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Inhibitors of heat shock protein 70 (Hsp70) with enhanced metabolic stability reduce tau levels

Hao Shao [a,*], Xiaokai Li [b,c], Shigenari Hayashi [c], Jeanette L. Bertron [b,c], Daniel M.C. Schwarz [b,c], Benjamin C. Tang [c], Jason E. Gestwicki [b,c,*]

[a] Hunan Key Laboratory of Molecular Precision Medicine, Department of Oncology, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China
[b] Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA 94158, United States
[c] Institute for Neurodegenerative Disease, University of California San Francisco, San Francisco, CA 94158, United States

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ABSTRACT

The molecular chaperone, Heat Shock Protein 70 (Hsp70), is an emerging drug target for neurodegenerative diseases, because of its ability to promote degradation of microtubule-associated protein tau (MAPT/tau). Recently, we reported YM-08 as a brain penetrant, allosteric Hsp70 inhibitor, which reduces tau levels. However, the benzothiazole moiety of YM-08 is vulnerable to metabolism by CYP3A4, limiting its further application as a chemical probe. In this manuscript, we designed and synthesized seventeen YM-08 derivatives by systematically introducing halogen atoms to the benzothiazole ring and shifting the position of the heteroatom in a distal pyridine. In microsome assays, we found that compound JG-23 has 12-fold better metabolic stability and it retained the ability to reduce tau levels in two cell-based models. These chemical probes of Hsp70 are expected to be useful tools for studying tau homeostasis.

Introduction

Tau is an intrinsically disordered, microtubule-associated protein that is abundantly expressed in neurons.[1,2] When tau's microtubule binding is disrupted, by either mutation[3,4] or post-translational modifications, such as phosphorylation[5,6] and acetylation[7,8], it is aberrantly localized to the cytosol. This dissociated tau is then prone to aggregate, where it can form neurofibrillary tangles (NFTs) that are associated with neuronal cell death[9] and neurodegenerative disorders, including Alzheimer's disease (AD) and frontotemporal dementia (FTD). These observations have driven interest in therapeutic approaches that modulate tau homeostasis,[10,11] including stabilizing microtubule[12], reducing tau post-transcriptional modifications[8,13], and enhancing tau degradation through inhibition of the molecular chaperones Hsp90 and Hsp70[14–16]. Scheme 1.

Heat Shock Protein 70 (Hsp70) is a molecular chaperone that binds to tau soon after its release from microtubules.[17] There are two major, cytoplasmic orthologs of Hsp70 that are thought to perform this function: Hsc70 (HSPA8) and Hsp72 (HSPA1A). These isoforms are highly conserved and, after they bind tau, they either stabilize its re-binding to microtubules or promote its degradation.[18] Despite the similarities between the orthologs, Hsp72 is more tightly linked to tau degradation.[19] Pharmacological studies have also supported this idea. Specifically, a brain penetrant, irreversible Hsp72 inhibitor, methylene blue (MB), reduces levels of insoluble tau and improves learning and memory in tauopathy mouse models.[20] Likewise, other classes of Hsp70 inhibitors, including dihydropyridines and rhodacyanines, which likely bind to both Hsp72 and Hsc70,[21] reduce tau levels in cellular and tissue models.[22–24] These molecules appear to work by stabilizing a conformation of Hsp70s that promotes tau degradation.[25] Unlike MB, which has promiscuous activity, the rhodacyanines are relatively selective for Hsp70 family members in cells,[26] so there is interest in further developing them as chemical probes.

One rhodacyanine, MKT-077, was first identified as an inhibitor of Hsp70s in cancer models, but it was later shown to decrease both phosphorylated and total tau levels in HeLaC3 cells.[23] We found that MKT-077 binds to an allosteric site that is conserved among Hsp70 isoforms[21] and it is considered to be a pan-Hsp70 inhibitor. Here, the term "inhibitor" is somewhat incomplete, because, while binding to this allosteric site inhibits ATP turnover, it also stabilizes the conformation

* Corresponding authors at: No 110, Xiangya Road, Kaifu District, Changsha, Hunan 410008, China, (H. Shao). 675 Nelson Rising Lane, San Francisco, CA 94158, United States (J.E. Gestwicki).
E-mail addresses: haoshaodream@hotmail.com, hao.shao@ucsf.edu (H. Shao), Jason.gestwicki@ucsf.edu (J.E. Gestwicki).

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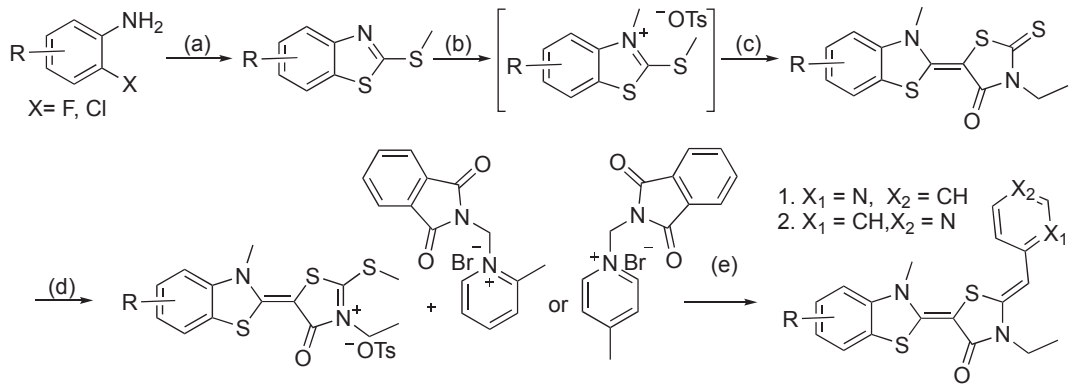


Scheme 1. Reaction conditions:(a) 1) potassium ethylxanthate, DMF, 140 °C, h; 2) MeI, Net_3, EtOH, 80 °C, 1 h; (b) *p*-TsOMe, anisole, 125 °C, 4 h; (c) 3-ethyl rhodanine, NEt_3, MeCN, 25 °C, h; (d) *p*-TsOMe, DMF, 135 °C, 3h; (e) 1) Net_3, MeCN, 70 °C, 3 h; 2) DCM/MeOH, aq·NH_3, r.t., 1 h.

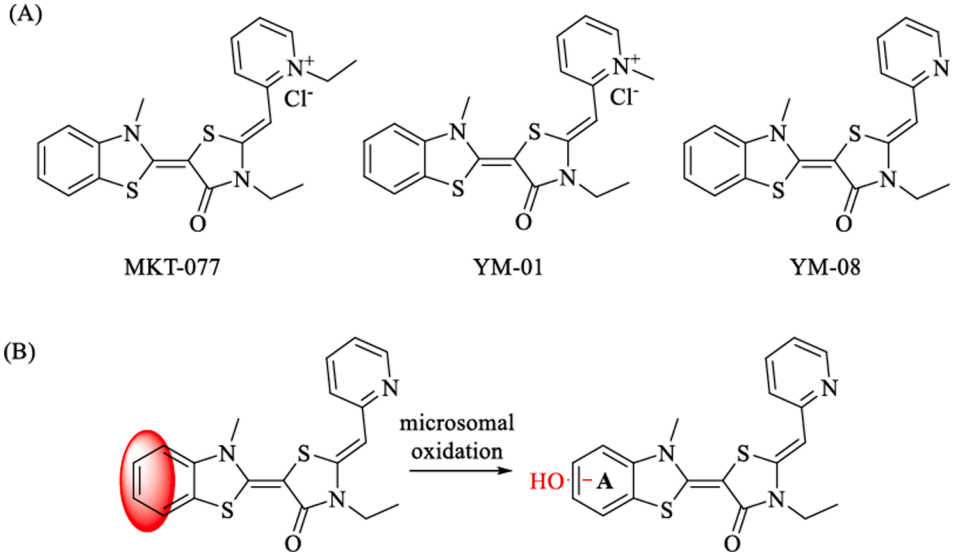


Fig. 1. Previously reported allosteric Hsp70 inhibitors and their metabolism. (A) Chemical structures of MKT-077, YM-01 and YM-08. (B). The benzothiazole is known to be the metabolically liable site.

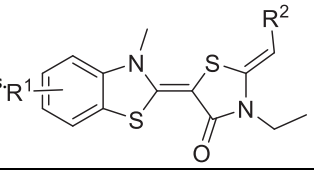
of Hsc70 and Hsp72 that promotes degradation of bound proteins, such as tau. Thus, molecules in the chemical series could be useful probes in neurodegenerative disease models because they accelerate tau degradation. Towards that goal, recent medicinal chemistry efforts have started to improve the potency of MKT-077. For example, a close MKT-077 derivative, YM-01, was found to have improved membrane permeability and it promotes tau degradation at concentrations ~1 μM. Importantly, treatment with YM-01 leads to preferential degradation of pathogenic tau, while normal, microtubule-associated tau is resistant, in a mouse brain slice model.[24]. While these findings are promising, the quaternary amine in YM-01 leads to its accumulation in mitochondria and limits its blood–brain barrier (BBB) permeability. To solve this issue, a neutral analog, YM-08 (Fig. 1), was synthesized by replacing the pyridinium with a pyridine. YM-08 retained binding to Hsp70s and it still had the ability to decrease tau levels in HeLaC3 cells, while it also had improved brain exposure.[16] However, YM-08 is rapidly metabolized and its half-life is less than three minutes in mouse liver microsome assays, significantly limiting its applications *in vivo*.

In a metabolite identification study, we had previously identified the benzothiazole in YM-08 as the major site for Phase I oxidation (Fig. 1).[16] However, it was not clear which site was oxidized. ed Here, we explored whether introducing halogens to specific sites on the benzothiazole ring may block this oxidation and increase the lifetime of YM-08 analogs. This approach seemed feasible because docking of YM-08 to Hsc70 suggested that the benzothiazole is located in a hydrophobic pocket that would accept modification by halogens. In this study, we reported the synthesis of seventeen YM-08 analogs and the selection of **JG-23** as a molecule with improved metabolic stability and tau-reducing activity.

Results and discussion

To understand which sites might enhance metabolic stability, we sought to systematically install halogens at the 3-, 4–5- and 6-ring positions in the benzothiazole. In addition, we envisioned swapping the position of the pyridine nitrogen to reveal any distal, electronic effects. Accordingly, seventeen YM-08 analogs were designed and synthesized using a previously reported method as shown in Scheme 1.[2627] Briefly, the synthesis started from the cyclization of substituted anilines with potassium ethyl xanthate, followed by methylation with methyl iodide under mild basic condition. The resulting substituted 2-(methylthio) benzothiazole was reacted with methyl *p*-toluenesulfonate to afford its methylthioiminium salt, which was subsequently condensed with 3-ethylrhodanine. The resulting compounds were activated by methyl *p*-toluenesulfonate, followed by the condensation of either 1-((1,3-dioxoisoindolin-2-yl)methyl)-2-methylpyridin-1-ium bromide or 1-((1,3-dioxoisoindolin-2-yl)methyl)-4-methylpyridin-1-ium bromide. The protecting group on the pyridine nitrogen was then removed in the presence of catalytic amount of aqueous ammonium hydroxide to yield

Table 1
Chemical structures and liver microsome stabilities of YM-08 analogs.



Compound	R_1	R_2	Percentage at T_{30min}
YM-08	H	2-pyridinyl	–
1	3-F	2-pyridinyl	12
2	4-F	2-pyridinyl	19
3	5-F	2-pyridinyl	5
4	6-F	2-pyridinyl	12
5	3-Cl	2-pyridinyl	38
6	4-Cl	2-pyridinyl	37
7	5-Cl	2-pyridinyl	13
8	6-Cl	2-pyridinyl	17
9	H	4-pyridinyl	6
10	3-F	4-pyridinyl	33
11	4-F	4-pyridinyl	23
12	5-F	4-pyridinyl	13
13	6-F	4-pyridinyl	30
14	3-Cl	4-pyridinyl	n/t
15 (JG-23)	4-Cl	4-pyridinyl	52
16	5-Cl	4-pyridinyl	39
17	6-Cl	4-pyridinyl	n/t

the final products **1**–**17** in overall yields between 20 and 30% and purity greater than 97%. The compounds were purified by flash chromatography and characterized by LC-MS and ^{1}H-NMR.

To probe which benzothiazole position might be most important for microsome stability, we first measured the amount of unmodified compound remaining after incubation with mouse liver microsomes for 30 min at 37 °C, using dextromethorphan as a positive control[28]. First, we confirmed that YM-08 is rapidly metabolized in this experiment, such that its levels were below the limit of detection at 30 min (Table 1). Then, we found that introduction of fluorine at any of the 3-, 4-, 5- or 6-positions on YM-08 (compounds **1**–**4**) modestly increased this stability (~5 to 20% remaining after 30 min). The analogs containing chlorine at the corresponding positions (compounds **5**–**8**) were generally more stable. For example, the **5** and **6** were approximately 2- to 3-fold more stable than **1** and **2** (37 to 38%). In addition, across this series, the compounds with substituents at the 3- and 4-positions were generally better than those with halogens at the 5- and 6-positions.

Next, we examined the effects of switching the pyridine nitrogen from the *ortho* (YM-08) to *para* position (compound **9**–**17**). In early experiments, we found that compound **9** was modestly more stable than **YM-08** (Table 1), so it seemed worth exploring this series in more detail. Introduction of a fluorine at any of the four positions (compounds **10**–**13**) improved microsome stability (~13 to 33% remaining). As in the previous series, replacement of fluorine with chlorine further improved metabolic stability. Specifically, 52% and 39% of compounds **15** (JG-23) and **16** were remaining, respectively. Compounds **14** and **17** had poor solubility and were thus excluded from further study (n/t; Table 1). Based on these results, we chose the top 4 compounds and measured their $T_{1/2}$ in mouse liver microsome assays (Fig. 2). The calculated $T_{1/2}$ values from these experiments correlated with the single time point measurements: the half-lives of compounds **5**, **6** and **16** are around 20 min, and **JG-23** (**15**) has the longest $T_{1/2}$ value (36 min).

Finally, we tested the ability of **JG-23** to decrease total tau (*t*-tau) levels in the HeLaC3 cell model. Cells were treated with **JG-23** for 24 h at the indicated concentrations, and the *t*-tau levels were analyzed by Western blot. We found that **JG-23** significantly decreased *t*-tau levels at concentrations above 10 μM (Fig. 3a). Also, **JG-23** did not activate cellular stress, as determined by the constant levels of Hsp72 and Hsp90 in the treated cells. Next, we asked whether **JG-23** might also reduce *t*-tau levels in SH-SY5Y cells. This experiment was important because the HeLaC3 model over-expresses 0N4R tau, while it is expressed at physiological levels in the SH-SY5Y cells. Satisfyingly, we found that **JG-23** had similar effects on *t*-tau, Hsp72 and Hsp90 in this model (Fig. 3b), reducing *t*-tau by ~80%.

Conclusions

Allosteric inhibitors of Hsp70, which favor a pro-degradation conformation of the chaperone, are potentially promising chemical probes for understanding tau homeostasis. However, the poor metabolic stability of earlier molecules, such as YM-08, had limited such studies. Here, guided by the knowledge that P_{450}-mediated metabolism occurs in the benzothiazole ring, we focused on introducing halogens at the 3-, 4-, 5- and 6-positions. Two series of compounds were designed and synthesized, revealing that a 4-chloro modified analog, **JG-23**, is 12-fold more stable than YM-08. One of the interesting aspects of these findings was that shifting the heteroatom in the distal pyridine generally improves stability, despite the fact that metabolism does not occur in this ring. It seems possible that the pyridine impacts either P_{450} binding or oxidation activity, perhaps mediated by electronic effects through the conjugated *pi* system. Importantly, these modifications did not seem to affect target binding, as compound **JG-23** retained the ability to promote *t*-tau degradation in two cellular models. Future work will focus on improving potency and brain exposure, with the goal of creating a next-generation Hsp70 probe for studying tau turnover in the brain.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

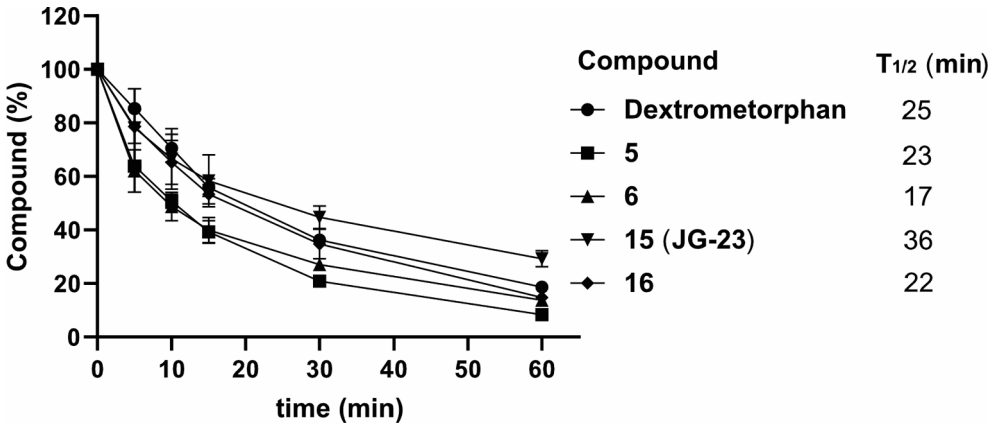


Fig. 2. Microsomal stability of selected compounds. Dextrometorphan is used as a positive control.

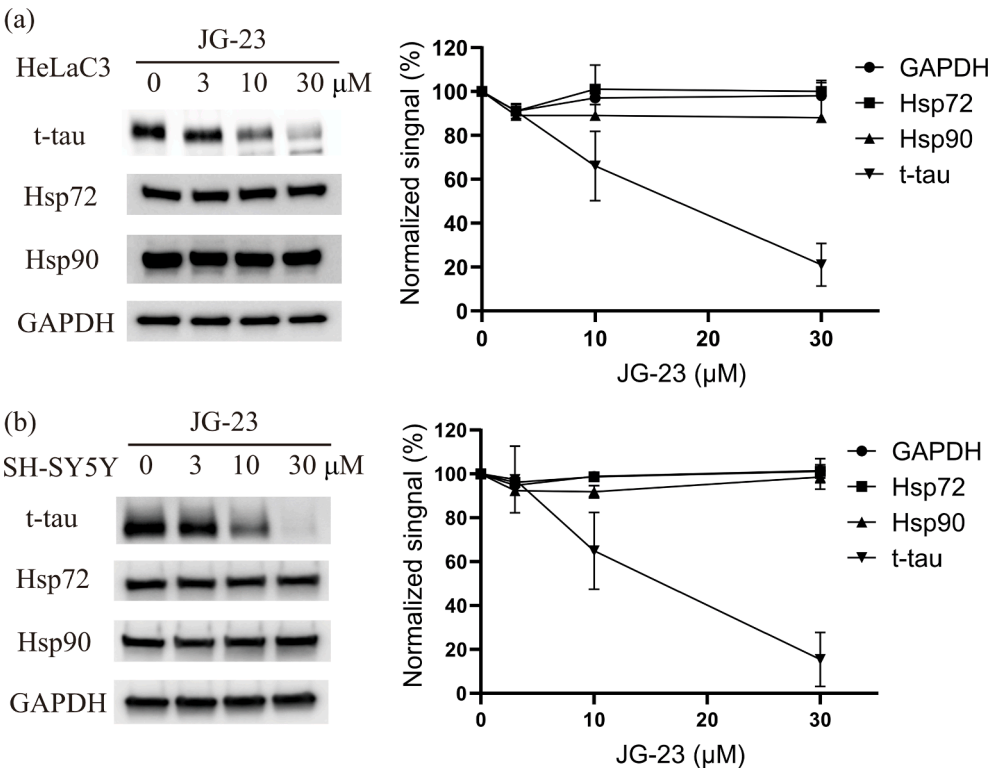


Fig. 3. Compound JG-23 led to degradation of t-tau without eliciting a heat shock response in HeLaC3 (a) and SH-S55Y (b) cells. HeLaC3 or SH-SY5Y cells were treated with JG-23 for 24 h at the indicated concentrations, lysed and western blots performed. Results are representative of at least two independent experiments.

the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128025.

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