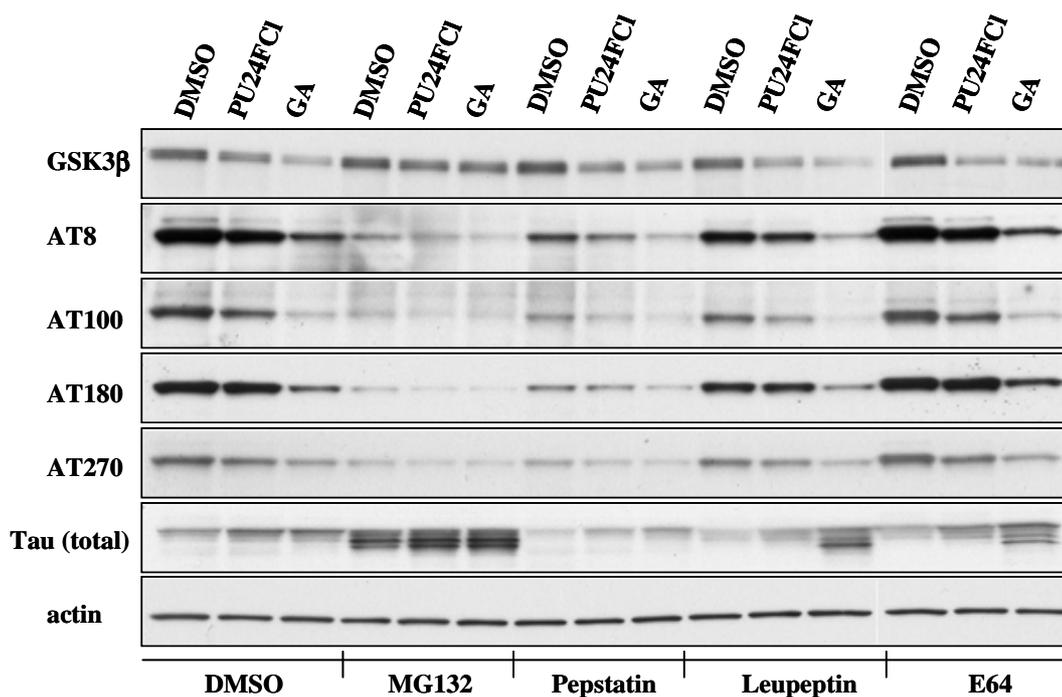


**Figure 2.** Inhibition of Hsp90 specifically decreases GSK3 $\beta$ . COS cells were treated with 10 $\mu$ M PU24FC1 or 400nM GA for 20h, in some experiments, cells were treated with novobycin at indicated concentration for 20h. Rat embryonic cortical primary neurons were treated under same conditions.

### 3.2 Degradation of GSK3 $\beta$ by Hsp90 inhibition is mediated by the proteasome

Protein degradation may be achieved in cells by several protease systems. However, proteins regulated by Hsp90 appear to be degraded majorly via the proteasome pathway upon chaperone

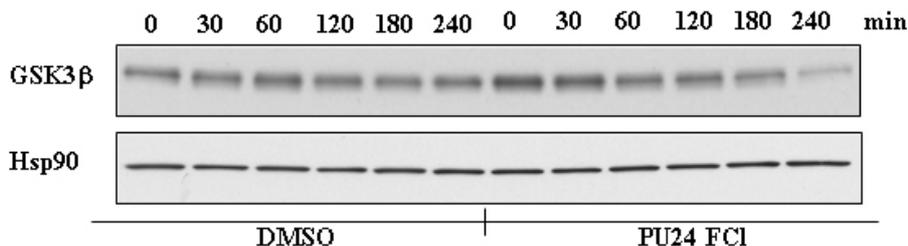
inhibition [25]. To examine whether the proteasome is accounted for the GSK3 $\beta$  degradation in our study, we explored the effect of several inhibitors of other proteases in preventing the degradation of GSK3 by Hsp90 inhibitors. Inhibitors of lysosome proteases (E64 and leupeptin), aspartate proteases (pepstatin) or caspases (ZVAD) failed to rescue GSK3 $\beta$ . Only a proteasome inhibitor (MG132) was able to prevent their degradation over a 20 h treatment with the Hsp90 inhibitor (Figure 3). These observations are in agreement with observed effects on other oncoprotein clients of Hsp90, which suggest that GSK3 $\beta$  are protected through Hsp90 chaperone complex formation, from ubiquitination and proteasomal degradation. In concordance with this result, the decrease of tau phosphorylation could not be prevented by those protease inhibitors as well except MG132 (Figure 3). The effect of MG132 on tau phosphorylation is complicate because the MG132 itself could cause the tau phosphorylation decrease [26]. Under this circumstance it's hard to distinguish the effect of Hsp90 inhibitor from proteolytic stress. Maybe both pathways share the same mechanism to reduce tau phosphorylation, since MG132 treatment could induce heat shock response in cells as well [26]. A rational hypothesis is that proteolytic stress results in a increase of misfolded proteins in cytosol, which competed with Hsp90 client proteins for this chaperone. Those client proteins of Hsp90 include HSF1 and GSK3, may head to different destination. HSF1 moves to nucleus and activates the expression of Hsps, while the GSK3 and other kinases head to ubiquitination.



**Figure 3.** Degradation of GSK3 $\beta$  by Hsp90 inhibitors is mediated by the proteasome. Only proteasome inhibitors (i.e. MG132) were able to prevent the degradation of GSK3 $\beta$  over a 20 h treatment with the Hsp90 inhibitors PU24FCI and GA. Primary neurons were treated with DMSO, 10  $\mu$ M PU24FCI or 400nM GA in the presence of the following inhibitors: 10  $\mu$ M MG132, 1 $\mu$ M pepstatin, 75 $\mu$ M leupeptin, 10  $\mu$ M E64 or 20  $\mu$ M ZVAD for 20h. Cells were collected, lysed and the resulting lysates analyzed by immunoblot. Proteins were analyzed by Western blot. The phosphorylation level of tau protein was analyzed by Western blotting using phospho-specific antibodies [AT8 (1:250), AT100 (1:250), AT180(1:250), AT270 (1:250)] anti total tau antibody (1:500). Actin were used as protein quantification control.

### 3.3 Hsp90 regulates the stability of GSK3β

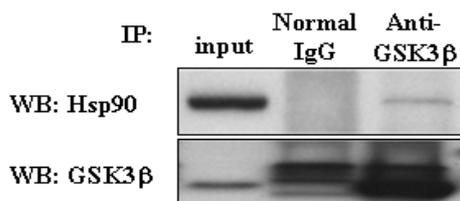
To examine if Hsp90 plays a direct role in maintaining the stability of GSK3β, we tested whether inhibition of Hsp90 function affected its half-life. Primary neuronal cultures were treated with inhibitor or vehicle in the presence of cycloheximide, a protein synthesis inhibitor. The half-life of the GSK3β protein was remarkably shortened in the cells treated with Hsp90 inhibitors (Figure 4). Moreover, the protein was degraded upon the Hsp90 inhibitor treatment even when induction of Hsp70 was blocked by the protein-synthesis inhibitor. These findings strongly implicate Hsp90 as a direct and important regulator of GSK3β stability.



**Figure 4.** Hsp90 inhibition affects the protein’s half-life. COS-7 cells were treated with DMSO or 10 μM PU24FCI in the presence of 30 μM cyclohexamide to halt translation. At the times indicated, cells were lysed and whole-cell lysates immunoblotted with anti-GSK3β antibody.

### 3. 4 GSK3β exist in a complex with Hsp90

We next immunoprecipitated endogenous GSK3β from 2mg of COS cells lysate and demonstrated by western blotting that Hsp90 was coimmunoprecipitated. During the experiment, 15μg of COS cells lysate was loading on the same gel and immunoblotted in parallel. The result showed that only a small fraction of cellular Hsp90 is associated with GSK3β. This observation is not surprising as the Hsp90 is one of the most abundant cellular proteins and is known to be associated with a large number of proteins.



**Figure 5.** GSK3β exist in a complex with Hsp90. GSK3β was immunoprecipitated from COS cell lysate, the immunoreactive materials were subjected to SDS-PAGE and analyzed by immunoblot using anti-Hsp90 antibody and anti-GSK3β antibody.

## Discussion

Hyperphosphorylation of tau leading to aggregated tau and tangle formation is a common pathological feature of tauopathies, including Alzheimer's disease. The toxic behavior of the abnormal tau is solely due to its hyperphosphorylation because dephosphorylation restores it into a normal-like protein [27]. The longest form of human brain tau contains 80 serine and threonine and 5 tyrosine residues. Thus, almost 20% of the protein has the potential to become phosphorylated. Several kinases,

including cyclin-dependent kinase 5 (Cdk5) and glycogen synthase kinase-3 beta (GSK3 $\beta$ ) have been implicated in phosphorylating tau at pathogenic sites. Abnormal phosphorylation of tau by kinases, in particular the GSK3 $\beta$  and Cdk5, has been proposed as a pathogenic mechanism in these diseases [28-30]. Cells co-transfected with GSK3 $\beta$  and tau showed abnormal microtubule bundles [31]. Recent animal studies have provided further support for the view that GSK3 $\beta$  is the most likely candidate for the protein kinase responsible for the abnormal phosphorylation state of tau in AD brain. Immunohistochemical studies located GSK3 in neurofibrillary tangles [32]. Moreover, GSK3 $\beta$  is elevated in AD human brain [33]. Maintenance of such anomalously activated kinases in a functional state requires a stabilizing mechanism. In oncogenic transformation, a corresponding role is assigned to molecular chaperones, in particular to the heat shock protein 90 (Hsp90) [34-36]. This chaperone allows cancer cells to tolerate the many components of dysregulated pathways in a transformation-specific manner by interacting with several client substrates, such as kinases, hormone receptors and transcription factors that are directly involved in driving multistep malignancy, and also with mutated oncogenic proteins required for the transformed phenotype. Under normal conditions, Hsp90 interacts with these client proteins in a dynamic, low-affinity manner. Upon mutation or dysregulated function many of these client proteins display unusually stable associations with Hsp90-containing chaperone complexes. These associations maintain them capable to function in the dysregulated state and appear to be essential for their transforming, aberrant activity. A similar role for Hsp90 in controlling kinases may be envisioned in neurodegenerative diseases. In this study we demonstrate that the heat shock protein 90 (Hsp90) maintains the stability and function of the GSK3 $\beta$ . By using both rat primary cortical neurons and COS-7 cells, we show that Hsp90 inhibitors lead to a destabilization of GSK3 $\beta$ , and that this effect is associated with both a decrease in tau phosphorylation at putative GSK3 $\beta$ . We further show that Hsp90 associates with the GSK3 $\beta$  regulating its stability and function and preventing its degradation by the proteasome.

Direct inhibition of kinases as a therapeutic modality in tauopathies has been recently investigated. Inhibition of GSK3 $\beta$  by lithium was demonstrated to reduced tauopathy and degeneration in the P301L tau transgenic mouse model [37]. These results support the idea that kinases are involved in tauopathy progression and that kinase inhibitors may be effective therapeutically. We propose here indirect kinase inhibition by interference with Hsp90 activity as another therapeutic strategy. Considering the facts that Hsp90 inhibitors have entered the phase I/II clinical trial for cancer, our work opens the door to the exciting possibility of using these emerging agents in the treatment of neurodegenerative diseases.

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