A High Throughput Drug Discovery for Intracellular Neurodegenerative Disease-Associated Targets using the LI-COR® Odyssey® Near Infrared Imaging System

Chad A. Dickey
Department of Neuroscience
Mayo Clinic

ABSTRACT

Recent evidence has indicated that although amyloid may initiate Alzheimer's disease (AD) progression, the intracellular accumulation of abnormal tau may ultimately be self-perpetuating. While this does implicate tau as a potentially critical pathological component for AD, it has yet to be definitively determined if tau would be a favorable clinical target. Perhaps the major reason for this uncertainty is the lack of significant drug discovery efforts toward identifying tau-altering compounds that would warrant clinical trial introduction, a process essential for validating the relevance of tau to the cognitive decline associated with AD. Here, we have used the dual near-infrared detection capability of the LI-COR® Odyssey® scanner to develop a novel, 96-well cell-based assay suited for high throughput detection of changes in tau protein levels that also allows for simultaneous first-pass estimations of toxicity by measuring glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels within the same well. Using this system, we have identified a panel of novel HSP90 inhibitors with potent tau-reducing activity. These compounds also provide proof of principle, suggesting that adaptation of this methodology for analysis of other pathologically relevant intracellular proteins is limited only by the availability of antibodies with sufficient specificity and sensitivity for use with the LI-COR Odyssey system. As intracellular targets have previously only been measurable by electrophoresis and immunocytochemistry techniques in the academic arena, the mainstreaming of this process could greatly expedite drug discovery efforts for neurodegenerative diseases of protein misfolding and accumulation.

Introduction

Alzheimer's disease (AD) pathology is primarily characterized by the accumulation of both the Aβ 1-42 peptide into extracellular plaques and the microtubule-associated protein tau into intracellular tangles with resulting neuronal loss and cognitive dysfunction. Current AD therapeutics are limited to cholinesterase inhibitors that enhance cholinergic function in AD synapses [1] and the NMDA receptor antagonist, memantine, thought to block excitotoxicity. Twenty years passed between the discovery of cholinergic synaptic dysfunction associated with AD and FDA approval of the first cholinesterase inhibitor, donepezil [2], a testament to the difficulty involved in drug discovery for age-related disorders. However, dramatic technological advances and scientific discoveries have been made over the past decade that hold the potential to greatly expedite therapeutic development for AD. While many such strategies are being investigated to reduce the accumulation of the Aβ peptide in AD [3-7], new evidence has emerged suggesting that these anti-Aβ therapies may only be clinically relevant early on in the pathogenesis [8]. Oddo et al. demonstrated that a pathogenic threshold is reached after which accumulation of tau into tangles is not reversible by removing Aβ alone. This finding, along with the discovery of tau mutations that lead to autosomal dominant forms of dementia in the absence of Aβ accumulation [9], indicates that the development of drugs able to reduce tau species should also be an important therapeutic target in AD and related tauopathies. Lowering total tau levels has been thought impractical due to its fundamental role in microtubule dynamics within neurons, and therefore, few anti-tau therapeutics have been generated or discovered. But with the recent generation of seemingly intact, viable tau knockout mice [10] and the finding that small molecule microtubule stabilizers can act as a surrogate for tau [11], it seems apparent that the brain may be tolerant of drugs that either lower total tau non-selectively, or alter only those aberrant forms of tau that accumulate in AD and other tauopathies. Recent studies have demonstrated that ubiquitination (a process of targeting proteins for removal and breakdown) and other degradation pathways may be impaired in diseases with aberrant protein aggregation, such as AD and Parkinson's disease [12-15]. In particular, elevated expression of heat shock proteins (HSPs) induced by treatment with a naturally occurring HSP90 inhibitor, Geldanamycin (GA), has been associated with reductions in tau protein levels and reduced aggregation of both ±-synuclein and huntingtin [16, 17]. Under normal circumstances, chaperones target misfolded or mutant proteins for either repair or degradation by the ubiquitin-proteasome system (UPS). While the functions of many of the components of this intricate system have been described, the mechanism by which disease-associated proteins are modified remains unknown. However, strides are being made in the field, as demonstrated by the recent finding that tau specifically binds to HSP27 and leads to decreases in phosphorylated tau and cell survival [18, 19]. Therefore, therapeutic strategies aimed at enhancing expression of chaperones have been an area of intense investigation, with particular emphasis being placed on inhibitors of HSP90. HSP90 demonstrates a regulatory function for the chaperone transcription factor heat shock factor-1 (HSF1), and inhibition of HSP90 releases HSF1, eliciting increased expression of other chaperones, such as HSP70, HSP40 and HSP27. This mechanism has allowed us for the first time to design assays to detect reductions in tau species with a working compound (HSP90 inhibitor) as a positive control. Unfortunately, the best characterized of these compounds is GA, which is a large molecule with poor stability, making its use in high throughput assays impractical. Therefore, GA derivatives and smaller HSP90 inhibitors were generated with much higher stability, providing an invaluable resource for confirming assay specificity. Using these HSP90 inhibitors as proof of concept has allowed us to develop an innovative cell-based assay format capable of detecting changes in tau species that can then be used to screen compound libraries for potentially novel therapeutic agents [20].

Materials & Methods

Cell Culture and Drug Treatments

Human H4 neuroglioma cells were maintained in flasks with Opti-MEM (Invitrogen) with 10% FBS and antibiotic. For preparation of homogenates for standard Western blot (SWB) analyses, cells were passaged into 6-well plates, incubated overnight, and treated the next day with drug or vehicle at a 1 µM concentration. Cells were then incubated 48 hours and harvested in lysis buffer containing (50 mM Tris-HCl pH 7.4, 1 M NaCl, 0.1% Triton-X, 5 mM EDTA) plus 1% SDS, PMSF, and both a protease and phosphatase inhibitor cocktail. For use

in the high throughput In-Cell Western (ICW) assay, cells were passaged into 96-well clear bottom plates (BD Falcon), incubated overnight and treated the next day in triplicate with a 1 μM concentration of 10 HSP90 inhibitors or vehicle. These plates were incubated for 48 hours and then prepared for ICW analysis described below.

Western Blot Analysis for Chaperones and Tau
Cell lysates were sonicated and protein concentrations were measured by a standard BCA assay (Pierce). The samples were then heated in Laemmli's buffer and equal amounts of protein were loaded into 12-well 10% Tris-HCl gels (Bio-Rad). Tau protein levels were then analyzed using rabbit anti-human tau (1:2000; DAKO) and mouse anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2000; Biodesign) antibodies. A mouse anti-HSP70 antibody was used to analyze chaperone protein induction following HSP90 inhibitor treatment (1:1000; Stressgen).

High Throughput ICW Blotting and Quantitation
H4 cells were treated with 10 compounds and vehicle in triplicate within a 96-well cell culture plate. After 48 hours, cells were directly fixed in the plate with 3.7% formaldehyde in PBS for 20 minutes and then permeabilized with three 10-minute washes in PBS + 0.1% Triton-X 100 using an ELX405 auto plate washer (BioTek Instruments, Inc.). LI-COR Blocking Buffer (LBB) was added for two hours, followed by overnight incubation with either rabbit anti-human tau (1:500) or mouse anti-HSP70 (1:100) and mouse anti-GAPDH (1:1500) antibodies in a 1:1 buffer of LBB and PBS + 0.2% Tween 20. After three 10-minute washes in PBS + 0.1% Tween 20, secondary detection was carried out using two infrared fluorescent dye conjugated goat antibodies in 1:1 buffer of LBB and PBS + 0.4% Tween 20; one absorbing at 680 nm (AlexaFluor® 680, Molecular Probes) and the other absorbing at 800 nm (IRDye™ 800CW, Rockland). After an hour of incubation and washing, the targets were simultaneously visualized using the Odyssey Infrared Imaging Scanner with the 680 nm fluorophore indicated by a red color and the 800 nm fluorophore indicated by a green color. Relative fluorescence units from the scanning allowed for quantitative analyses of both labeled proteins. Relative fluorescent units for HSP90 inhibitor-treated samples were divided by vehicle controls to determine percent change in expression of both tau and GAPDH levels relative to control. The student t-test was used to determine significant toxicity effects due to drug treatment based on the expression levels of GAPDH, and then a separate student t-test was used to determine significant differences between tau and GAPDH levels in treated cells.

RESULTS & DISCUSSION

Here, we have tested the effects of a panel of novel synthetic HSP90 inhibitors (Conforma Therapeutics, San Diego, CA) on total tau levels using the ICW assay and have demonstrated that tau protein levels are reduced following a 48-hour incubation at a 1 μM concentration (Figure 1). Significant reductions were observed in seven of the ten compounds (A-G). In particular, three compounds (B-D) displayed dramatic tau-lowering effects of more than 60% (p values of B = 0.013, C = 0.013, and D = 0.006 by t-test). Using the ICW assay, we were able to simultaneously assess toxicity in a quantitative fashion using GAPDH levels, a feature not available by standard Western blot due to protein concentration adjustment and, indeed, compounds B - D did not cause significant toxicity; however, while inhibitor F had the most pronounced effect on tau levels with a greater than 80% reduction ($p = 0.001$ by t-test), GAPDH expression was also decreased by ~30% ($p = 0.019$ by t-test). Compounds A and E did elicit less potent reductions in tau without changes in GAPDH, but compounds H - J showed no reductions in either tau or GAPDH. SWB analysis was used to confirm the ICW findings, and a very similar pattern of tau reduction was observed (Figure 1).

To determine whether chaperone induction by the HSP90 inhibitor compounds was coincidental with the reductions in tau levels, we assayed for HSP70, the most robustly elevated chaperone following HSP90 inhibition. We demonstrate that those compounds with the greatest capacity to lower tau did cause the largest induction of HSP70 expression by both ICW analysis and SWB (Figure 1), suggesting that induction of a heat shock response is likely capable of reducing endogenous tau levels. This likely perpetuates tau degradation via intracellular chaperone-mediated trafficking of aberrant tau species for subsequent degradation [14, 18].

Finally, due to the quantitative capability of near-infrared imaging, analyses of changes in tau, GAPDH and HSP70 levels for drug-treated cells relative to vehicle-treated cells were performed and are presented in figure 2.

Recently, Pickhardt et al. used an *in vitro* assay of tau aggregation to specifically look for inhibitors of tau accumulation, and were able to demonstrate that anthroquinones, a class of anti-tumor drugs, were able to not only inhibit the formation of PHFs, but also to disrupt them [21]. Perhaps more importantly, this study also demonstrated that microtubule stabilization by tau was unaffected by these inhibitors, suggesting that cells may be tolerant to the reduction or redistribution of tau within the cell. In light of findings such as these, along with the viability of tau knockout mice, tau-lowering compounds may indeed hold promise for sufferers of tauopathies. However, with the development of an ICW methodology in which the cells are fixed directly in the assay plate, preserving any secondary and tertiary protein structures, we can now quantitatively measure levels of tau in specific pathogenic states, as it has been demonstrated that specific conformational and phosphorylation changes are associated with the aggregation of tau into filaments and with neuronal cell death.

Because our In-Cell assays fix these epitopes in a similar manner to immunohistochemical studies, we should be able to quantitatively analyze levels of these crucial aggregation-competent species. Given the innovation of the ICW assay format, we are confident that we will now be able to 1) rapidly identify novel compounds capable of specifically reducing aberrant forms of tau that are implicated in disease progression, and 2) develop similar cell-based protocols for other disease-associated (i.e. synuclein and huntingtin) proteins of interest using unique cell lines and antibodies. Overall, compounds discovered to be capable of favorably altering tau levels could also be rapidly introduced into the clinic, particularly for use in patients with hereditary mutations in the tau gene that lead to its over-production and aggregation. Screening assays, such as the ICW described here, will likely be critical for future drug development against diseases of intracellular protein accumulation, such as AD, PD and Huntington's Disease.

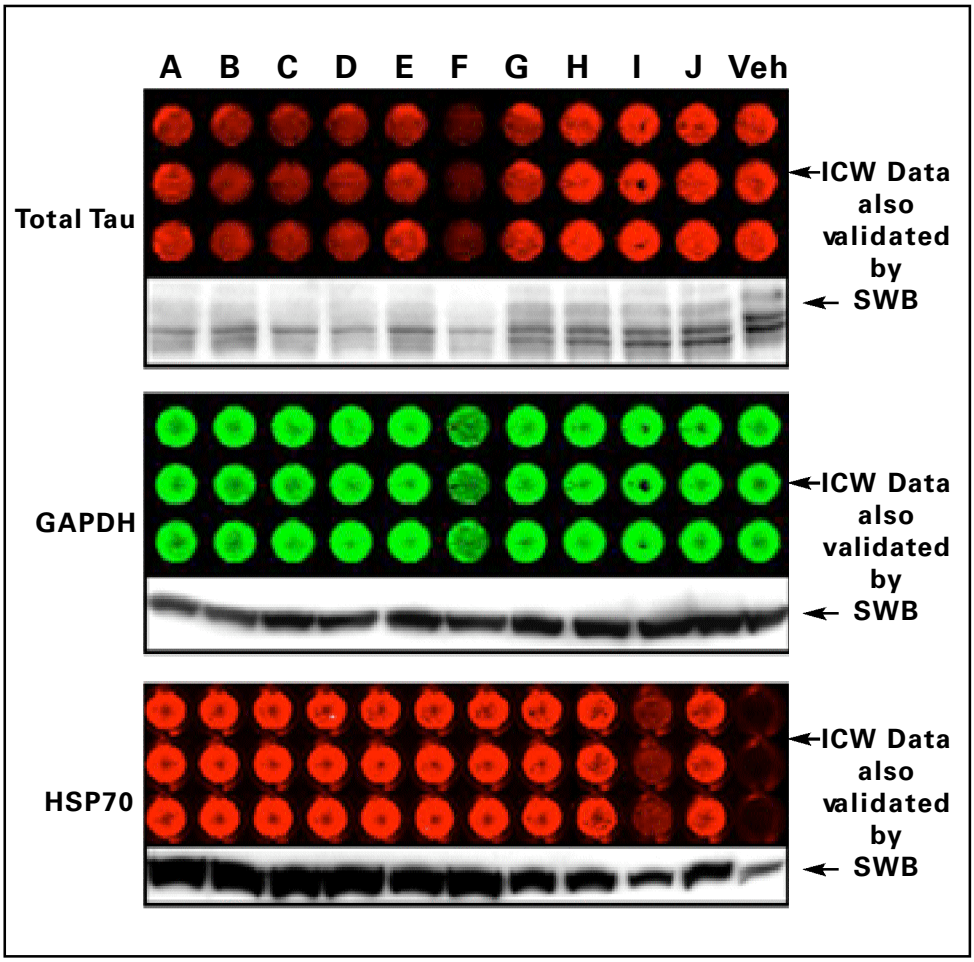

Figure 1.
HSP90 inhibitors induce HSP70 protein levels and reduce total tau levels, largely in the absence of toxicity. H4 cells were treated with a 1 µM concentration of small molecule HSP90 inhibitors for 48 hours. Cells were treated in triplicate for In-Cell Western (ICW) analysis and validated by Standard Western blot (SWB) in separate experiments. Compounds A - J are 10 novel and distinct compounds with putative HSP90 inhibiting activity. Secondary detection for both tau and HSP70 were done respectively with AlexaFluor® 680 conjugated goat anti-rabbit and anti-mouse antibodies. For secondary detection of GAPDH, IRDye™ 800CW-labeled goat anti-mouse antibody was used. Veh indicates DMSO treated cells only.

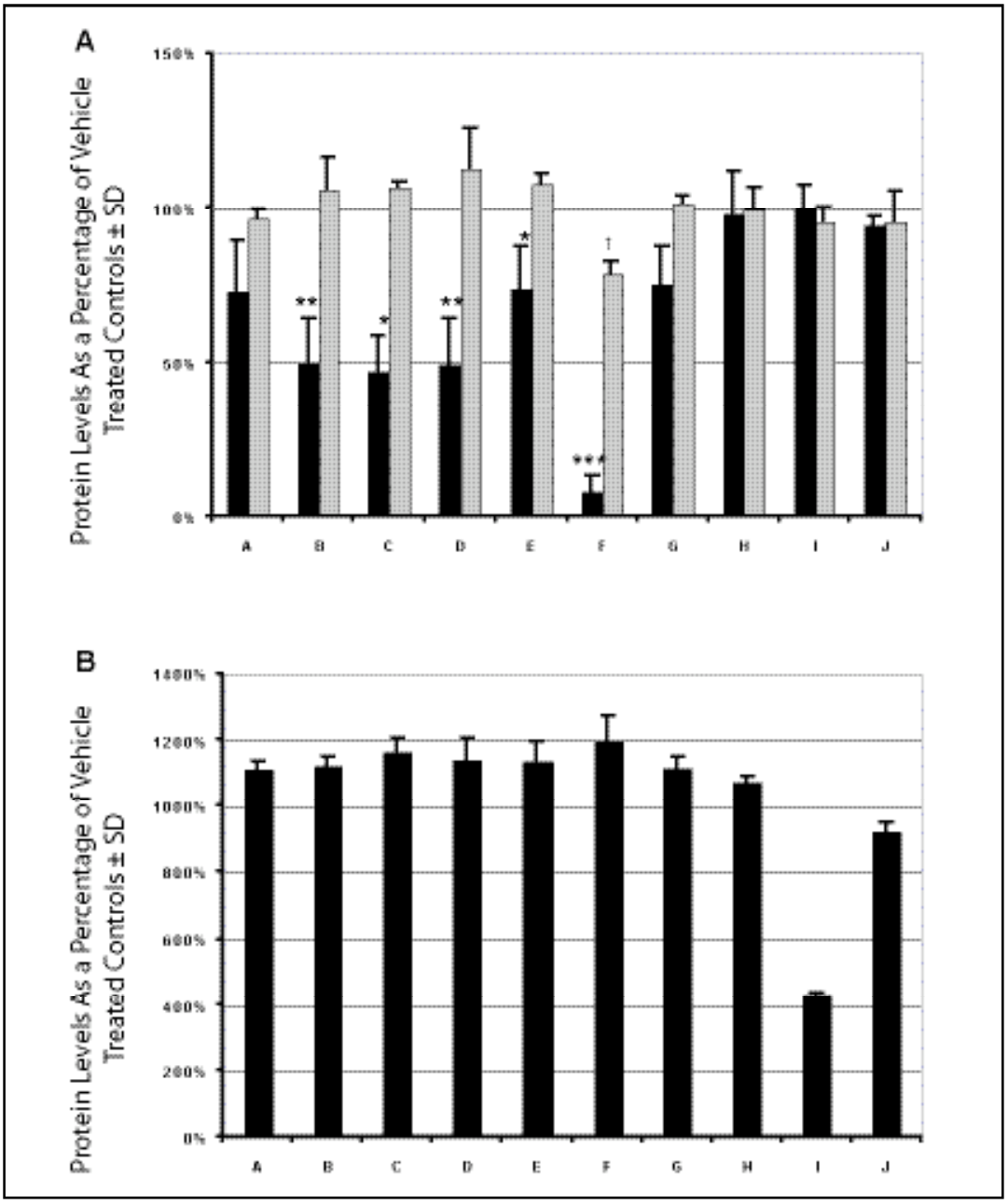

Figure 2.
Predominantly non-toxic novel HSP90 inhibitors lower tau protein levels at a tolerable dosage following 48-hour exposure. Human H4 neuroglioma cells were treated in triplicate for 48 hours with both a 1 μM concentration of 10 HSP90 inhibitors (identified by letter on the x-axis) and vehicle for 48 hours.

PANEL A: Tau (black bars) and GAPDH (gray bars) protein expression were simultaneously measured with rabbit anti-human tau and mouse anti-GAPDH antibodies using an ICW analysis. Expression of both tau and GAPDH are presented here as a percentage of their respective vehicle control levels. Significance was measured for tau levels by comparing GAPDH to tau expression. As determined by student t-test, "*" indicates a p-value of < 0.05, "" indicates a p-value of < 0.01 and "***" indicates a p-value of < 0.001. Toxicity was determined by comparing GAPDH levels in drug treated cells to vehicle treated cells by student t-test. "† " indicates a p-value of < 0.05.**

PANEL B: HSP70 levels were measured in a separate ICW assay using an anti-mouse HSP70 antibody. All 10 compounds elevated HSP70 significantly compared to vehicle as determined by Student t-test (p< 0.05); however, drugs that caused tau reductions elicited a much more robust HSP70 induction than those compounds that did not lower tau levels.

REFERENCES

1. Davies, P., Maloney, A. J.: **Selective loss of cen tral cholinergic neurons in Alzheimer's disease**. *Lancet* 1976, **2**(8000):1403.

2. Rogers, S. L., Friedhoff, L. T.: **The efficacy and safety of donepezil in patients with Alzheimer's disease: results of a US Multicentre, Randomized, Double-Blind, Placebo-Controlled Trial. The Donepezil Study Group**. *Dementia* 1996, **7**(6):293-303.

3. Marjaux, E., Hartmann, D., De Strooper, B.: **Presenilins in memory, Alzheimer's disease, and therapy.** *Neuron* 2004, **42**(2):189-192.

4. Ohno, M., Sametsky, E. A., Younkin, L. H., Oakley, H., Younkin, S. G., Citron, M., Vassar, R., Disterhoft, J. F.: **BACE1 Deficiency Rescues Memory Deficits and Cholinergic Dysfunction in a Mouse Model of Alzheimer's Disease**. *Neuron* 2004, **41**(1):27-33.

5. Weggen, S., Eriksen, J. L., Das, P., Sagi, S. A., Wang, R., Pietrzik, C. U., Findlay, K. A., Smith, T. E., Murphy, M. P., Bulter, T., et al: **A subset of NSAIDs lower amyloidogenic Abeta42 inde pendently of cyclooxygenase activity**. *Nature* 2001, **414**(6860):212-216.

6. Cuajungco, M. P., Faget, K. Y., Huang, X., Tanzi, R. E., Bush, A. I.: **Metal chelation as a potential therapy for Alzheimer's disease.** *Ann N Y Acad Sci* 2000, **920**:292-304.

7. Carson, J. A., Turner, A. J.: **Beta-amyloid catab olism: roles for neprilysin (NEP) and other met allopeptidases?** *J Neurochem* 2002, **8**1(1):1-8.

8. Oddo, S., Billings, L., Kesslak, J. P., Cribbs, D. H., LaFerla, F. M.: **Abeta immunotherapy leads to clearance of early, but not late, hyperphos phorylated tau aggregates via the proteasome.** *Neuron* 2004, **43**(3):321-332.

9. Hutton, M., Lendon, C. L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., et al: **Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17.** *Nature* 1998, **393**(6686):702-705.

10. Tucker, K. L., Meyer, M., Barde, Y. A.: **Neurotrophins are required for nerve growth during development.** *Nat Neurosci* 2001, **4**(1):29-37.

11. Kar, S., Fan, J., Smith, M. J., Goedert, M., Amos, L. A.: **Repeat motifs of tau bind to the insides of microtubules in the absence of taxol.** *EMBO J* 2003, **22**(1):70-77.

12. Eriksen, J. L., Dawson, T. M., Dickson, D. W., Petrucelli, L.: **Caught in the act: alpha-synuclein is the culprit in Parkinson's disease.** *Neuron* 2003, **40**(3):453-456.

REFERENCES (CONTINUED)

13. Shimura, H., Schwartz, D., Gygi, S. P., Kosik, K. S.: **CHIP-Hsc70 complex ubiquitinates phos phorylated tau and enhances cell survival**. *J Biol Chem* 2004, **279**(6):4869-4876.

14. Petrucelli, L., Dickson, D., Kehoe, K., Taylor, J., Snyder, H., Grover, A., De Lucia, M., McGowan, E., Lewis, J., Prihar, G., et al: **CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation**. *Hum Mol Genet* 2004, **13**(7):703-714.

15. Dou, F., Netzer, W. J., Tanemura, K., Li, F., Hartl, F. U., Takashima, A., Gouras, G. K., Greengard, P., Xu, H.: **Chaperones increase association of tau protein with microtubules**. *Proc Natl Acad Sci U S A* 2003, **100**(2):721-726.

16. McLean, P. J., Klucken, J., Shin, Y., Hyman, B. T.: **Geldanamycin induces Hsp70 and prevents alpha-synuclein aggregation and toxicity in vitro**. *Biochem Biophys Res Commun* 2004, **321**(3):665-669.

17. Sittler, A., Lurz, R., Lueder, G., Priller, J., Lehrach, H., Hayer-Hartl, M. K., Hartl, F. U., Wanker, E. E.: **Geldanamycin activates a heat shock response and inhibits huntingtin aggre gation in a cell culture model of Huntington's disease**. *Hum Mol Genet* 2001, **10**(12):1307-1315.

18. Shimura, H., Miura-Shimura, Y., Kosik, K. S.: **Binding of tau to heat shock protein 27 leads to decreased concentration of hyperphosphorylat ed tau and enhanced cell survival**. *J Biol Chem* 2004, **279**(17):17957-17962.

19. Bagatell, R., Whitesell, L., Shimura, H., Miura-Shimura, Y., Kosik, K. S., Zhou, Y., Gu, G., Goodlett, D. R., Zhang, T., Pan, C., et al: **Altered Hsp90 function in cancer: a unique therapeutic opportunity**. *Mol Cancer Ther* 2004, **3**(8):1021-1030.

20. Dickey, C. A., Eriksen, J., Kamal, A., Burrows, F., Kasibhatla, S., Eckman, C. B., Hutton, M., Petrucelli, L.: **Development of a High Throughput Drug Screening Assay for the Detection of Changes in Tau Levels - Proof of Concept with HSP90 inhibitors**. *Curr Alz Res* 2005, **2**(2):231-238(8).

21. Pickhardt, M., Gazova, Z., von Bergen, M., Khlistunova, I., Wang, Y., Hascher, A., Mandelkow, E. M., Biernat, J., Mandelkow, E.: **Anthraquinones inhibit tau aggregation and dissolve alzheimer paired helical filaments *in vitro* and in cells**. *J Biol Chem* 2004.

LI-COR®
Biosciences

4308 Progressive Ave. • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
North America: 800-645-4267 • International: 402-467-0700 • FAX: 402-467-0819
LI-COR GmbH*: +49 (0) 6172 17 17 771 • www.licor.com
(Serving Austria, Czech Republic, Germany, Hungary, Slovakia, and Switzerland)

Doc # 979-08133
0705