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Supplemental Information

Differential Scanning Fluorimetry (DSF) Screen to Identify Inhibitors of Hsp60 Protein-Protein Interactions

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Experimental

Protein expression and purification

The purification of Hsp60 was modified from a previously reported method.[1] Briefly, human Hsp60 was expressed in *E. coli* BL21 (DE3) cells. The BL21 cells in 1L of terrific broth (TB) were grown at 37 °C with shaking until the OD_{600} reached 0.6. Then, the cultures were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration 300 μM) and grown at 37 °C for 4 hours. For protein purification, cell pellets were re-suspended in His-binding buffer (50 mM TRIS, 10 mM Imidazole, 500 mM NaCl, pH 8) supplemented with protease inhibitors. Cells were then lysed by sonication, pelleted by centrifugation, and the supernatant was incubated with Ni-NTA His-Bind Resin for 1 hour at 4 °C (Novagen, Germany). The resin was washed with His-binding buffer, followed by His-washing buffer (50 mM TRIS, 30 mM Imidazole, 300 mM NaCl, pH 8). The protein was then removed from the resin using His-elution buffer (50 mM TRIS, 300 mM Imidazole, 300 mM NaCl, pH 8). The N-terminal His-tags were removed by incubating the protein and TEV protease with 1 mM DTT for 4 hours at room temperature, followed by dialysis in 4 L of SEC buffer (50 mM Tris-HC1, 0.3 M NaC1, 10 mM $MgC1_2$, pH 7.70) overnight. The second day, the uncleaved protein was removed by a reverse Ni-NTA purification and concentrated. To assemble Hsp60 oligomers, the following were mixed:

573 µL of Hsp60 (purified as monomer), 13 µL of 1M KC1, 13 µL of 1M magnesium acetate, and 52 µL of 0.05M ATP. After a 90 min incubation at 30 °C, the protein mixture was purified by SEC200 column (Running buffer: 50 mM Tris-HC1, 0.3 M NaC1, 10 mM $MgC1_2$, pH 7.7). The pooled oligomers are concentrated to 15-20 mg/mL, aliquoted, flash- frozen, and stored at -80 °C.

Differential Scanning Fluorimetry Assay

DSF experiments were performed in a BioRad CFX connect or Roche LC480 Light Cycler II qPCR machine in the FRET channel. Solutions of protein (assay buffer: 50 mM Tris, 20 mM KCl, 10 mM $MgCl_2$, 200 mM NaCl, PH 7.4), Sypro Orange (Thermo Fisher Scientific, S6650) and tested compounds or DMSO were added to the wells of a 96-well PCR plate (USA Scientific, 1402-9590) or 384-well microtiter plate (Axygen, PCR-284- LC480WNFBC). The plates were sealed with transparent films (EXCEL Scientific, TS- RT2-100) and heated in the qPCR machine from 25 to 95 °C in increments of 1.0 °C. The 96 well-plate assay data was analyzed using DSFworld (Wu et al submitted) using the first derivative and Boltzmann models. The 384-well assay data was analyzed using Roche LC480 Light Cycler II software. Technical note: In our experiments, we have noted that signal intensity is highly dependent on the plastic used in microtiter plates, even showing differences between “lots”. Using different qPCR machines also cause signal intensity variability. So, one should not read into that value between experiments.

Malachite Green ATPase Assay

The ATPase activity assay was adopted from a previous method, with modifications.[2] The

malachite green reagent was prepared from stock solutions three hours before use (malachite green (0.081% w/v), polyvinyl alcohol (2.3% w/v), ammonium heptamolybdate tetrahydrate (5.7% w/v in 6 M HCl) and water (ratio of 2:1:1:2). To each well of a 96-well plate, 1 μL compounds or DMSO were added to 19 μL Hsp60/Hsp10 solution (2.63 μM and 5.26 μM respectively) in assay buffer (100 mM Tris, 20 mM KCl, 10 mM $MgCl_2$, 0.017% Triton, pH 7.4). To this solution, 5 μL of 5 mM ATP was added to initiate the reaction, such that the final reaction volume was 25 μL and the conditions were 2.0 μM Hsp60, 4 μM Hsp10, 4% DMSO, 0.01% Triton X-100, and 1 mM ATP. After 3 h incubation at 37 °C, 80 μL of malachite green reagent was added into each well, followed by 10 μL 32% sodium citrate to limit chemical hydrolysis of ATP. The plate was incubated at 37 °C for 15 min before measuring OD_{620} on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

Native Gel Assay

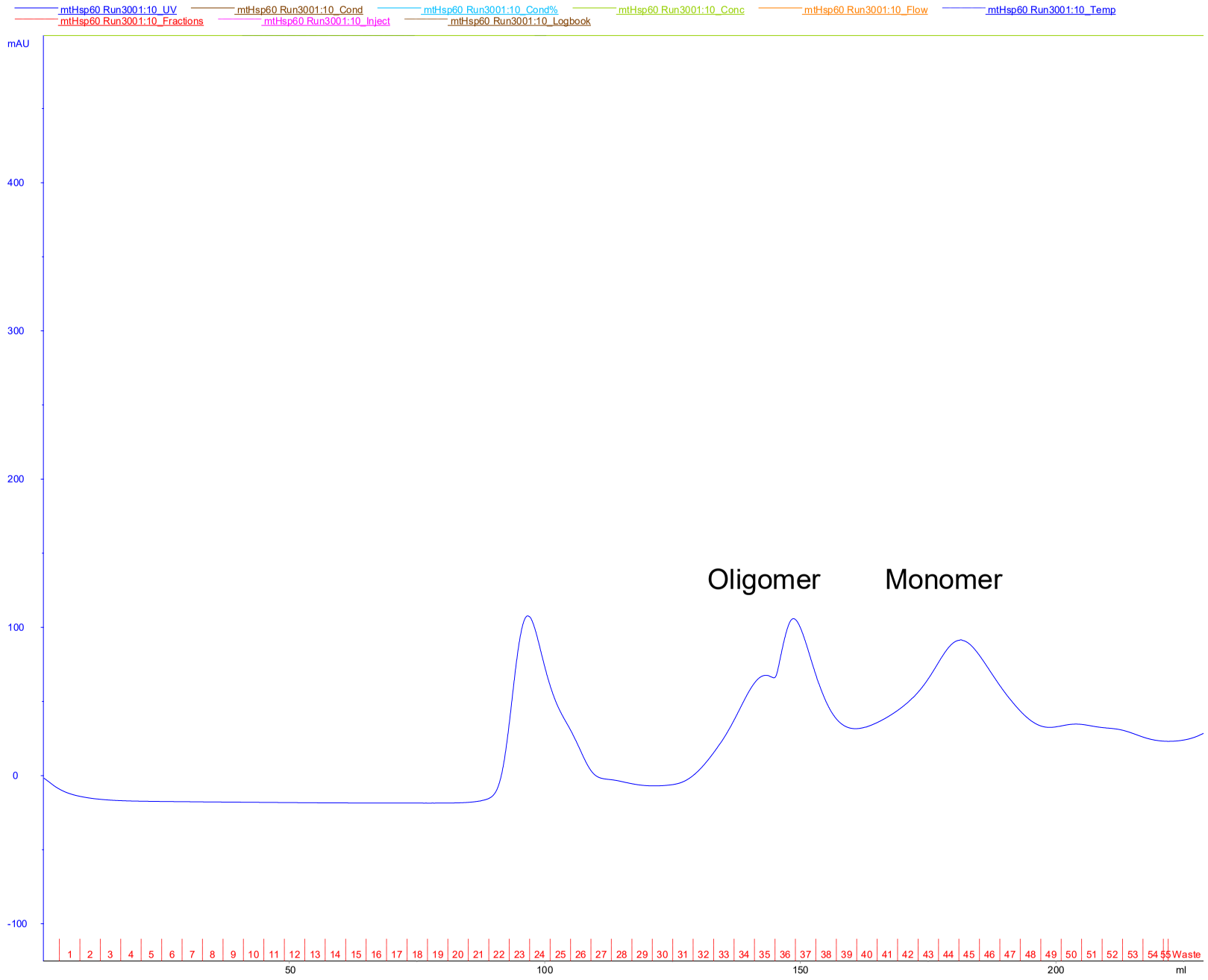
A solution of Hsp60 (5 μM) was treated with compounds at the indicated concentrations for 30 mins. Then sample buffer (5X: 0.05% Ponceau Red, 50% Glycerol, 250 mM 6- aminohexanoic acid, 50 mM Bis-Tris, pH 7.0) was added. An aliquot of 15 μL and molecular weight marker (NativeMark Unstained Protein Standard-Invitrogen) were loaded on precast 4–16% Novex NativePAGE Bis-Tris gels. The electrophoresis was performed at 150 V constant voltage for 4 hours at room temperature with 50 mM Bis Tris (pH 7.0) as anode buffer and 15 mM Bis Tris and 50 mM Tricine (PH 7.0) as Cathode buffer. Gel was stained by Coomassie G-250 and scanned using ChemiDoc Touch Imager.

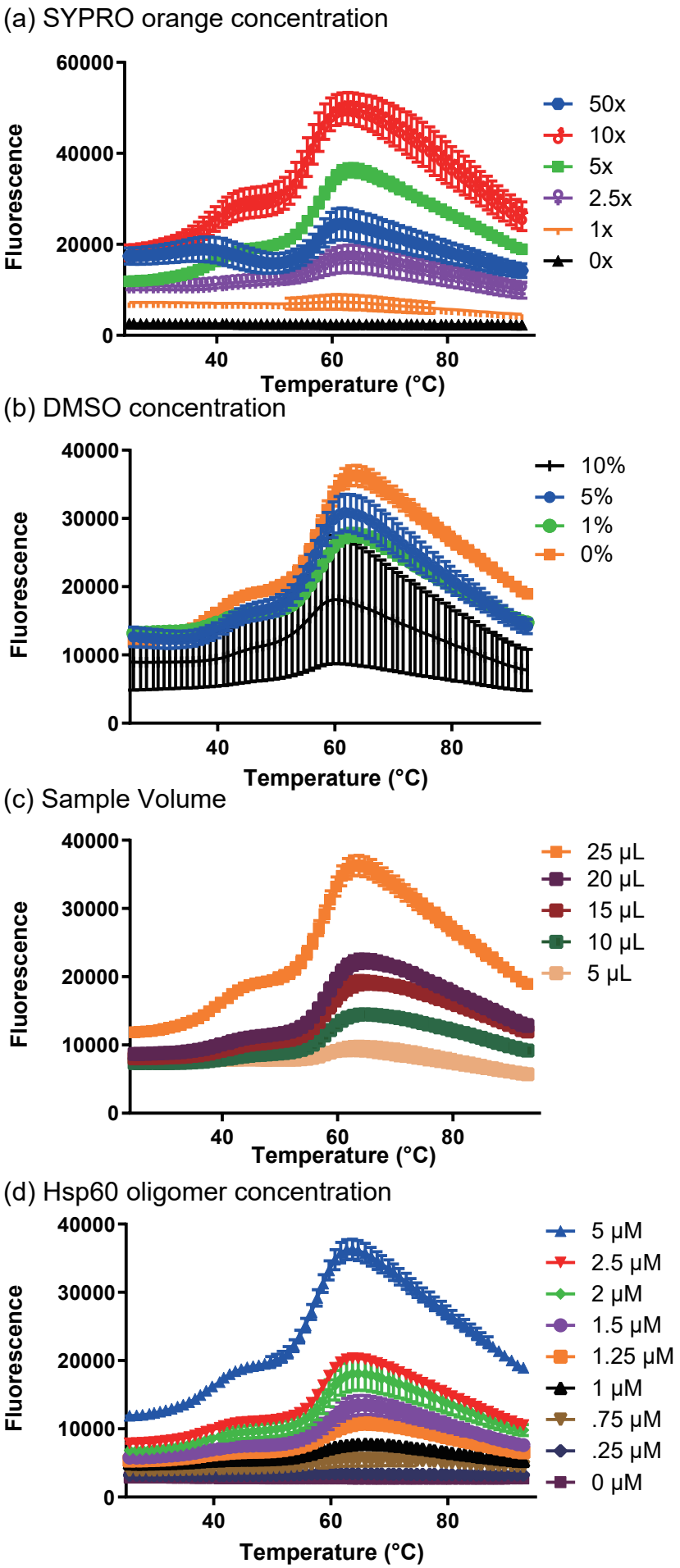
Refolding of Denatured MDH

The assay was adopted from a previously published protocol with modification.[3] Briefly, malate dehydrogenase (MDH, Sigma-10127256001) was denatured by incubating 94 μM MDH with one equivalent guanidine buffer (7 M guanidine•HCl, 200 mM Tris, pH 7.4) for 1 h at room temperature. Then, the denatured MDH (120 nM) was added to Hsp60 (3.33 μM) in refolding buffer (100 mM Tris pH 7.4, 20 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT). After ~10 mins at room temperature, Hsp10 (6.67 μM) was added. Then, 30 μL of this solution was dispensed into clear 384-well plates (CORNING, 3702), followed by addition of 1 μL compound A10 or DMSO. Refolding was initiated by addition of 20 μL of 2.5 mM ATP solution (concentrations during refolding cycle: 2 μM Hsp60, 4 μM Hsp10, 72 nM denatured MDH, 1 mM ATP). Finally, after incubating at 37 °C for an hour, the reaction was quenched by adding 10 μL of 500 mM EDTA solution. To determine the amount of MDH that was re-folded, reaction progress was monitored by measuring NADH absorbance at 340 nM for 60 minutes in assay buffer (20 mM sodium mesoxalate monohydrate and 2.4 mM NADH in reaction buffer, Sigma-Aldrich). A_{340} measurements were recorded at T_0 and then the time point at which 90% of substrate was consumed was reported. The IC_{50} values were calculated using GraphPad Prism.

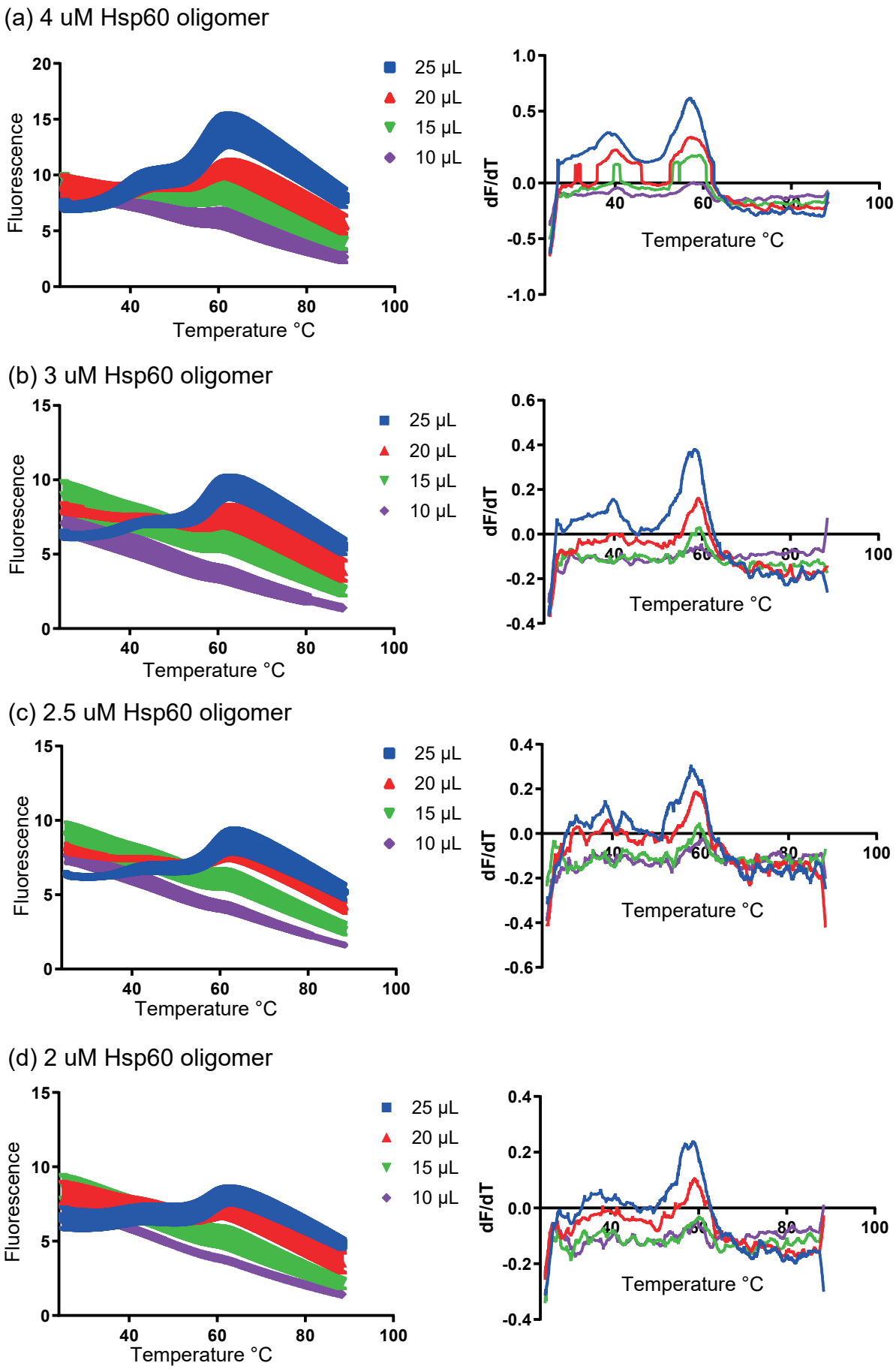
1. Viitanen PV, Lorimer G, Bergmeier W, Weiss C, Kessel M, Goloubinoff P. Purification of mammalian mitochondrial chaperonin 60 through in vitro reconstitution of active oligomers. *Method Enzymol.* 1998;290: 203-217.
2. Chang L, Bertelsen EB, Wisen S, Larsen EM, Zuiderweg ERP, Gestwicki JE. High-throughput screen for small molecules that modulate the ATPase activity of the molecular chaperone DnaK. *Anal Biochem.* 2008;372(2): 167-176.
3. Johnson SM, Sharif O, Mak PA, et al. A biochemical screen for GroEL/GroES inhibitors. *Bioorganic & medicinal chemistry letters.* 2014;24(3): 786-789.

Supplemental Figure 1: SEC purification traces for Hsp60 oligomer and monomer.



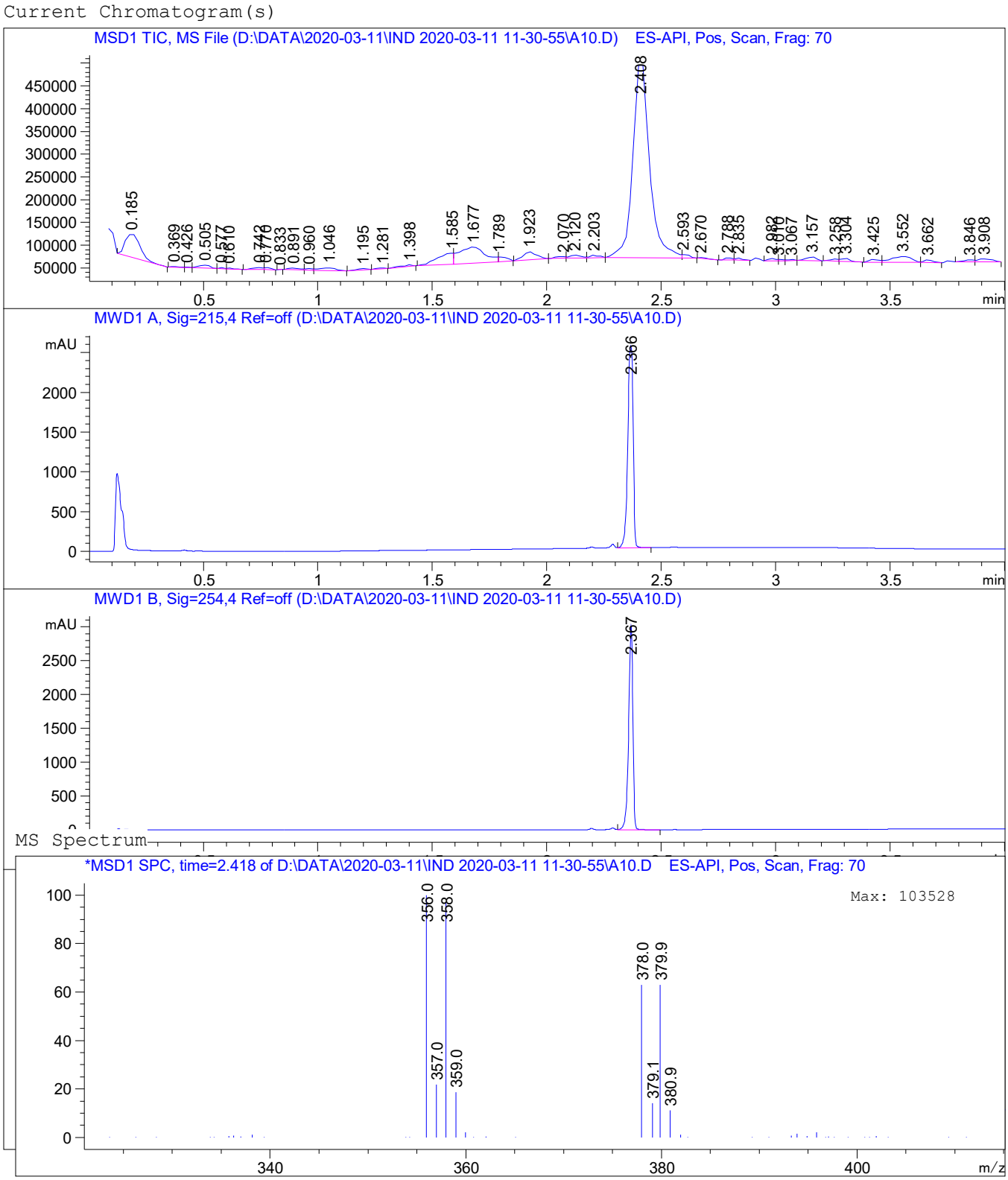


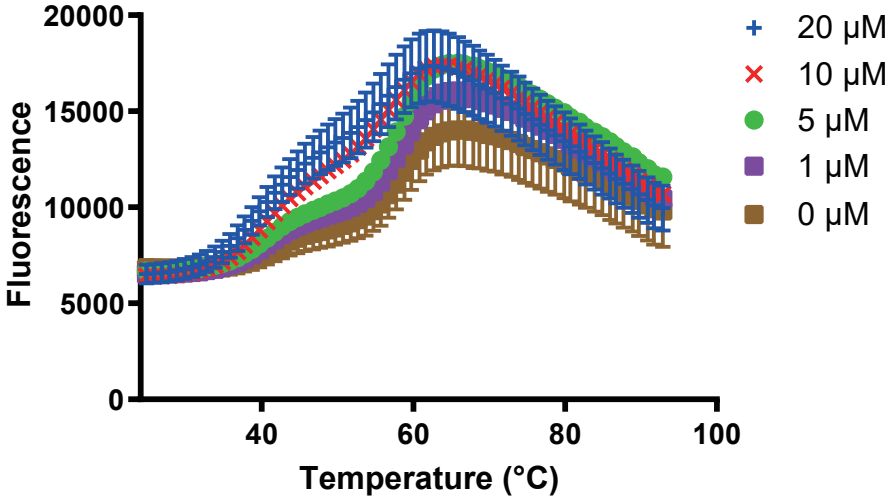
Supplemental Figure 2: Optimization of DSF assay in 96 well plate. Investigate (a) SYPRO Orange concentration, (b) DMSO concentration, (c) sample volume, and (d) Hsp60 oligomer concentration's effect on Tm. Results are the average of experiments performed in triplicate. Error bars represent SD.



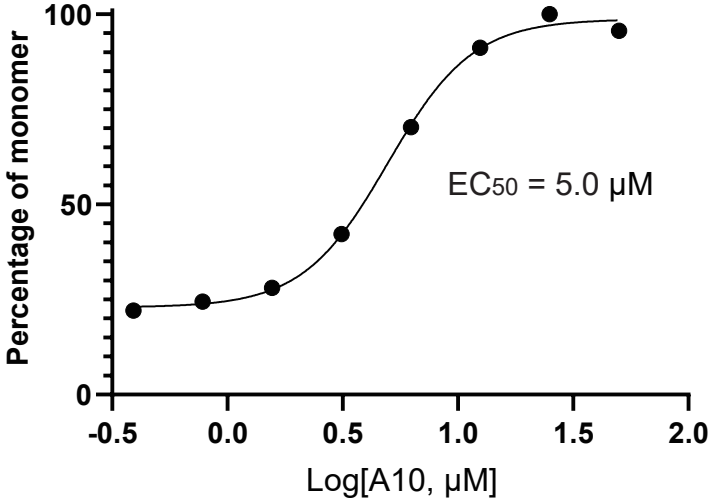
Supplemental Figure 3: Optimization of DSF assay in 384 well plate. Results are the average of experiments performed in triplicate. Error bars represent SD.

Supplemental Figure 4: LC-MS spectrum of compound A10

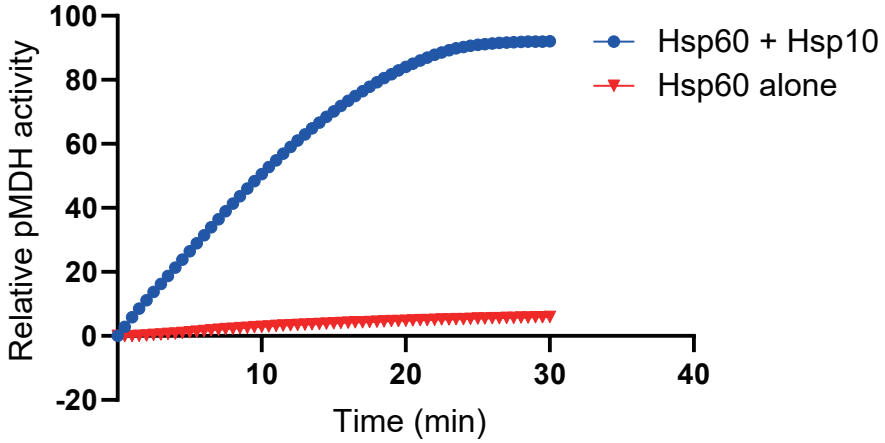




Supplemental Figure 5: The melting curves of Hsp60 oligomer treated with compound A10 at indicated concentrations. Results are the average of experiments performed in triplicate. Error bars represent SD.



Supplemental Figure 6: A10 increased monomer population in a dose-dependent manner.



Supplemental Figure 7: Hsp60 works with Hsp10 to refold denatured pMDH.