Supplemental Information

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] and Jason E. Gestwicki[b, c,*]

[a]Hunan Key Laboratory of Molecular Precision Medicine, Department of Oncology, Xiangya Hospital, Central South University, Changsha, Hunan, China, 410008

[b]Department of Pharmaceutical Chemistry and the [c]Institute for Neurodegenerative Disease, University of California San Francisco, San Francisco, CA 94158

Experimental section

Compound Characterization

Solvents and starting materials were purchased from commercial sources and used without further purification. ^{1}H-NMR and ^{13}C-NMR were recorded on Varian 400 MHz, 500 MHz, or 600 MHz spectrometer. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; q, quartet. ESI-MS was recorded on Micromass LCT Time-of-Flight mass spectrometer.

(2*Z*,5*E*)-3-ethyl-5-(4-fluoro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-2-(pyridin-2-ylmethylene)thiazolidin-4-one (**Compd 1**). Purity: 97%. ^{1}H *d6*-DMSO (400 MHz) 8.49 (d, J = 4.4 Hz, 1H), 7.60 (t, J = 7.6 Hz, 1H), 7.46 (d, J = 7.6 Hz, 1H), 7.25-7.05 (m, 3H), 6.95-6.88 (m, 1H), 6.19 (s, 1H), 4.02 (s, 3H), 3.87 (q, J = 7.2 Hz, 2H), 1.17 (t, J = 7.2 Hz, 3H). ^{13}C *d6*-DMSO (150 MHz) 165.21, 155.75, 150.03, 147.43, 139.73, 135.90, 129.38,

123.58, 122.11, 117.83, 117.64, 114.22, 100.15, 94.11, 90.43, 85.79, 40.06, 37.26, 12.13. ESI-MS: m/z calculated for $[C_{19}H_{17}FN_3OS_2]^+$, 386.079, found 386.102 $[M+H]^+$.

(2*Z*,5*E*)-3-ethyl-5-(5-fluoro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-2-(pyridin-2-ylmethylene)thiazolidin-4-one (**Compd 2**). Purity: 96%. ^{1}H *d6*-DMSO (400 MHz) 8.54 (s, 1H), 7.72-7.57 (m, 2H), 7.35-7.20 (m, 2H), 7.02-6.90 (m, 2H), 6.21 (s, 1H), 3.94 (s, 3H), 3.90 (q, *J* = 7.2 Hz, 2H), 1.20 (t, *J* = 7.2 Hz, 3H). ^{13}C *d6*-DMSO (100 MHz) 165.17, 160.50, 155.82, 151.69, 147.30, 142.14, 139.78, 135.94, 134.36, 122.78, 122.01, 121.21, 117.61, 109.29, 98.48, 93.79, 37.20, 34.60, 12.12. ESI-MS: m/z calculated for $[C_{19}H_{17}FN_3OS_2]^+$, 386.079, found 386.065 $[M+H]^+$.

(2*Z*,5*E*)-3-ethyl-5-(6-fluoro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-2-(pyridin-2-ylmethylene)thiazolidin-4-one (**Compd 3**). Purity: 97%. ^{1}H *d6*-DMSO (500 MHz) 8.53 (d, *J* = 4.0 Hz, 1H), 7.66-7.59 (m, 2H), 7.32 (dd, *J* = 9.0, 4.0 Hz, 1H), 7.25-7.17 (m, 2H), 6.94 (t, *J* = 6.0 Hz, 1H), 6.19 (s, 1H), 3.93 (s, 3H), 3.88 (q, *J* = 7.0 Hz, 2H), 1.19 (t, *J* = 7.0 Hz, 3H). ^{13}C *d6*-DMSO (150 MHz) 165.14, 158.68, 157.34, 156.06, 149.95, 147.41, 140.09, 137.65, 135.86, 127.76, 122.00, 117.43, 111.31, 108.72, 93.62, 84.30, 37.17, 34.57, 12.11. ESI-MS: m/z calculated for $[C_{19}H_{17}FN_3OS_2]^+$, 386.079, found 386.082 $[M+H]^+$.

(2*Z*,5*E*)-3-ethyl-5-(7-fluoro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-2-(pyridin-2-ylmethylene)thiazolidin-4-one (**Compd 4**). Purity: 96%. ^{1}H *d6*-DMSO (400 MHz) 8.54 (d, *J* = 4.8 Hz, 1H), 6.33 (td, *J* = 8.0, 1.6 Hz, 1H), 7.43-7.35 (m, 1H), 7.25 (d, *J* = 8.0 Hz,

1H), 7.19 (d, *J* = 8.0 Hz, 1H), 7.04 (t, *J* = 8.4 Hz, 1H), 6.98-6.92 (m, 1H), 6.23 (s, 1H), 3.96 (s, 3H), 3.90 (q, *J* = 7.2 Hz, 2H), 1.21 (t, *J* = 7.2 Hz, 3H). ^{13}C *d6*-DMSO (100 MHz) 165.70, 157.46, 156.15, 155.05, 149.02, 147.86, 144.31, 140.18, 136.35, 128.85, 122.55, 118.07, 112.83, 109.31, 107.36, 94.62, 37.71, 35.13, 12.54. ESI-MS: m/z calculated for $[C_{19}H_{17}FN_3OS_2]^+$, 386.079, found 386.048 $[M+H]^+$.

(2*Z*,5*E*)-5-(4-chloro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-3-ethyl-2-(pyridin-2-ylmethylene)thiazolidin-4-one (**Compd 5**). Purity: 97%. ^{1}H *d6*-DMSO (400 MHz) 8.54 (d, *J* = 4.4 Hz, 1H), 7.67-7.58 (m, 2H), 7.35 (d, *J* = 8.0 Hz, 1H), 7.27 (d, *J* = 8.0 Hz, 1H), 7.14 (t, *J* = 8.0 Hz, 1H), 6.98 (t, *J* = 6.0 Hz, 1H), 6.26 (s, 1H), 3.99 (s, 3H), 3.90 (q, *J* = 7.2 Hz, 2H), 1.21 (t, *J* = 7.2 Hz, 3H). ^{13}C *d6*-DMSO (100 MHz) 165.13, 155.60, 150.74, 147.44, 139.53, 135.98, 129.70, 128.60, 124.19, 122.22, 120.58, 117.87, 116.39, 94.73, 88.63, 40.17, 40.06, 37.30, 17.17. ESI-MS: m/z calculated for $[C_{19}H_{17}ClN_3OS_2]^+$, 402.050, found 402.020 $[M+H]^+$.

(2*Z*,5*E*)-5-(5-chloro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-3-ethyl-2-(pyridin-2-ylmethylene)thiazolidin-4-one (**Compd 6**). Purity: 99%. ^{1}H *d6*-DMSO (400 MHz) 8.54 (d, *J* = 4.4 Hz, 1H), 7.67 (d, *J* = 8.0 Hz, 1H), 7.63 (t, *J* = 8.0 Hz, 1H), 7.46 (s, 1H), 7.25 (d, *J* = 8.0 Hz, 1H), 7.17 (d, *J* = 8.4 Hz, 1H), 6.95 (t, *J* = 5.2 Hz, 1H), 6.22 (s, 1H), 3.94 (s, 3H), 3.90 (q, *J* = 7.2 Hz, 2H), 1.20 (t, *J* = 7.2 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}ClN_3OS_2]^+$, 402.050, found 402.021 $[M+H]^+$.

(2*Z*,5*E*)-5-(6-chloro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-3-ethyl-2-(pyridin-2-ylmethylene)thiazolidin-4-one (**Compd 7**). Purity: 97%. ^{1}H *d6*-DMSO (400 MHz) δ 8.53 (d, *J* = 4.8 Hz, 1H), 7.80 (d, *J* = 2.4 Hz, 1H), 7.62 (td, *J* = 8.0, 2.4 Hz , 1H), 7.37 (dd, *J* = 8.8, 2.0 Hz, 1H), 7.30 (d, *J* = 8.8 Hz, 1H), 7.24 (d, *J* = 8.0 Hz, 1H), 6.97-6.92 (m,1H), 6.19 (s, 1H), 3.92 (s, 3H), 3.89 (q, *J* = 7.2 Hz, 2H), 1.20 (t, *J* = 7.2 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}ClN_3OS_2]^+$, 402.050, found 402.078 $[M+H]^+$.

(2*Z*,5*E*)-5-(7-chloro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-3-ethyl-2-(pyridin-2-ylmethylene)thiazolidin-4-one (**Compd 8**). Purity: 98%. ^{1}H *d6*-DMSO (400 MHz) δ 8.53 (d, *J* = 4.0 Hz, 1H), 7.64 (t, *J* = 7.6 Hz, 1H), 7.38 (t, *J* = 7.6 Hz , 1H), 7.30-7.15 (m, 3H), 6.96 (t, *J* = 5.2 Hz, 1H), 6.23 (s, 1H), 3.94 (s, 3H), 3.90 (q, *J* = 6.8 Hz, 2H), 1.21 (t, *J* = 6.8 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}ClN_3OS_2]^+$, 402.050, found 402.062 $[M+H]^+$.

(2*Z*,5*E*)-3-ethyl-5-(3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-2-(pyridin-4-ylmethylene) thiazolidin-4-one (**Compd 9**). Purity: 97%. ^{1}H *d6*-DMSO (500 MHz) δ 8.43 (d, *J* = 5.0 Hz, 2H), 7.71 (d, *J* = 7.5 Hz, 1H), 7.42-7.38 (m, 2H), 7.29 (d, *J* = 5.0 Hz, 2H), 7.18 (t, *J* = 7.0 Hz , 1H), 6.02 (s, 1H), 3.94 (s, 3H), 3.90 (q, *J* = 7.6 Hz, 2H), 1.19 (t, *J* = 7.6 Hz, 3H). ^{13}C *d6*-DMSO (100 MHz) 164.42, 150.56, 149.29, 143.86, 140.73, 139.76, 126.60, 125.65, 122.72, 121.61, 120.76, 120.40, 110.82, 109.55, 92.71, 79.58, 37.17, 33.99, 12.06. ESI-MS: m/z calculated for $[C_{19}H_{18}N_3OS_2]^+$, 368.089, found 368.063 $[M+H]^+$.

(2*Z*,5*E*)-3-ethyl-5-(4-fluoro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-2-(pyridin-4-ylmethylene)thiazolidin-4-one (**Compd 10**). Purity: 96%. ^{1}H *d6*-DMSO (400 MHz) 8.44 (dd, *J* = 4.4 , 1.6 Hz, 1H), 7.53 (dd, *J* = 7.6, 1.2 Hz, 1H), 7.30 (dd, *J* = 4.4, 2.0 Hz, 1H), 7.26-7.12 (m, 2H), 6.07 (s, 1H), 4.04 (s, 3H), 3.89 (q, *J* = 7.2 Hz, 2H), 1.19 (t, *J* = 7.2 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}FN_3OS_2]^+$, 386.079, found 386.504 $[M+H]^+$.

(2*Z*,5*E*)-3-ethyl-5-(5-fluoro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-2-(pyridin-4-ylmethylene)thiazolidin-4-one (**Compd 11**). Purity: 98%. ^{1}H *d6*-DMSO (400 MHz) δ 8.44 (apparent d, *J* = 6.0 Hz, 2H), 7.71 (dd, *J* = 8.4, 5.6 Hz, 1H), 7.41 (dd, *J* = 10.4, 2.4 Hz, 1H), 7.30 (apparent d, *J* = 6.4 Hz, 2H), 7.02 (td, *J* = 8.8, 2.4 Hz, 1H), 6.04 (s, 1H), 3.93 (s, 3H), 3.89 (q, *J* = 7.2 Hz, 2H), 1.18 (t, *J* = 7.2 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}FN_3OS_2]^+$, 386.079, found 386.038 $[M+H]^+$.

(2*Z*,5*E*)-3-ethyl-5-(6-fluoro-3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)-2-(pyridin-4-ylmethylene)thiazolidin-4-one (**Compd 12**). Purity: 96%. ^{1}H *d6*-DMSO (400 MHz) δ 8.41 (dd, *J* = 4.8, 1.6 Hz, 2H), 7.65 (dd, *J* = 8.0, 2.4 Hz, 1H), 7.38 (dd, *J* = 8.4, 4.0 Hz, 1H), 7.27 (dd, *J* = 4.8, 1.6 Hz, 2H), 7.20 (td, *J* = 8.8, 2.8 Hz, 1H), 6.00 (s, 1H), 3.91 (s, 3H), 3.86 (q, *J* = 7.2 Hz, 2H), 1.16 (t, *J* = 7.2 Hz, 3H). ^{13}C *d6*-DMSO (100 MHz) 164.51, 151.53, 149.41, 149.23, 143.71, 139.78, 137.49, 127.10, 120.80, 113.68, 113.43, 111.66, 109.09, 108.82, 92.91, 79.78, 37.21, 34.32, 12.07. ESI-MS: m/z calculated for $[C_{19}H_{17}FN_3OS_2]^+$, 386.079, found 386.089 $[M+H]^+$.

(*2Z*,*5E*)-3-ethyl-5-(7-fluoro-3-methylbenzo[*d*]thiazol-2(*3H*)-ylidene)-2-(pyridin-4-ylmethylene)thiazolidin-4-one (**Compd 13**). Purity: 95%. ^{1}H *d6*-DMSO (400 MHz) δ

8.44 (apparent d, *J* = 6.0 Hz, 2H), 7.46-7.39 (m, 1H), 7.32 (apparent d, *J* = 6.0 Hz, 2H), 7.28 (d, *J* = 8.0 Hz, 1H), 7.07 (t, *J* = 8.8 Hz, 1H), 6.06 (s, 1H), 3.96 (s, 3H), 3.90 (q, *J* = 7.2 Hz, 2H), 1.19 (t, *J* = 7.2 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}FN_3OS_2]^+$, 386.079, found 386.009 $[M+H]^+$.

(*2Z*,*5E*)-5-(4-chloro-3-methylbenzo[*d*]thiazol-2(*3H*)-ylidene)-3-ethyl-2-(pyridin-4-ylmethylene)thiazolidin-4-one (**Compd 14**). Purity: 98%. ^{1}H *d6*-DMSO (400 MHz) δ 8.46 (d, *J* = 5.5 Hz, 2H), 7.70 (d, *J* = 7.7 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.36 (d, *J* = 4.9 Hz, 2H), 7.18 (t, *J* = 7.8 Hz, 1H), 6.14 (s, 1H), 4.03 (s, 3H), 3.91 (q, *J* = 7.1 Hz, 2H), 1.20 (t, *J* = 7.0 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}ClN_3OS_2]^+$, 402.050, found 401.878 $[M+H]^+$.

(*2Z*,*5E*)-5-(5-chloro-3-methylbenzo[*d*]thiazol-2(*3H*)-ylidene)-3-ethyl-2-(pyridin-4-ylmethylene)thiazolidin-4-one (**Compd 15**). Purity: 98%. ^{1}H *d6*-DMSO (400 MHz) δ 8.44 (apparent d, *J* = 6.0 Hz, 2H), 7.72 (d, *J* = 8.0 Hz, 2H), 7.56 (s, 1H), 7.31 (apparent d, *J* = 5.6 Hz, 2H), 7.28 (apparent d, *J* = 8.4 Hz, 1H), 6.05 (s, 1H), 3.94 (s, 3H), 3.89 (q, *J* = 7.2 Hz, 2H), 1.18 (t, *J* = 7.2 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}ClN_3OS_2]^+$, 402.050, found 402.011 $[M+H]^+$.

(*2Z*,*5E*)-5-(6-chloro-3-methylbenzo[*d*]thiazol-2(*3H*)-ylidene)-3-ethyl-2-(pyridin-4-ylmethylene)thiazolidin-4-one (**Compd 16**). Purity: 97%. ^{1}H *d6*-DMSO (400 MHz) δ 8.43 (apparent d, *J* = 6.0 Hz, 2H), 7.83 (s, 1H), 7.37 (apparent s, 2H), 7.28 (d, *J* = 5.6 Hz,

2H), 6.01 (s, 1H), 3.90 (s, 3H), 3.87 (q, *J* = 7.2 Hz, 2H), 1.18 (t, *J* = 7.2 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}ClN_3OS_2]^+$, 402.050, found 401.850 $[M+H]^+$.

(*2Z*,*5E*)-5-(7-chloro-3-methylbenzo[*d*]thiazol-2(*3H*)-ylidene)-3-ethyl-2-(pyridin-4-ylmethylene)thiazolidin-4-one (**Compd 17**). Purity: 98%. ^{1}H *d6*-DMSO (400 MHz) δ 8.46 (d, *J* = 5.7 Hz, 2H), 7.82 (apparent d, *J* = 3.9Hz, 1H) 7.46-7.41 (m, 3H), 7.31 (apparent s, 1H), 6.20 (s, 1H), 3.98 (s, 3H), 3.96 (q, *J* = 8.0 Hz, 2H), 1.21 (t, *J* = 7.0 Hz, 3H). ESI-MS: m/z calculated for $[C_{19}H_{17}ClN_3OS_2]^+$, 402.050, found 401.934 $[M+H]^+$.

Metabolic stability assay

Metabolic stability was assessed after incubation with mouse liver microsomes. Briefly, test compounds (1 μM) were incubated with mouse liver microsomes (Xenotech Sekisui) at 0.5 mg/mL in 100 mM potassium phosphate buffer at 37 ℃ with an NADPH regeneration system. Aliquots are collected at various time points and quenched in an ice cold methanol:acetonitrile mixture (1:1 v/v) containing niflumic acid as an internal standard. Quenched solutions were then filtered (Captiva, Agilent) before analysis by LC/MS-MS. Stability was quantified by determination of the ratio of the peak areas for the compound peak : internal standard, normalized to the relevant initial (t = 0) time point. Half-lives were obtained by fitting the time course data. Samples without the NADPH regeneration system were used as negative controls.

Cell culture and immunoblotting

HeLaC3 and SH-SY5Y cells were cultured in supplemented Opti-MEM media at 37 °C, as described[1]. Cells were cultured in 6-well plates and treated with compounds at the indicated concentrations for 24 h before lysis with RIPA buffer supplemented with protease inhibitor (Sigma). After quantification of total protein concentration, normalized amounts of lysate were separated by 10% SDS-PAGE, transferred to nitrocellulose and then the proteins of interests were identified by Western blot, as described[1].

Reference

1. Young ZT, Rauch JN, Assimon VA, et al. Stabilizing the Hsp70-Tau Complex Promotes Turnover in Models of Tauopathy. *Cell Chem Biol.* 2016;23(8): 992-1001.