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Stabilizing the Hsp70-Tau Complex Promotes Turnover in Models of Tauopathy

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Summary

Heat shock protein 70 (Hsp70) is a chaperone that normally scans the proteome and initiates the turnover of some proteins (termed “clients”) by linking them to the degradation pathways. This activity is critical to normal protein homeostasis, yet it appears to fail in diseases associated with abnormal protein accumulation. It is not clear why Hsp70 promotes client degradation under some conditions, while sparing that protein under others. Here, we use a combination of chemical biology and genetics strategies to systematically perturb the affinity of Hsp70 for the model client, tau. This approach revealed that tight complexes between Hsp70 and tau are associated with enhanced turnover while transient interactions favored tau retention. These results suggest that client affinity is one important parameter governing Hsp70-mediated quality control.

Graphical abstract

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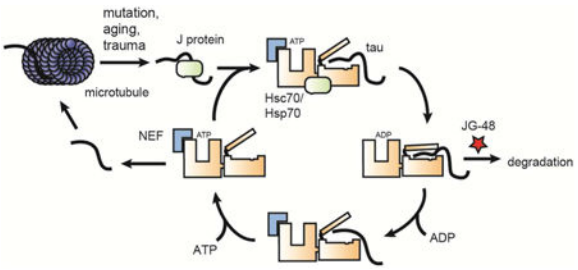

Introduction

Heat shock protein 70 (Hsp70/HSPA1A) and heat shock cognate 70 (Hsc70/HSPA8) are highly conserved molecular chaperones that are expressed in the cytosol of all eukaryotic cells. These factors are often referred to as “triage” chaperones because they bind to misfolded proteins and somehow choose to shuttle them to the lysosome-autophagy pathway or ubiquitin-proteasome system (UPS) for degradation (Mayer, 2013). Although they play a complex and important role in proteostasis, members of the Hsp70 family have a relatively simple structure, composed of a 45 kDa nucleotide-binding domain (NBD) and 25 kDa substrate-binding domain (SBD) (Jiang et al., 2005; Zhuravleva et al., 2012). The NBD has a deep cleft for binding ATP, while client proteins bind in a β-sandwich region of the SBD (Bertelsen et al., 2009). These two domains are allosterically linked, with nucleotide turnover in the NBD controlling the affinity of SBD-client interactions (Bauer et al., 2015; Clerico et al., 2015; Palleros et al., 1993; Zhuravleva and Gierasch, 2015). In the ATP-bound form, clients bind Hsp70s with fast-on, fast-off kinetics, while hydrolysis to the ADP-bound form stabilizes the SBD-client interaction by slowing the off-rate (Ha and McKay, 1995).

Co-chaperones bind members of the Hsp70 family to regulate their nucleotide cycling (Mayer, 2013). These co-chaperones include J-proteins, which accelerate ATP hydrolysis, and nucleotide exchange factors (NEFs) that promote the discharge of ADP. Together, J proteins and NEFs coordinate turnover, ultimately regulating the affinity for clients. Then, additional co-chaperone families, including the tetratricopeptide repeat (TPR) domain proteins, bind Hsp70s and help direct bound clients into specific pathways. For example, CHIP is an E3 ubiquitin ligase that directs Hsp70 clients to the proteasome (Dickey et al., 2006; Shimura et al., 2004). Other co-chaperones, including members of the Bag family of NEFs, link Hsp70 and its clients to the lysosome-autophagy pathway (Demand et al., 2001). Together, Hsp70, Hsc70 and their co-chaperones cooperate to identify misfolded proteins and somehow enact the “decision” to degrade them. This process is central to health and proteostasis because it limits protein accumulation; however, the molecular mechanisms are not clear.

Microtubule-binding protein tau (MAPT/tau) has served as an important client for understanding chaperone-mediated quality control (Miyata et al., 2011). The accumulation of aggregated tau is a pathological feature of many neurodegenerative disorders, including Alzheimer's disease (AD), frontotemporal dementia (FTD) and progressive supranuclear palsy (PSP). Tau is an intrinsically disordered protein (Narayanan et al., 2010) that normally stabilizes microtubules. As a result of alternative splicing, tau exists as six major isoforms

(Ballatore et al., 2007), which are named based on the inclusion of variable numbers of microtubule-binding repeats (either 3R or 4R) and the number of N-terminal extensions (0N, 1N or 2N). The aggregation-prone regions of tau are located in the microtubule-binding repeats, such that free tau (*i.e.* the pool that is not bound to microtubules) is considered to be most likely to aggregate. Indeed, mutations, such as P301L and A152T (Coppola et al., 2012; Hong et al., 1998; Kara et al., 2012; Vogelsberg-Ragaglia et al., 2000), and/or post-translational modifications (PTMs), such as hyperphosphorylation and acetylation (Cook et al., 2014; Hanger et al., 2009; Morris et al., 2015), seem to disrupt the affinity of tau for microtubules and promote its aggregation.

Members of the Hsp70 family are well known to play an important role in tau homeostasis. Hsc70 and Hsp70 share the same binding sites within the aggregation-prone regions of the microtubule-binding repeats (Jinwal et al., 2013; Thompson et al., 2012) and they both block tau aggregation *in vitro* (Voss et al., 2012). However, Hsc70 and Hsp70 are thought to do more than simply limit aggregation. Over-expression of Hsc70 favors association of tau with microtubules, while over-expression of Hsp70 leads to its degradation (Jinwal et al., 2013). Hsp70 more effectively recruits CHIP to tau (Jinwal et al., 2013), which might be why this paralog is more closely linked to degradation. Accordingly, Hsc70 might interfere with the ability of Hsp70-CHIP to degrade tau, leading to the observed increase in microtubule binding. The difference between these paralogs is important because Hsc70 is constitutively expressed, while expression of Hsp70 is stress-inducible. So, the ratio of the paralogs is likely to change in response to stress and this might impact tau homeostasis. Differences in the ability of Hsc70 and Hsp70 to recruit CHIP are surprising because they are more than 85% identical and they have the same affinity for CHIP *in vitro* (Smith et al., 2013). However, over-expression of a dominant negative form of Hsc70 has recently been found to enhanced turnover of tau (Fontaine et al., 2015), suggesting that Hsc70 and Hsp70 are both capable of directing tau to the degradation pathway under some conditions. Together, these results show a close relationship between Hsp70s and tau homeostasis, but the molecular mechanisms are not clear. Most importantly, it isn't clear how members of the hsp70 family “decide” to shuttle tau to the degradation pathways.

We wanted to use a chemical biology approach to address this question. Inhibitors that bind to both Hsc70 and Hsp70, such as YM-01, are known to activate the degradation of tau in models of tauopathy (Rousaki et al., 2011). In these findings, we saw an opportunity to use YM-01 as a chemical probe. However, we first had to synthesize JG-48, an analog which did not interfere with chaperone activity assays. We found that JG-48, but not a closely related compound, JG-273, stabilized the ADP-bound state and blocked the ability of NEFs to facilitate ADP and tau release. The net effect of this allosteric perturbation was that the affinity for tau was increased *in vitro* and in cells. Guided by those findings, we used chemical and genetic approaches to show that stabilizing the complex tended to enhance tau turnover, while weakening it favored tau retention. While many questions remain, these results suggest that client-binding affinity is one feature used by Hsp70 family chaperones to initiate their degradation.

Results

JG-48 reduces tau accumulation in multiple models and restores long-term potentiation

YM-01 and its analogs are known to enhance tau turnover through interactions with Hsc70 and Hsp70 (Abisambra et al., 2013; Rousaki et al., 2011). However, these compounds contain a cationic pyridinium that precludes many *in vitro* studies because they absorb at the same wavelengths that are used in common chaperone assays (*e.g.* 535 to 620 nm). To provide a molecule suitable for use in these platforms, we assembled JG-48 using a previously established synthetic route (Miyata et al., 2013) (Supplemental Figure 1A). At the same time, we synthesized a control compound, JG-273, that lacks the benzyl group predicted to be important for binding (Li et al., 2013). As anticipated, the neutral molecules, JG-48 and JG-273, lacked the strong absorbance in the region of 535 to 620 nm, which previously hindered use of YM-01 (Supplemental Figure 1B).

Before using JG-48 as a chemical probe, we needed to establish whether it retained the ability of YM-01 to reduce tau levels in models of tauopathy. In the first experiments, we used HeLaC3 cells that had been stably transfected with human 4R0N tau. These cells were treated with JG-48 or JG-273 for 24 hours and their total tau levels analyzed by Western blot. We found that JG-48, but not JG-273, reduced tau levels by ~50% at 30 μM (Figure 1A). This decrease in tau levels required the lysosome under these conditions (Supplemental Figure 1C). To confirm whether JG-48 could also reduce endogenous (rather than over-expressed) tau, we used SHSY-5Y neuroblastoma cells. JG-48 decreased total and phosphorylated tau levels in that system as well (Figure 1A).

Another feature of YM-01 is that it reduces tau levels in brain slices. To see if JG-48 shared this activity, we treated hippocampal brain slices from the rTg4510 transgenic mouse model (Spires et al., 2006). Brain slice cultures from these mice were treated for six hours with JG-48 and we found that, like YM-01 (Abisambra et al., 2013), the levels of total and phosphorylated tau were reduced (Figure 1B). Brain slices contain both neurons and support cells, such as astrocytes. To understand if the reduction in tau occurred in neurons, we made brain aggregate cultures from the rTg4510 mice. This system was used because, compared to brain slices, the aggregate model provides a more convenient platform for immunofluorescence and imaging. We found that tau levels in tubulin-positive neurons were significantly reduced by either YM-01 or JG-48 (10 μM) (Figure 1C). These results suggest that JG-48 shares YM01's ability to reduce tau levels.

JG-48 binds the NBD of Hsc70 but does not inhibit ATP binding

Our strategy was to use JG-48 as a chemical probe to better understand the mechanisms of chaperone-mediated tau quality control. The first question was whether JG-48 might compete with ATP for binding to the chaperones, which would provide a potential clue to its mechanism-of-action. NMR titrations had previously shown that compounds similar to JG-48, such as MKT-077 and JG-98, bind in a deep, conserved pocket of the NBD (Amick et al., 2014; Li et al., 2013; Rousaki et al., 2011). This site is 100% conserved between Hsc70 and Hsp70, so these compounds bind both paralogs equally (Li et al., 2013). To confirm that JG-48 also binds to this site, we repeated the HSQC titration study with ^{15}N

Hsc70 NBD in the presence of ADP. In these studies, we used $Hsc70_{NBD}$, rather than $Hsp70_{NBD}$, because the peak assignments were available. Consistent with the previous work, JG-48 caused selective chemical shift perturbations (CSPs) in residues adjacent to a deep cleft composed of hydrophobic and anionic residues (Figure 2A). Guided by the CSPs, we performed docking simulations of JG-48 bound to $Hsc70_{NBD}$ (PDB: 3C7N) and found that the most favorable poses positioned it in a cleft between subdomains IIA and IIB (Figure 2A). In the docked binding mode, the benzothiazole ring system of JG-48, which is missing in JG-273, was predicted to make a number of important interactions.

The docked pose of JG-48 suggested that the molecule might not directly interfere with nucleotide binding. Indeed, there appeared to be a channel through the protein, with nucleotide on one end and JG-48 on the other (Figure 2A). To directly test this prediction experimentally, we adapted a fluorescence polarization (FP) assay (Williamson et al., 2009) in which competition with a tight binding (K_D ~ 400 nM) probe, N^6-(6-Amino)hexyl-ATP-6-FAM (or ATP-FAM), is measured. We first confirmed that unlabeled ATP and a known competitive inhibitor, VER-155008 (VER) (Williamson et al., 2009), interrupted tracer binding with IC_{50} values of 200 ± 19 nM and 390 ± 28 nM, respectively (Figure 2B). In contrast, JG-48 had no effect on ATP-FAM binding, suggesting that it does not compete with ATP. Rather, its activities appear to be allosteric.

JG-48 inhibits ATPase activity

We then turned to a series of biochemical assays to understand the impact of JG-48 *in vitro*. First, the ability of JG-48 to inhibit steady state ATPase activity was measured using a colorimetric assay (Chang et al., 2008). In these studies, used a combination of Hsc70, a J protein (DnaJA2) and a nucleotide-exchange factor (BAG-1). Consistent with the literature (Rauch and Gestwicki, 2014), titration of BAG-1 into the combination of Hsc70 (1 μM) and DnaJA2 (0.2 μM) first stimulated ATPase activity and then, at higher concentrations, it decreased the signal as it favors the apo-state of Hsc70 (Figure 3A). Using this paradigm, we treated with JG-48, JG-273 and the characterized inhibitors, VER (Williamson et al., 2009) and myricetin (Chang et al., 2011). We found that JG-48, myricetin and VER (50 μM), inhibited the ATPase activity of this chaperone system (Figure 3A), while JG-273 had no effect (Supplemental Figure 2A-B). JG-48 caused a rightward shift in the BAG-1 concentration profile, perhaps consistent with a weakened affinity of that co-chaperone for Hsc70 (see below). A similar conclusion was made when we replaced DnaJA2 and BAG-1 with other co-chaperones, including DnaJA1, DnaJB1, DnaJB4, BAG2 and BAG3 (Supplemental Figure 2A-B), suggesting that this effect is not restricted to only one set of co-chaperones.

JG-48 inhibits substrate refolding by Hsc70

To explore whether JG-48 might impact Hsc70-client interactions, we used a well-established assay in which Hsc70, DnaJA2 and BAG-1 cooperate to refold denatured firefly luciferase *in vitro*. This assay requires dynamic interactions between luciferase and the chaperones, providing a functional read-out of client cycling (Sharma et al., 2011). Similar to the design of the ATPase assays, we titrated BAG-1 into the combination of Hsc70 (1 μM) and DnajA2 (0.2 μM), showing that it first stimulates and then inhibits luminescence (Figure

3B). In this system, we found that treatment with JG-48 and VER (50 μM), but not JG-273 (50 μM), reduced the amount of recovered luminescence by approximately 60% (Figure 3B and Supplemental Figure 2C). Again, similar findings were observed when we replaced DnaJA2 and BAG-1 with other co-chaperones (Supplemental Figure 2D). In addition, we found that replacing Hsc70 with Hsp70 did not impact the ability of JG-48 to inhibit luciferase refolding (Figure 3B). In control experiments, we found that none of the compounds directly interfered with native luciferase activity (Supplemental Figure 2C), ruling out this possible artifact.

JG-48 inhibits client release from Hsc70

The effects of JG-48 on luciferase refolding suggested that it might impact client-chaperone interactions, perhaps through BAG-1. To test this idea more directly, we turned to a fluorescence polarization (FP) assay in which binding to a FAM-labeled client peptide, HLA, is measured (Ricci and Williams, 2008). We found that Hsc70 binds the tracer with an apparent K_D of 5.1 ± 0.9 μM in the presence of 1 mM ADP, consistent with previous reports (Rauch and Gestwicki, 2014). Titration of BAG-1 into this mixture released the HLA-FAM tracer with an EC_{50} of 3.7 ± 0.8 μM (Figure 4A). Then, we measured the ability of JG-48 to inhibit BAG-1 activity. We found that JG-48, but not JG-273, limited the ability of BAG-1 to release the tracer. The net effect of JG-48 was to stabilize the client interaction by 6.7-fold (EC_{50} 25 ± 8.6 μM) (Figure 4A). Thus, JG-48 does appear to stabilize Hsc70-client interactions by restricting the ability of BAG-1 to release it.

Members of the Bag family of NEFs promote release of clients from Hsc70 and Hsp70 by binding to the NBD (Briknarova et al., 2001; Liu et al., 2010; Sondermann et al., 2001; Xu et al., 2008). To test if JG-48 could directly impact the protein-protein interaction between NEFs and Hsc70, biotinylated Hsc70 was immobilized on beads and these were incubated with fluorescently labeled BAG-1 in a flow cytometry protein interaction assay (FCPIA) format. For these experiments, BAG-1 was used because it appears to be particularly important for tau (Elliott et al., 2007; Luders et al., 2000). We found that JG-48, but not JG-273 or VER, inhibited BAG-1 binding (Figure 4B). Thus, JG-48 appeared to partially limit BAG-1 binding.

Chemical and genetic manipulation of tau affinity reveals a correlation with tau turnover in cells

Next, we wanted to test if JG-48 would increase binding to tau, as it did for the model HLA-FAM client. To do this, an ELISA (Thompson et al., 2012) was used in which Hsc70 or Hsp70 is immobilized in microtiter wells and binding to tau is measured with a labeled antibody. We found that both Hsc70 and Hsp70 have an affinity of ~5 μM for tau in the presence of ADP in this format, consistent with previous reports (Thompson et al., 2012). Replacing ADP with either ATP or non-hydrolyzable ATPγS weakened the apparent affinity of Hsc70 for tau to 12 ± 0.9 and 16 ± 1.7 μM, respectively, showing that this system is sensitive to nucleotide-driven changes in client affinity. DMSO alone had no effect on the strength of the complex in the presence of ADP, but JG-48 increased binding by ~2-fold (K_D = 2.4 ± 0.3 μM) (Figure 5A). A parallel experiment showed that JG-48 also increased binding of Hsp70 for tau in this format (K_D = 2.6 ± 0.8 μM). These results suggest that,

similar to what was observed with the model client, JG-48 stabilizes chaperone-tau complexes. To test this idea in cells, we performed immunoprecipitations of V5-tagged tau from HeLa C3 cells after treatment with JG-48. Consistent with the ELISA, JG-48 enhanced binding of Hsp70 to tau, by ~ 4-fold (Figure 5B). Interestingly, treatment with JG-48 did not change the amount of Hsc70 that was co-immunoprecipitated with tau (Figure 5B), perhaps because of competition between these chaperones for binding (Fontaine et al., 2015).

Together, these results suggested a possible model in which stabilization of the tau complex by JG-48 might possibly be a signal for promoting its turnover. To test this idea, we wanted to systematically control the affinity for tau and then measure the corresponding effects on its levels in cells. Towards this goal, we assembled a collection of known inhibitors, including VER (Williamson et al., 2009), MAL3-101 (Fewell et al., 2004) and YM-01 (Abisambra et al., 2013; Wang et al., 2013). Importantly, these compounds belong to three distinct chemical series and bind non-overlapping sites on Hsc70, so they provide a more rigorous test of the model than using only a single chemotype. In the ELISA, we found that MAL3-101 and YM-01 enhanced binding of Hsc70 to tau by approximately 2-fold (Figure 5A and Supplemental Figure 3A), similar to what we found for JG-48. Treatment with VER mildly destabilized the Hsc70-tau complex, decreasing its affinity by ~36%, perhaps consistent with its design as an ATP mimetic (Williamson et al., 2009). With these tools in-hand, we treated HeLaC3 cells and measured total tau levels by Western blot (Supplemental Figure 3). The results suggested that, consistent with the model, if a compound stabilized the Hsc70-tau complex *in vitro*, it tended to decrease tau stability in cells. For example, YM-01 enhanced Hsc70 binding by approximately 2-fold *in vitro* and reduced tau levels by about 75% compared to the controls.

To complement these chemical perturbations, we also wanted to use point mutations in Hsc70 to alter the affinity for tau. We considered this experiment to be important because the selectivity of small molecules in cells is often uncertain, so combining small molecules with genetics is a way to independently assess the model. As mentioned previously, a point mutant in Hsc70, E175S, had recently been shown to stabilize the Hsc70-tau interaction and lead to enhanced turnover (Fontaine et al., 2015). This result is consistent with the prediction, but to further interrogate this system we mutated residues in JG-48's binding site on Hsc70 (see Fig. 2) to see if any of these residues, when mutated, might also disrupt affinity for tau. In the ELISA platform, we found that L228A weakened the affinity for tau by ~80%, while R76A and Y149A had little impact on tau affinity (Figure 5A). Next, we over-expressed Hsc70, Hsp70 and the Hsc70 mutants in HelaC3 cells and measured their effects on tau levels. The results were consistent with the chemical genetic studies. For example, over-expression of the L228A mutant, which weakened the interaction *in vitro*, caused a reproducible increase in tau levels (Figure 5A and Supplemental Figure 3C). Taken together, these results suggested a rough correlation ($R^2 = 0.58$) between apparent affinity and tau turnover (Figure 5C).

Discussion

A network of chaperones, co-chaperones and stress response pathways is tasked with maintaining normal proteostasis (Powers et al., 2009a). In this network, members of the

Hsp70 family, such as Hsc70 and Hsp70, are considered to be the “triage” chaperones that initiate degradation of many clients (Hohfeld et al., 2001). This system is emerging as a potential drug target in a number of diseases, including cancer and neurodegeneration (Evans et al., 2010; Powers et al., 2009b; Pratt et al., 2015). Therefore, there is great interest in better understanding the molecular mechanisms of chaperone-initiated protein degradation.

In this study, we have taken advantage of chemical probes, combined with new point mutants, to explore these mechanisms. The key enabling observation was that Hsp70 inhibitors, such as YM-01, promote the turnover of tau (Abisambra et al., 2013). These findings suggested to us that YM-01 might be a chemical probe for understanding the role of Hsp70 family members in tau turnover. Before embarking on such a study, we first had to resolve problematic features of YM-01, removing spectral properties that interfered with many biochemical measurements. This exercise yielded JG-48, which retained the ability to reduce tau in cultured cells and brain slices (see Figure 1). By examining many possible mechanisms (*e.g.* effects on ATPase activity, protein-protein interactions, *etc*), we eventually found that the stability of the tau complex, as measured *in vitro*, seemed to best predict the effects of the compound on tau turnover in cells. This prediction was then tested using a collection of independent chemical and genetic perturbations, leading to a model (Figure 5D) in which one of the factors governing client degradation (at least for tau) appeared to be its affinity for the chaperone. This model has implications for the discovery of compounds that act through Hsp70 family members to accelerate tau turnover. Namely, it suggests that tau binding affinity might be a good metric by which to guide the optimization of anti-tau compounds.

This model, like many, leads to unanswered questions. What are the key factors that determine affinity for clients in the cell? In the case of tau, it seems possible that mutations or PTMs might signal degradation through altering affinity for chaperone. For instance, mutations or PTMS that tau's weaken affinity for microtubules might create a tighter affinity for Hsp70 by increasing the pool of free client, communicating a “desire” to destroy these potentially dangerous variants. Other mutations or PTMs in tau might directly enhance (or suppress) binding to the chaperone. Another open question involves the complex roles of Hsc70 and Hsp70 in tau turnover. In our co-immunoprecipiations, we noted that Hsp70, but not Hsc70, accumulated on tau in response to JG-48 (see Figure 5B). The origin of this paralog-selective response is mysterious. It is possible that additional factors, such as CHIP, might amplify the recruitment of Hsp70 at the expense of Hsc70. Future work will need to explore this important, next question. For the moment, we favor the conservative model in which both paralog are able to link to the degradation pathways, but that Hsp70 plays a dominant role.

According to this model, the decision to degrade tau should also be impacted by the availability of co-chaperones. NEFs might be particularly important because they would be expected to help release tau from the Hsp70 complex. Under the model, this activity would be predicted to enhance tau levels by avoiding turnover. This general concept is supported by the findings that BAG-1 overexpression increases total tau levels in an Hsp70 dependent manner (Elliott et al., 2007). Beyond tau, BAG-2 and HspBP1 are known to prevent

ubiquitination of the other Hsp70 clients Raf-1 and the cystic fibrosis transmembrane conductance regulator (CFTR) (Alberti et al., 2004; Arndt et al., 2005). These findings support the idea that the release of clients is associated with retention and that NEFs are important in "tuning" client dwell time. However, it is important to note that BAG-1 coordinates with ubiquitin ligases to target some Hsc70 clients for proteasomal degradation (Demand et al., 2001; Luders et al., 2000), illustrating the difficulty in identifying clear "rules" for protein quality control. Despite the need for additional studies, this work suggests that one important feature might be the intrinsic affinity of the client for the chaperone.

Significance

Accelerating the clearance of abnormal tau is an attractive therapeutic strategy because it would address tauopathies at their source. Members of the Hsp70 family are potential targets for such a strategy because they bind tau and assist in its degradation. Here, we probed the mechanisms by which an inhibitor, JG-48, promotes the turnover of tau. These results suggest that stabilizing the tau complex is a signal to initiate tau turnover. Although JG-48 itself may not be a clinical lead for such studies, these results provide a potential roadmap for the discovery of molecules to normalize tau homeostasis.

Methods

Cell culture and immunoblotting

HeLa C3 cells were stably transfected with V5-4R0N tau as previously described (Fontaine et al., 2015). Cells were cultured in supplemented Opti-MEM media. Cells were plated into 6-well plates (Corning) or 12-well plates (Corning) and treated with compound at indicated concentrations for 24 hrs before lysis with RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with protease inhibitor, PMSF and NaF. Following quantification, lysates were loaded to 10% gels (BioRad) and proteins identified via Western blot. For Hsc70 mutant transfections, HeLa C3 cells were plated into 12-well plates (Corning) and 4 μg of plasmid was added with Lipofectamine 2000 (Invitrogen). Transfection was allowed to proceed for 21 hrs before lysis with RIPA buffer. Cell or tissue lysates were separated and immonoblotted via Western blot. Phosphorylated tau (pTau) was measured with an antibody that recognizes the paired helical filament (PHF) form present in tauopathy patients (Otvos et al., 1994). All quantification of Western blots was performed with NIH Image J or Biorad Image Lab analysis.

Brain aggregate cultures

Brain aggregates are prepared from E15 days rTg4510 mouse brains. Briefly, E15 day mouse brains are dissociated through two nylon meshes and plated in a 96-well plate. A sphere-shaped brain aggregate formed in each well is cultured in DMEM H21 supplemented with glucose (6 g/L), gentamicin (50 mg/L) and 10% FBS. At 15 days in culture, they are infected with 5 μL of 10% brain homogenates of rTg4510 mice for 10 days. They are treated with 10 μM of YM-01 or JG-48 for 24 hrs before harvesting at 35 days in culture. They are fixed with 4% paraformaldehyde for 3 days. Fixed brain aggregates are washed with PBS for 1 hr (3×) and incubated with blocking buffer (0.3% Triton X-100, 0.1% Tween 20, 2%

bovine serum albumin and 10% normal goat serum in PBS) overnight and stained with primary antibodies (PHF-tau (MN1020, Thermo Scientific) and anti-beta tubulin (H-235, Santa Cruz Biotech); 1:100) for 3 days. After incubation with primary antibodies, they are washed with PBS and incubated with secondary antibodies (goat anti-rabbit IgG conjugated with Alexa633 and goat anti-mouse conjugated with Alexa568, Jackson Immunoresearch Laboratory; 1:800) overnight. After washing with PBS for 1 hr (3×), they were coversliped with Vectashield mounting media containing DAPI (H-1500, Vector laboratories). Confocal images are taken with a Leica SP8 confocal microscope and 15-20 z-sectioned images are stacked together before measuring phospho-tau intensity with Image J.

Slice cultures

Hippocampal slices from 4 month old rTg4510 or wild type mice were perfused with JG-48 (30 μM) or 1% DMSO vehicle control for 6 hours as previously described (Abisambra et al., 2013).

Protein expression and purification

Hsc70, Hsp70 and their co-chaperones were expressed and purified using previously reported methods (Rauch and Gestwicki, 2014). Apo chaperone was prepared from several days of dialysis in assay buffer (0.017% Triton X-100, 100 mM Tris-HCl, 20 mM KCl, and 6 mM $MgCl_2$, pH 7.4) at 4 °C to remove nucleotide.

NMR

Titration studies were carried out as previously described (Li et al., 2013). NMR samples of 160 μM ^{15}N labeled $Hsc70_{NBD}$ in 25 mM TrisHCl, 10 mM KCl, 8 mM $MgCl_2$, 10 mM PO_4^{2-}, 0.015% NaN_3, 5% D_2O and pH 7.0 was treated with either DMSO alone or JG-48 (200 μM). TROSY-HSQC data was processed in NMR PIPES and converted to SPARKY format.

Fluorescence Polarization

ATP-FAM—Fluorescence polarization experiments were performed in 384-well, black, low volume, round-bottom plates (Corning) using a SpectraMax plate reader. Increasing amounts of compound were incubated with nucleotide-free Hsc70 (300 nM) and ATP-FAM (20 nM) (Jena Bioscience) for three hours at room temperature in assay buffer (0.017% Triton X-100, 100 mM Tris-HCl, 20 mM KCl, and 6 mM $MgCl_2$, pH 7.4) prior to measurement by plate reader (Ex. 485 nm, Em. 535nm, 530nm cutoff). The final DMSO concentration in each well was 5% and compounds were serially diluted two-fold in 100% DMSO. All data was subsequently analyzed by Prism (Graphpad Software).

HLA-FAM—Experiments were adapted from previously reported methods (Rauch and Gestwicki, 2014). HLA-FAM is a 10mer peptide conjugated to fluorescein (FAM) that is used as an Hsp70 binding tracer in fluorescence polarization experiments. Briefly, 5 μM Hsc70 in the presence of 1 mM ADP was incubated with 25 nM HLA-FAM and treated with increasing concentrations of NEF proteins in 384-well, black, low volume, round-bottom plates (Corning) in the presence and absence of compound for 2 hrs at RT in assay buffer

(0.017% Triton X-100, 100 mM Tris-HCl, 20 mM KCl, and 6 mM $MgCl_2$, pH 7.4). Following incubation, fluorescence polarization was measured using a SpectraMax plate reader.

ATPase and Luciferase Assays

ATPase activity was measured by malachite green assays, as previously reported (Chang et al., 2008). Firefly luciferase refolding was measured using a previously reported method (Chang et al., 2010).

Flow Cytometry Protein Interaction Assay (FCPIA)

Procedure is adapted from previously reported methods (Rauch et al., 2013). Biotinylated Hsc70 was immobilized on polystyrene streptavidin coated beads (Spherotech), incubated with Alexa-Fluor 488 labeled NEF (50 nM) and increasing amounts of compound in buffer A (25 mM HEPES, 5 mM $MgCl_2$, 10 mM KCl, 0.03% Tween-20 pH 7.5). Protein complex inhibition was detected by measuring bead-associated fluorescence using an Accuri C6 Flow Cytometer. DMSO is used as a negative control and excess unlabeled Hsc70 (1 μM) is used as a positive control.

Tau Binding ELISA

Method was adapted from a previous report (Thompson et al., 2012). Briefly, 1 μM human Hsc70 (30 μL) was immobilized overnight at 37 °C in clear, non-sterile 96-well plates (Thermo) in 50mM MES (pH 5.5) and 0.5 mM DTT with 1 mM ADP. Wells were washed with 100 μL of PSB-T (3 × 3 min., rocking) prior to the addition of 30 μL of 4RON tau solution in binding buffer (25 mM HEPES, 40 mM KCl, 8 mM $MgCl_2$, 100 mM NaCl, 0.01% Tween, pH 7.4) with 1 mM ADP and 1 μL of either DMSO or compound for 3 hrs at RT. After blocking in 5% milk, quantification of tau binding was performed using rabbit anti-tau (H150) primary antibody (Santa Cruz, sc-5587, 1:2000 in TBS-T, 50 μL/well) and goat anti-rabbit HRP-conjugated secondary (Anaspec, 28177, 1:2000 in TBS-T, 50 μL/well). TMB substrate (Cell Signaling, 7400L) and 1N HCl were used to detect binding. Absorbance was measured using a SpectraMax plate reader (OD_{450}). Minimal, non-specific binding of 4R0N tau to empty wells was subtracted as background and curves were fit using non-zero intercept hyperbolic fits in Prism (GraphPad Software).

Immunoprecipitation of V5-Tau

The co-immunoprecipitation procedure was adopted from previously described methods (Thompson et al., 2012). Briefly, HeLa C3 cells were treated with bortezomib in a final concentration of 5 μM for 4 hours prior to lysis in mammalian protein extraction reagent (Thermo Scientific). Following protein quantification, 5 mg of lysate was incubated with 40 μL of goat anti-V5 conjugated to agarose beads (Bethyl Laboratories, S190-119) and 50 μM JG-48 or DMSO (5%) at 4 °C in the dark overnight. Lysate was incubated with normal goat IgG with DMSO (4%) as a negative control. Protein A/G agarose beads (20μL) (Santa Cruz, sc-2003) were added to samples for 4 hours. Beads were then washed 3 times with 100 μL of PBS (Gibco) at 1000×g for 1 minute. Proteins were eluted by boiling at 96°C in 40μL of 1× SDS loading dye. 10 μL of sample were separated on 10% Tris-trycine gels (Bio-rad) and

transferred to nitrocellulose membrane. The membranes were blocked in nonfat milk (5% milk in TBS, 0.5% Tween) for 1 hour, incubated with primary antibodies for Tau (Santa Cruz, sc-5587) and Hsp72 (Enzo, ADI-SPA-811) overnight at 4 °C in the dark, washed, and then incubated with a horseradish peroxidase-conjugated secondary antibody (Anaspec, 28177) for 1 hour. Finally, membranes were developed using chemiluminescence (Thermo Scientific, Supersignal® West Pico).

Protein Dynamics and Molecular Modeling

Computational modeling of JG-48 binding to $Hsc70_{NBD}$ (PDB: 3C7N) was obtained using similar methods as previously described (Miyata et al., 2013; Rousaki et al., 2011). Briefly, AUTODOCK-4.2 was used for the docking of JG-48 to $Hsc70_{NBD}$ with the following parameters: GA runs = 100, initial population size = 1500, max number of evaluations = long, max number of surviving top individuals = 1, gene mutation rate = 0.02, rate of crossover = 0.8, GA crossover mode: two points, Caushy distribution mean for gene mutation = 0, Cauchy distribution variance for gene mutation = 1, number of generations for picking worst individuals = 10. The docked structures were clustered and then evaluated using PyMOL. All calculations were completed on a Apple MacBookPro computer equipped with a 64-bit 2.4 GHz Intel Core 2 Duo processor running MacOSX 10.6.8.

Synthesis and Characterization of JG-48 and JG-273

JG-48: ^{1}H NMR (400 MHz, DMSO) δ 8.55 (d, J = 4.1 Hz, 1H), 8.12 (s, 1H), 7.67 (dd, J = 7.9, 5.2 Hz, 1H), 7.63 (dd, J = 7.4, 1.8 Hz, 1H), 7.46 (d, J = 8.7 Hz, 1H), 7.27 (d, J = 8.0 Hz, 1H), 6.97 (ddd, J = 7.3, 4.9, 1.1 Hz, 1H), 6.25 (s, 1H), 3.98 (s, 3H), 3.91 (q, J = 7.0 Hz, 2H), 1.21 (t, J = 7.1 Hz, 3H). ESI-MS: calculated for $C_{20}H_{17}F_3N_3OS_2^+$ 436.08, found 436.04.

JG-273: ^{1}H NMR (400 MHz, DMSO) δ 8.47 (d, J = 4.3 Hz, 1H), 7.59 (td, J = 7.8, 1.7 Hz, 1H), 7.19 (d, J = 8.0 Hz, 1H), 6.90 (dd, J = 6.7, 5.4 Hz, 1H), 6.08 (s, 1H), 3.81 (d, J = 7.1 Hz, 2H), 3.65 (t, J = 7.3 Hz, 2H), 3.32 (s, 3H), 3.08 (t, J = 7.2 Hz, 2H), 1.14 (t, J = 7.0 Hz, 3H). ESI-MS: calculated for $C_{15}H_{18}N_3OS_2^+$ 320.09, found 320.13.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Citations

Abisambra J, Jinwal UK, Miyata Y, Rogers J, Blair L, Li X, Seguin SP, Wang L, Jin Y, Bacon J, et al. Allosteric Heat Shock Protein 70 Inhibitors Rapidly Rescue Synaptic Plasticity Deficits by Reducing Aberrant Tau. Biological psychiatry. 2013

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Highlights

- The Hsp70 inhibitor JG-48 reduces tau levels in models of tauopathy
- JG-48 traps tau in an Hsp70-bound complex
- Perturbations that promote binding to tau also trigger its degradation

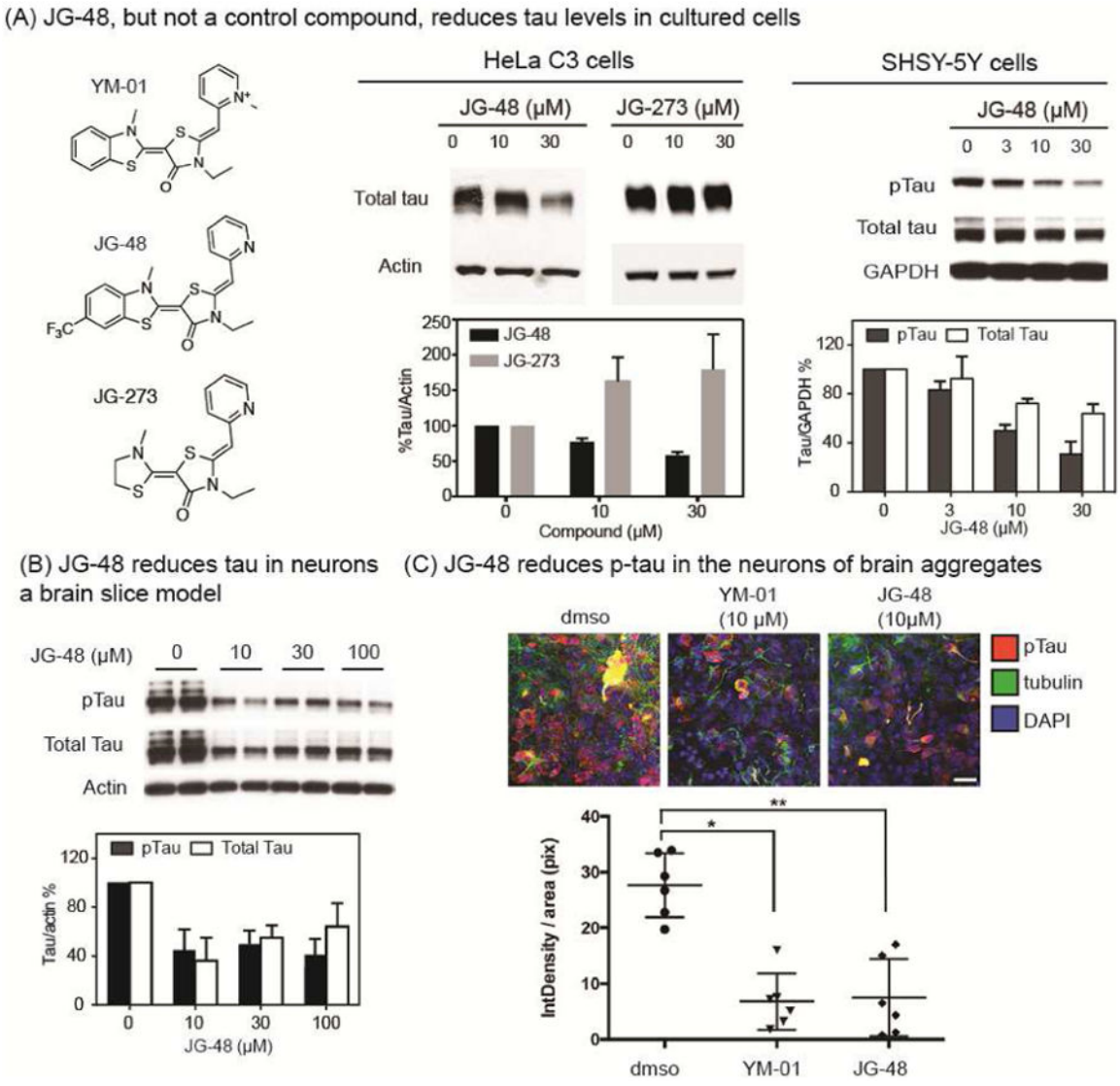

Figure 1.

JG-48 reduces tau levels in cellular and brain tissue models of tauopathy. (A) JG-48, but not JG-273, reduces tau in HeLa C3 cells stably transfected with 4R0N tau (left). It also reduces endogenous tau in SHSY-5Y neuroblastoma cells (right). Cells were treated for 24 hours. Eror bars represent SEM. JG-48 reduced tau and p-tau levels at 10 and 30 μM (p-value< 0.001). (B) Acute hippocampal slice cultures from 3-4 month old rTg4510 tau transgenic mice were treated with JG-48 for 6 hours. Results from two independent experiments are shown. At all concentrations, JG-48 reduced tau and p-tau ($p<0.001$). The quantifications are an average of the two independent experiments and the error bars are SEM. (C) Brain aggregates were infected with 10% rTg4510 brain homogenates from 15 to 25 days in culture and treated with YM-01 or JG-48 (10 μM) for 24 hrs prior to harvesting at 35 days in culture. They were stained with anti-phospho-tau (red), anti-beta tubulin (green) and DAPI (blue). 15-20 confocal images were stacked and phospho-tau intensity was measured with Image J. N=6. *$p<0.0001$ **$p<0.0003$. Bar = 25 μm.

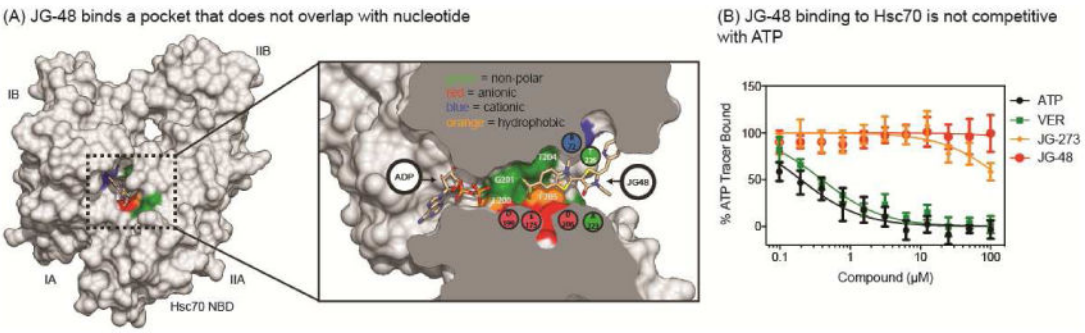

Figure 2.

JG-48 does not compete with ATP for binding to Hsc70. (A) Docking of JG-48 to the NBD of Hsc70. Based on NMR chemical shift perturbations, the binding site of JG-48 was modeled. JG-48 is predicted to bind a deep cleft in Hsc70, which is not overlapping with the ATP/ADP-binding cleft. Hydrophobics in orange, apolar in green, anionic in red, cationic in blue. (B) Positive controls, ATP and VER-155008 (VER) compete with a fluorescent ATP tracer for binding to the NBD of Hsc70. Results are the average of three independent experiments performed in triplicate each and the error bars represent the standard deviations.

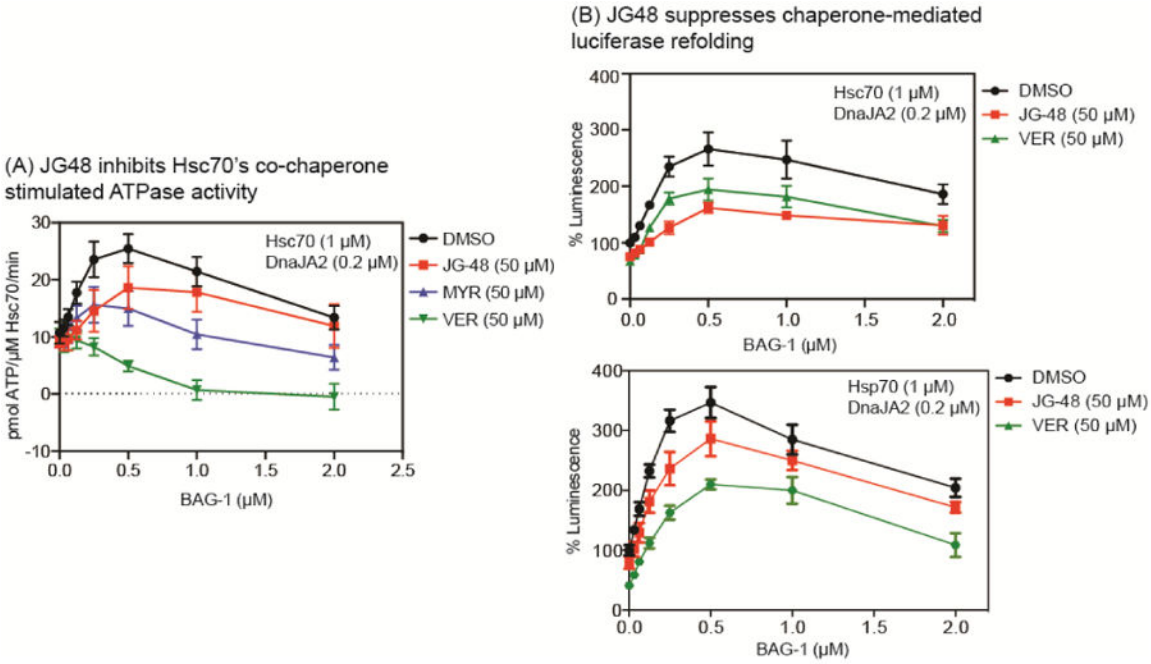

Figure 3.

JG-48 is an inhibitor of chaperone functions. (A) Effects of JG-48 (and the negative control, JG-273) on steady state ATPase activity of Hsc70 (1 μM), DnaJA2 (0.2 μM) and titration with BAG-1. MYR = myricetin VER = VER-155008. All compounds at 50 μM. Results are the average of at least three independent experiments performed in triplicate each and the error bars represent the SD. See the Supplemental Information for results with other co-chaperones. (B) Effects of JG-48, JG-273 and VER on the refolding of luciferase by Hsc70 (1 μM), DnaJA2 (0.2 μM) and BAG-1. All compounds at 50 μM. Results are the average of at least two independent experiments performed in triplicate each and error bars represent SD. See the Supplemental Information for additional details.

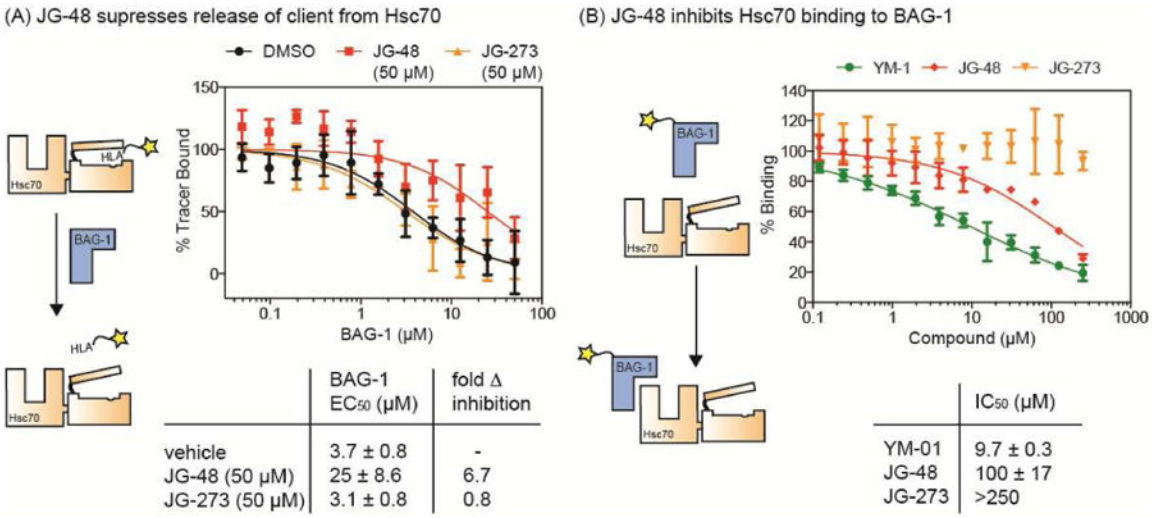

Figure 4.

JG-48 limits NEF binding and activity. (A) BAG-1 accelerated release of a peptide tracer (HLA-FAM) from the SBD of Hsc70 was measured and the effects of 50μM compounds determined. The apparent EC50 values for BAG-1 are shown in the table. Results are the average of three independent experiments performed in triplicate each. Error bars represent the SD. (B) The interaction between Hsc70 and BAG-1 was measured by FCPIA. YM-01 and JG-48, but not the negative control, JG-273, partially block the protein-protein interaction. Results are the average of three independent experiments performed in triplicate each and the error bars represent SD. Some error bars are smaller than the symbols.

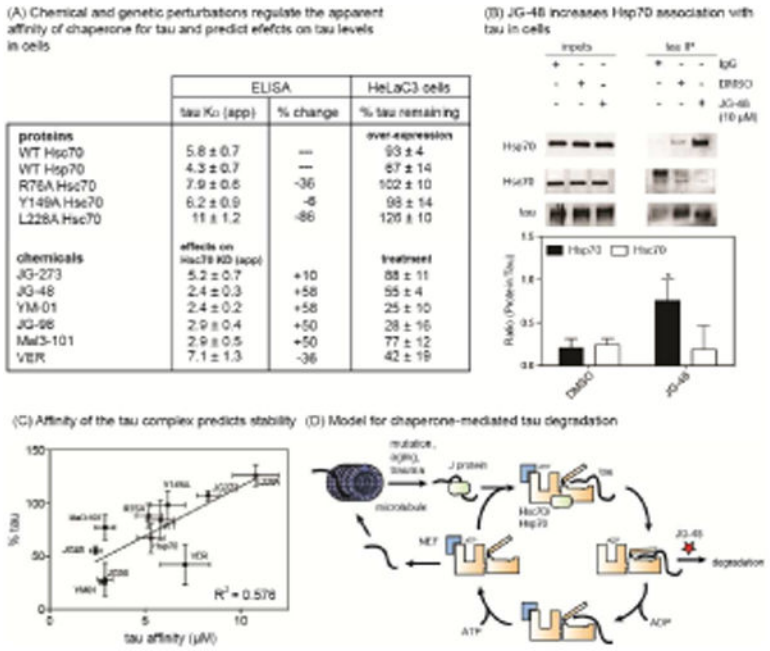

Figure 5.

Tight binding to tau correlates with increased turnover in cells. (A) Summary of the results from ELISA and Western blot experiments. Binding to tau was measured in vitro, using an ELISA. The effects of compounds and mutations on the apparent EC50 value were then determined. Results are the average of at least three independent experiments performed in triplicate each and the error is SD. For the cell-based studies, compounds were added to HeLaC3 cells at their respective EC50 values for 24hrs and the percent change in tau levels quantified by Western blots. Results are the average of at least three independent experiments and the error is SD. See the Supplemental information for additional details and raw data. (B) JG-48 (10 μM) increases co-immunoprecipitation of Hsp70, but not Hsc70, with tau. A representative blot of two independent experiments is shown. The quanitification is an average of both experimetns and the error bars represent SEM. * p-value < 0.0005. (C) Correlation between the relative affinity of the Hsc70-tau complex from the ELISA with the levels of tau in treated cells (data from panel A). (D) Model for how members of the Hsp70 family might control tau homeostasis. In this model, the strength of the chaperone-tau complex is predicted to determine whether the client is degraded. If tau interacts transiently with the complex, then it might be retained.