Supporting Information

Ligands for glaucoma-associated myocilin discovered by a generic binding assay

Susan D. Orwig[a], Pamela V. Chi[a], Yuhong Du[b], Shannon E. Hill[a], Marchello A. Cavitt[a], Amrithaa Suntharalingam[c], Katherine C. Turnage[a], Chad A. Dickey[c], Stefan France[a], Haian Fu[b], Raquel L. Lieberman[a]*

[a]School of Chemistry & Biochemistry, Georgia Institute of Technology, 901 Atlantic Drive NW, Atlanta, GA 30332-0400 [b]Department of Pharmacology, Emory University School of Medicine, 1510 Clifton Road, Atlanta, GA 30322 [c]Department of Molecular Medicine and Byrd Alzheimer's Research Institute, University of South Florida, 4001 E. Fletcher Ave. Tampa, FL 33613

Supplemental Table S1. Screening summary.

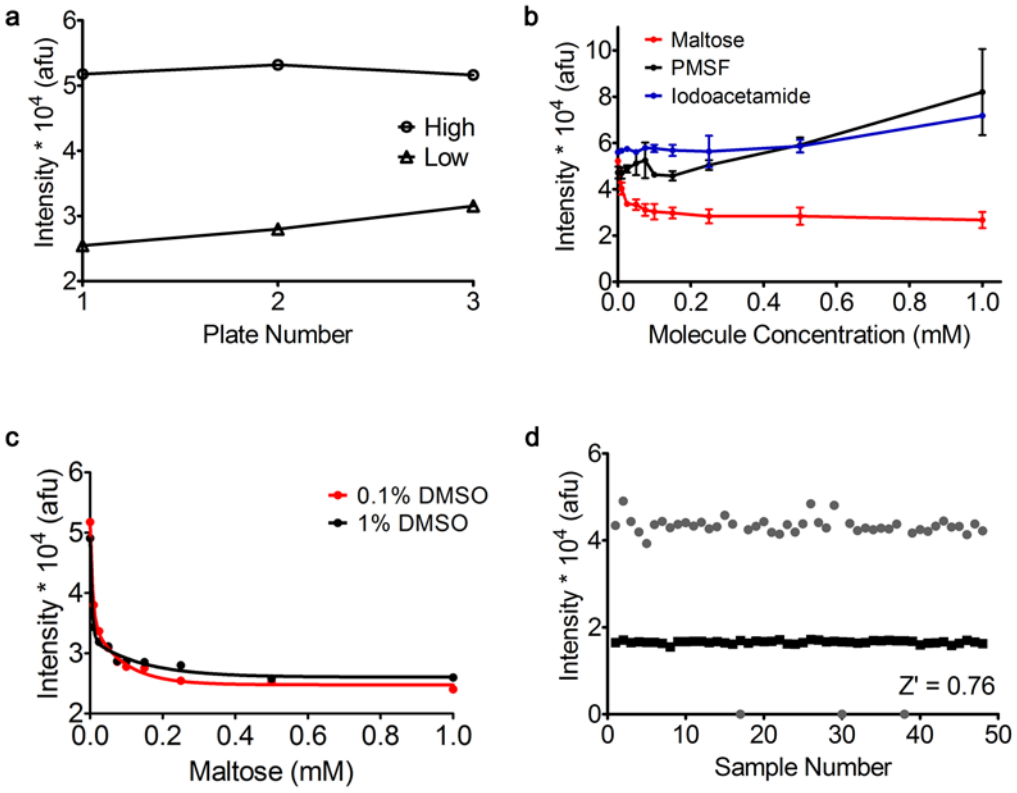
Category	Parameter	Description
Assay	Type of assay	Ligand-binding (biochemical/in vitro)
	Target	myoc-OLF or other purified (soluble) protein
	Primary measurement	Fluorescence emission of Sypro Orange
	Key reagents	Master Mix: 0.54 M GdnHCl, 4.5X SO, PBS, pH 7.2 Library Compounds: 1 mM in DMSO Positive control: 0.8 M TMAO Protein: 4 µM in PBS, pH 7.2
	Assay protocol	See Table 1. Dispense Master Mix (20 µl), add compound (0.5 µl), then protein. Centrifuge 800 x g, read fluorescence.
Library	Library size	1280
	Library composition	LOPAC
	Source	Sigma-Aldrich
Screen	Format	384-well microtiter plate (Costar)
	Concentration(s) tested	15.7 µM compound
	Plate controls	No compound, + 0.8 M TMAO in each tray
	Reagent/ compound dispensing system	Sciclone ALH 3000 Workstation with 384-well channel cannula array (Caliper LifeScience); Multidrop Combi.
	Detection instrument and software	2103 EnVisition Multilabel Plate Reader (Perkin Elmer) (λex = 480 nm; λem = 572 nm)
	Assay validation/QC	Z', S/B, CV
	Correction factors	None
	Normalization	None
Post-HTS analysis	Hit criteria	50% or better fluorescence decrease compared to TMAO
	Hit rate	1.25 %
	Additional assay(s)	Fibrillization assay, cellular secretion of mutant myocilin

Supplemental Table S2. DSF data for 1 μM MBP destabilized with 0.6 M GdnHCl with 1mM known ligands maltose, maltotetraose and maltitol.

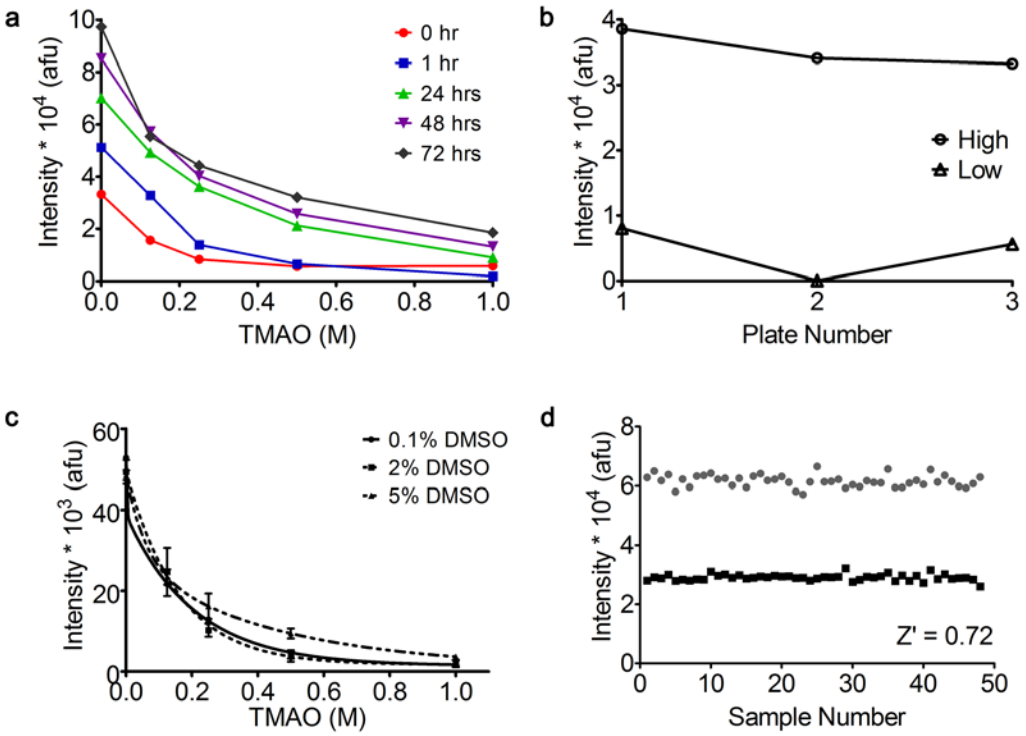
MBP + 0.6 M GdnHCl	47.7 ± 0.3 °C
MBP + 0.6 M GdnHCl + 1 mM maltose	56.6 ± 0.1 °C
MBP + 0.6 M GdnHCl + 1 mM maltotetraose	57.4 ± 0.2 °C
MBP + 0.6 M GdnHCl + 1 mM maltitol	48.6 ± 0.1 °C

Supplemental Table S3. DSF data for 1 μM myoc-OLF with 1 M TMAO, destabilized or not with 0.6 M GdnHCl

myoc-OLF	54.6 ± 0.1 °C
myoc-OLF + 1 M TMAO	59.8 ± 0.1 °C
myoc-OLF + 0.6 M GdnHCl	39.7 ± 0.1 °C
myoc-OLF + 0.6 M GdnHCl + 1 M TMAO	45.9 ± 0.9 °C

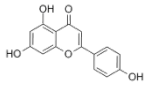
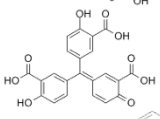
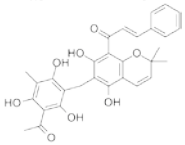
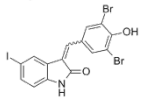
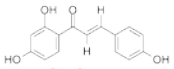
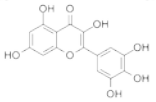
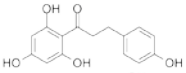
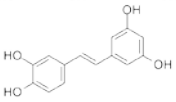
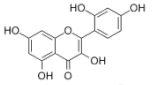
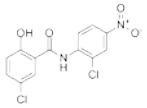
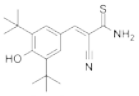
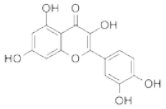
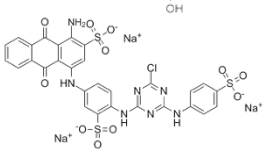
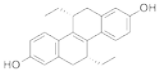
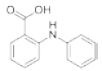
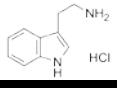


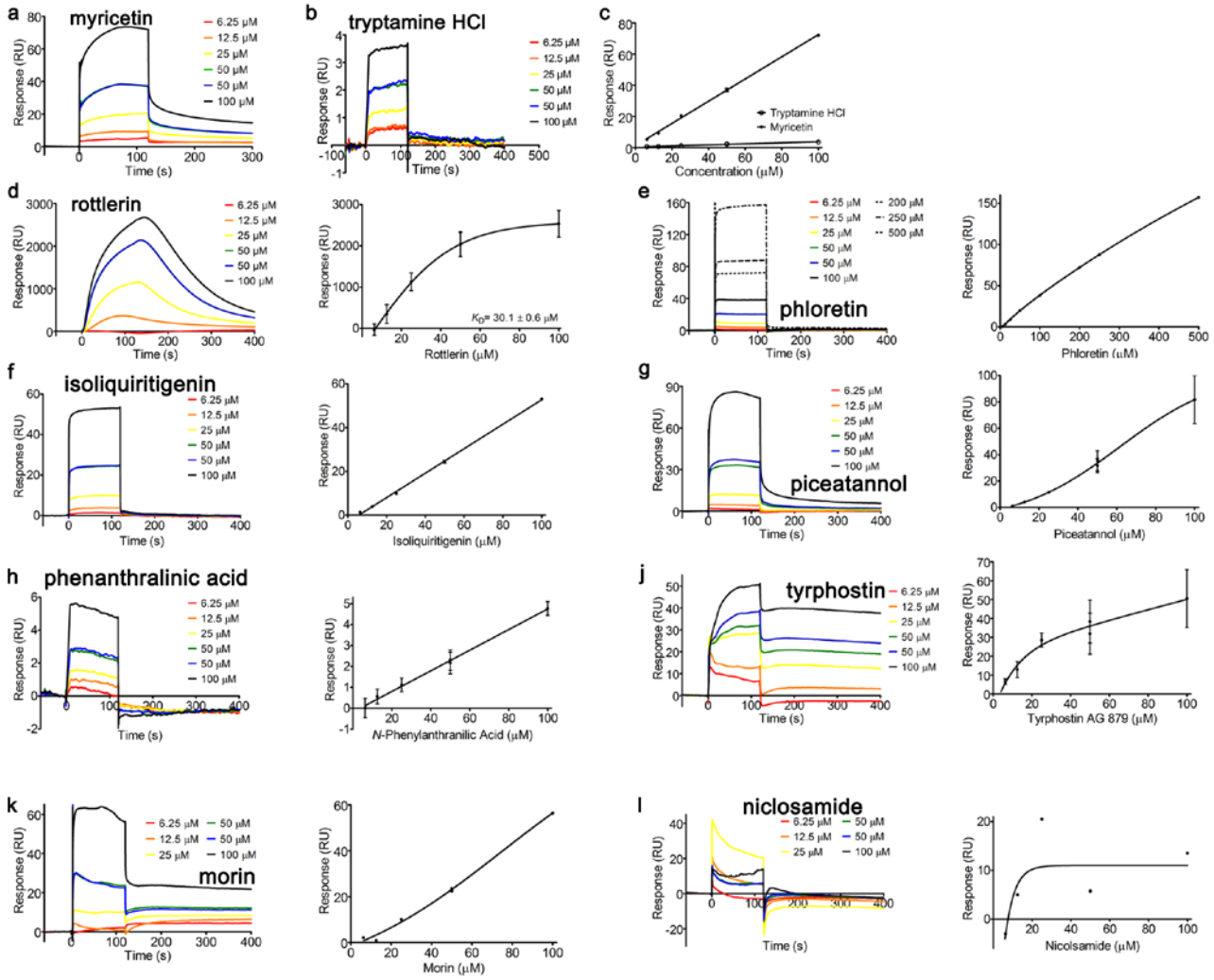
Supplemental Fig. S1. Control experiments conducted for assay development with MBP in 96-well format. (A) Plate-to-plate variability conducted using MBP without (high fluorescence) and with (low fluorescence) addition of 1 mM maltose. (B) negative controls conducted with PMSF (black) and iodoacetamide (blue). Red curve for MBP + maltose is included for comparison. (C) Effects of DMSO on MBP stabilization with maltose. (D) Well-to-well variability for MBP without (high fluorescence) and with (low fluorescence) 1 mM maltose.



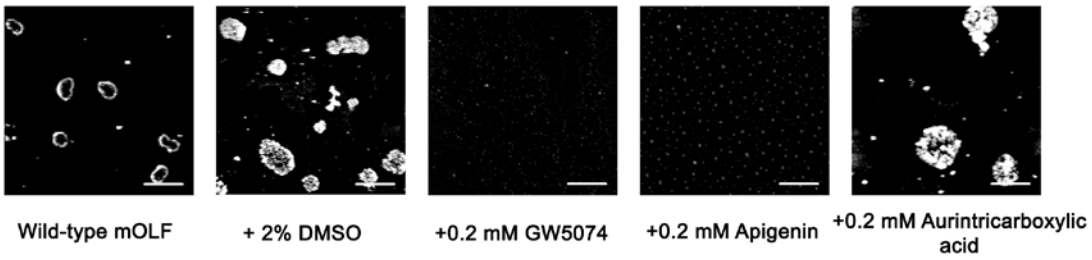
Supplemental Fig. S2. Control experiments conducted for myoc-OLF. (A) Effect of fluorescence readout as a function of time for myoc-OLF stabilization by TMAO. (B) Plate-to-plate variability conducted using myoc-OLF without (high fluorescence) and with (low fluorescence) 1M TMAO. (C) Effect of DMSO on myoc-OLF stabilization by TMAO. (D) Well-to-well variability for myoc-OLF without (high fluorescence) and with (low fluorescence) 1 M TMAO.

Supplemental Table S4. Hit compound chemical structures as reported by LOPAC.

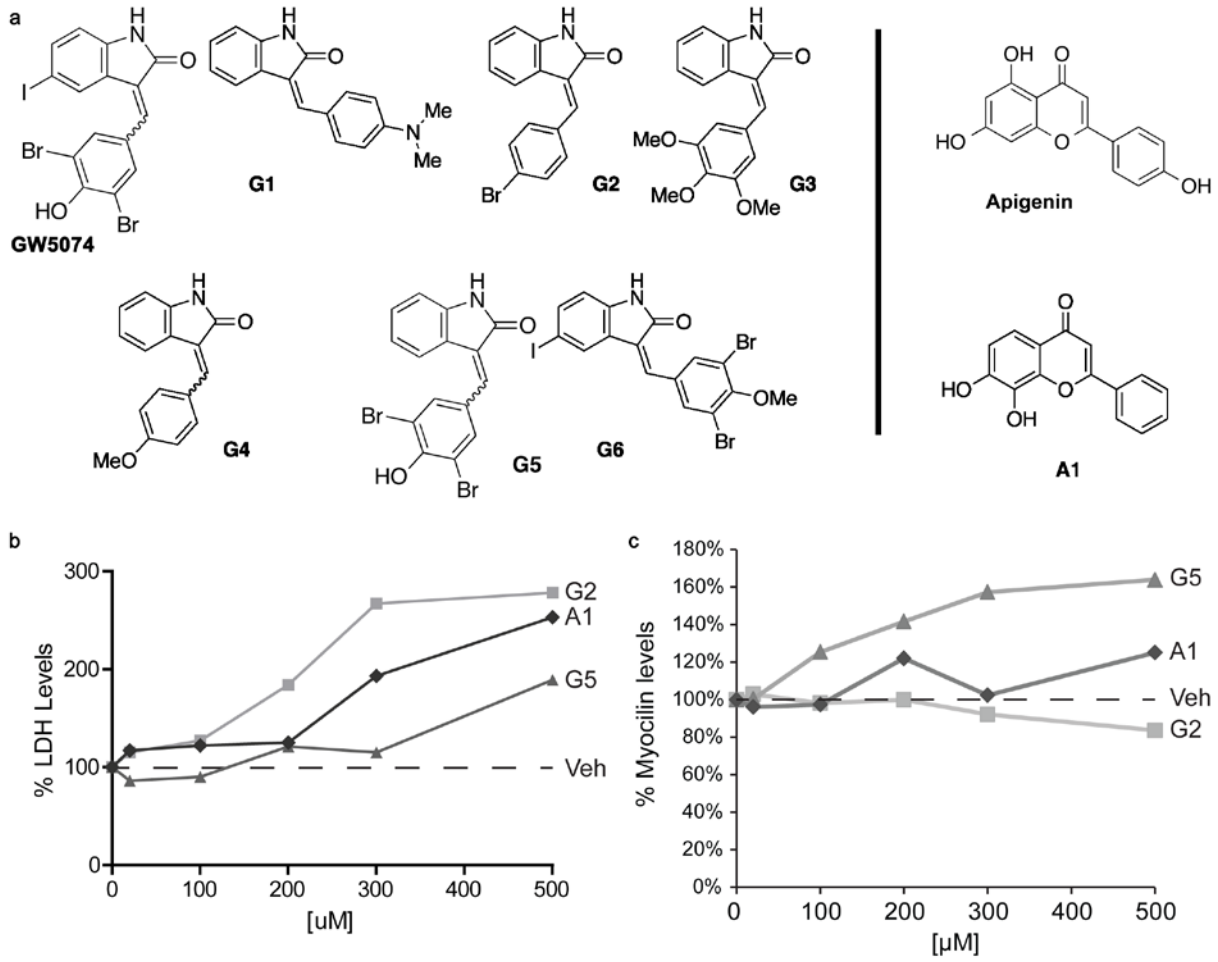
Compound	Structure
Apigenin	
Aurintricarboxylic acid	
Rottlerin	
GW5074	
Isoliquiritigenin	
Myricetin	
Phloretin	
Piceatannol	
Morin	
Niclosamide	
Tyrphostin AG 879	
Quercetin dihydrate	
Reactive Blue 2	
(R,R)-cis-Diethyl tetrahydro-2,8-chrysenediol	
N-phenylanthanilic Acid (neg. control)	
Tryptamine HCl (neg. control)	



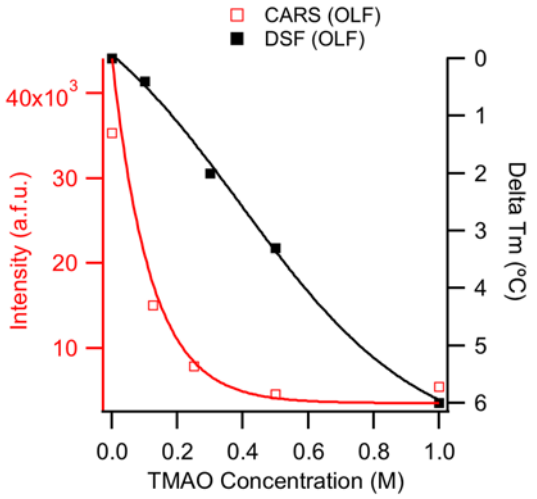
Supporting Fig. S3. Sensorgrams and binding curve SPR data for compounds not included in main text. Tyrphostin, morin and niclosamide data were conducted with surfactant P-20 whereas the remaining compounds, rottlerin, phloretin, isoliquiritigenin, piceatannol, phenanthralinic acid were not.



Supplemental Fig. S4. AFM images associated with in vitro myoc-OLF fibrillization assay. For myoc-OLF incubated with 0.2 mM GW5074 or Apigenin, no amyloid-like deposits were observed at the conclusion of the fibrillization assay (72 h). Scale bar = 1 μm, height = 0 (black) to 13 nm (white).



Supplemental Fig. S5. SAR study and enhancement of I477N-mutant myocilin cellular secretion. (a) Compounds synthesized in first SAR study. (b) Toxicity profile of G2, G5, and A1 compared to vehicle (DMSO). After 24 h the media was collected and analyzed via LDH cytotoxicity assay (Promega). (c) Levels of secreted myocilin upon treatment with selected derivatives compared to vehicle (DMSO) based on intensities from dot blot in Fig. 6c. Values shown are % of vehicle treatment ± SEM.



Supplemental Fig. S6. CARS vs DSF sensitivity comparison. Change in SO fluorescence intensity (red, CARS) and thermal stability (black, DSF) for myoc-OLF plus TMAO. Changes in SO fluorescence in CARS are apparent at the lowest concentrations of compound added where the ΔT_m is within error of DSF measurement. Protein concentration is 1 μM.

Supplemental Methods

Molecular Biology, Protein Expression, and Purification. MBP was expressed in *E. coli* Rosetta-gami 2(DE3)pLysS cells (Novagen) using the pMAL-c4x vector (New England Biolabs). Cells were grown at 37 °C in Luria broth (Fisher Scientific) to an OD_{600} = 0.6, induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside, and harvested after 3h by centrifugation. Cells were lysed by passage through French Press, and cell debris was pelleted by ultracentrifugation. MBP was purified using a high-flow amylose resin (New England Biolabs) column using phosphate buffer (PBS, 10 mM sodium phosphate dibasic, 10 mM potassium phosphate monobasic, 200 mM NaCl pH 7.2) with 1 mM EDTA, and elution buffer containing additional 10 mM maltose.

Chemical Stability Assay Development. Increasing concentrations of GdnHCl (0-2 M) were added to 10 μM MBP or myoc-OLF in PBS, and 5X SO (Invitrogen) to determine the optimal denaturant concentration. The samples were assembled at room temperature and delivered to 96-well microplates (Grenier). Fluorescence data were acquired on a Biotek Synergy 2 instrument equipped with a 485/20 nm excitation filter and a 590/35 nm emission filter. Data were acquired in triplicate and blank subtracted.

CD. CD was performed on a Jasco J-810 spectropolarimeter with myoc-OLF (45 μM-50 μM) in PBS, 5X Sypro Orange, with or without 0.6M GdnHCl. Temperature was maintained at 24 ºC. Wavelength scans were measured with a 0.1 cm cuvette from 250 nm to 320 nm at a rate of 50 nm/min and a data pitch of 1nm. Each measurement was an average of 10 scans performed in triplicate directly after adding the protein and background subtracted. The spectrum reported is the average of these 3 measurements converted to mean residue ellipticity.

AFM imaging. A few hours after the ThT amyloid aggregation assay, 40 μL of the sample was removed from the bottom of the 1.5 mL centrifuge tube and deposited onto freshly cleaved mica for 30 minutes, rinsed for 3 seconds with deionized water, and left to dry overnight in a Petri dish. After drying, the samples were imaged in air with a MFP-3D atomic force microscope (Asylum Research) using PPP-FMR (NanoAndMore) silicon tips with nominal tip radii less than 7 nm. The cantilever was driven at 60–70 kHz in alternating current mode and a scan rate of 0.5 Hz with 512 x 512-pixel resolution. Raw image data were corrected for image bow and slope using the software provided by Asylum Research.

ANS fluorescence. ANS (Fluka Analytical) fluorescence measurements were performed on a Shimadzu RF-530/PC spectrofluorophotometer with myoc-OLF (10 μM), ANS (100 μM), and increasing concentrations of GdnHCl (0-1.2M) in PBS. Samples were prepared by first mixing 50 μL of 12 μM myoc-OLF and the appropriate concentration of GdnHCl at room temperature for 5 minutes, then adding 10 μL of a stock solution containing 600 μM ANS in the appropriate concentration of GdnHCl and waiting 5 additional minutes at room temperature before taking measurements using a small volume cuvette with a 3 mm pathlength. The emission spectrum (excitation wavelength of 380 nm (slit width 3 nm) and an emission range of 400-600 nm (slit width 3 nm) is an average of 5 scans that were background subtracted.

Absorbance Assay to Detect Aggregation. Absorbance was measured on a Biotek Synergy 2 instrument in a clear 96-well microplate (Nest Biotech Co.) at room temperature at 620 nm every 10 minutes for 20 hours. Each well was assembled, sealed with MicroAmp PCR film (Applied Biosystems), and measured immediately in PBS containing 1 μM myoc-OLF, 5X SO, and 0.6 M GdnHCl or 0 M GdnHCl. Data for each sample was acquired in duplicate and blank subtracted.

HT Screening of Small Molecule Library. For the pilot screen, 1,280 compounds (LOPAC® 1280, Sigma Aldrich) were tested at the Emory University Chemical Biology Discovery Center. Assay volumes were adjusted to a 384-well format (30 µL reaction volume). Myoc-OLF concentration (1-10 µM) was re-optimized for the 384-well format, and a concentration of 4 µM was utilized during the library screen. Master mix solution (0.54 M GdnHCl, 4.5X SO, PBS, and water) was dispensed via a MultiDrop Combi (Thermo Scientific) to a 384-well microtiter plate (Costar). The concentration of 0.8 M TMAO was used as positive control for screening based on dispensing considerations. The Sciclone ALH 3000 Workstation with 384-channel cannula array (Caliper LifeSciences) was used to transfer 0.5 µL of compound (1 mM stock) to the dispensed master mix solution (final concentration 16.7 µM). myoc-OLF was added last, again using the MultiDrop Combi. The plates were centrifuged for five minutes at 800 x g to remove air bubbles prior to fluorescence reading in a 2103 EnVision Multilabel Plate Reader (Perkin Elmer) (λ_{ex} = 480 nm; λ_{em} = 572 nm) (Table 1).

To evaluate false positive hits, the compounds (Sigma Aldrich) were tested for potential quenching of SO in the absence of myoc-OLF using 96-well format (100 µL total volume), where reagent concentrations were identical to those used in the HT assay. Fluorescence intensities were measured in triplicate on the aforementioned Biotek Synergy 2 fluorescence plate reader.

The quality of the assay, evaluated by the Z′ factor, the CV, and S/B were calculated as described previously (1) from a 96-well plate containing destabilized MBP or myoc-OLF in 0.6 M GdnHCl and the same solution without protein. Data analysis was conducted using GraphPad Prism, and figures were generated in GraphPad Prism or Igor Pro.

Amyloid aggregation assay. ThT (Sigma Aldrich) was prepared as a 1 mg/mL stock solution in water and diluted to a working stock of 200 µM in PBS. Compounds were prepared as 10mM stock solutions in neat DMSO, then diluted to the appropriate concentration in PBS (final concentration of DMSO = 2%). Solutions of 10 µM ThT, 30 µM myoc-OLF, and 200 µM or 20 µM compound concentration were first prepared in 1.5 mL centrifuge tubes and then transferred (150 µL sample volume) to a 96-well microplate (Grenier) sealed with clear MicroAmp PCR film (Applied Biosystems). ThT fluorescence measurements were performed every 10 minutes over a 96 hour period in a Biotek Synergy microplate reader equipped with a 440 nm excitation filter and 485 nm emission filter. Each sample was background subtracted using the appropriate ThT and compound concentration in PBS. Myoc-OLF control experiments revealed 2% DMSO slightly enhanced aggregation, thus all control, sample, and background wells contained 2% DMSO. Immediately after the ThT aggregation assay, samples were removed and placed in 1.5 mL centrifuge tubes.

SPR. Myoc-OLF was immobilized on a CM5 sensor (GE Healthcare) chip using the amine coupling method (2). The chip was equilibrated in PBS followed by activation by injecting 0.2 M N-ethyl-N-dimethylaminopropylcarbodiimide and 50 mM N-hydroxysuccinimide. myoc-OLF (50 µg/mL) in 10 mM sodium acetate, pH 4.0 was then injected and followed by the deactivation of residual NHS esters with 1 M ethanolamine, pH 8.5. The protein was diluted from a 3.75 mg/mL stock to the final concentration immediately before the immobilization procedure. The reference cell was activated and deactivated in the same manner but without immobilization in order to minimize non-specific binding. Final immobilization levels ranged between 3500-4200 resonance units (RU). In cases of saturated binding curves, stoichiometries were evaluated by comparing observed maximal RU signal (R_{max}) to the calculated R_{max} = ($MW_{compound}/MW_{myoc\text{-}OLF}$) x immobilization level (RU) x stoichiometric ratio, where MW is the molecular mass.

Compounds were prepared as 10 mM stock solutions in neat DMSO, then diluted to 500 µM in PBS (final concentration of DMSO = 5%) or in PBS with 0.005% surfactant P-20 (2%

DMSO), and again to 100 µM in the same buffer. The 100 µM stock was serially diluted to 6.25 µM for subsequent binding experiments. Different concentrations of each compound (0-100 µM) were injected over the reference and myoc-OLF flow cells at a flow rate of 20 µL/min with a 120 s contact time, 600 s dissociation time, and 300 s stabilization period. Experiments were run in at least duplicate at 25 °C with 5% DMSO in PBS running buffer. No surface regeneration strategies were employed. Data obtained in the reference flowcell was subtracted from that obtained in the myoc-OLF cell. These responses were corrected for DMSO effects by use of solvent calibration curves generated at the beginning and end of each run. BIAcore evaluation software version 1.0 (GE Healthcare) using the steady-state model was employed for data analysis and determination of the K_d where applicable.

Compound synthesis. Compounds were either purchased (GW5074, Sigma Aldrich; 7,8-dihydroxyflavone (A1), TCI chemicals, or synthesized as follows. All reactions were carried out in pre-dried glassware from the oven and any additional moisture was removed by flame-drying the reaction vessel under vacuum. Each reaction proceeded under a nitrogen atmosphere with anhydrous solvents, unless stated otherwise. Aboslute ethanol was used. All other reagents were purchased from Acros, Sigma-Aldrich, Fluka, VWR, Merck, Alfa Aesar, TCI and Strem (for metal catalysts) and used without further purification. Chromatographic purification was performed as flash chromatography with Dynamic Adsorbents silica gel (32-65µm) and solvents indicated as eluent with 0.1-0.5 bar pressure. For quantitative flash chromatography, technical grades solvents were utilized. Analytical thin-layer chromatography (TLC) was performed on EMD silica gel 60 F_{254} TLC glass plates. Visualization was accomplished with UV light, aqueous basic potassium permanganate ($KMnO_4$) solution, iodine, aqueous acidic dinitrophenylhydrazine (DNP) solution, aqueous acidic *p*-anisaldehyde (PAA) solution, and an ethanol solution of phosphomolybdic acid (PMA) followed by heating. Melting points were determined by and Electrothermal Mel-Temp. Each yield refers to an isolated, analytically-pure material. Infrared (IR) spectra were obtained using a Shimadzu IRPrestige21 FTIR instrument. The IR bands are characterized as weak (w), medium (m), and strong (s). Proton and carbon nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR) were recorded on a Varian Mercury Vx 300 MHz spectrometer, Varian Mercury Vx 400 MHz spectrometer, Bruker 400 MHz spectrometer, or Bruker 500 MHz spectrometer with solvent resonances as the internal standard (^{1}H NMR: $CDCl_3$ at 7.26 ppm or DMSO-d_6 at 2.50 ppm; ^{13}C NMR: $CDCl_3$ at 77.0 ppm or DMSO-d_6 at 39.50 ppm). ^{1}H NMR data are reported as follows: chemical shift (ppm), multiplicity (s = singlet, d = doublet, dd = doublet of doublets, dt = doublet of triplets, ddd = doublet of doublet of doublets, t = triplet, m = multiplet), coupling constants (Hz), and integration. Mass spectra were obtained using a VG-70SE instrument. Uncorrected melting points were measured with a digital melting point apparatus (DigiMelt MPA 160).

GW5074 [3-(3,5-dibromo-4-hydroxybenzylidene)-5-iodoindolin-2-one] (GW5074) was purchased from Sigma Aldrich (Lot #010M4606V) as a 1:1.25 mixture of E:Z isomers, apigenin was purchased from Sigma Aldrich and used without further analysis. 7,8-Dihydroxyflavone (A1) was purchased from TCI Chemicals.

*General Procedure***:** 2-Indolinone (1.0 equiv), aldehyde (1.0-1.2 equiv), piperidine (0.2-2 equiv), and absolute ethanol (10 mL) were charged to a flask equipped with a condenser and stir bar. The mixture was heated to a reflux for 16 hours. The resulting solid was filtered, washed repeatedly with low boiling petroleum ether, collected, and dried *in vacuo* overnight. Each sample was characterized, examined for purity by NMR, and compared to the literature(3).

(*Z*)-3-[4-(Dimethylamino)benzylidene]indolin-2-one (**G1**): The general procedure was followed using indolin-2-one (415 mg, 3.12 mmol), 4-(dimethylamino)benzaldehyde (541 mg, 3.63 mmol),

piperidine (517 mg, 6.07 mmol), and ethanol (15 mL). The reaction proceeded for 16 h. **^{13}C NMR** (75 MHz ,DMSO-d_6) δ ppm = 167.6, 151.7, 139.5, 138.0, 134.7, 127.0, 126.1, 122.1, 120.6, 120.3, 118.4, 111.0, 108.9. The characterization of the compound was in accordance with reported data(3).

(*E*)-3-(4-Bromobenzylidene)indolin-2-one (**G2**): The general procedure was followed using indolin-2-one (415 mg, 3.11 mmol), 4-bromobenzaldehyde (668 mg, 3.61 mmol), piperidine (517 mg, 6.07 mmol), and ethanol (15 mL). The reaction proceeded for 12 h. The characterization of the compound was in accordance with the reported data. This compound isomerizes in DMSO. The ^{1}H NMR matched the literature values for the *E* isomer. **^{13}C NMR** (101MHz,DMSO-d_6) δ = 168.5, 143.1, 134.4, 133.7, 131.8, 131.3, 130.4, 128.2, 122.9, 122.5, 121.2, 120.6, 110.2. The other characterization was in accordance with the reported data(3).

(*E*)-3-(3,4,5,-trimethoxybenzylidene)indolin-2-one (**G3**): The general procedure was followed using indolin-2-one (301 mg, 2.26 mmol), 3,4,5-trimethoxybenzaldehyde (488 mg, 2.49 mmol), piperidine (431 mg, 5.06 mmol), and ethanol (15 mL). The reaction proceeded for 12 h. The characterization of the compound was in accordance with the reported data(3).

3-(4-Methoxybenzylidene)indolin-2-one (**G4**): **[(**E:Z ratio) = 6.3:1] The general procedure was followed using indolin-2-one (499 mg, 3.75 mmol), 4-methoxybenzaldehyde (615 mg, 4.52 mmol), piperidine (34 mg, 0.405 mmol), and ethanol (10 mL). The reaction proceeded for 6.5 h. **^{13}C NMR** (75MHz, CHLOROFORM-d) δ ppm = 170.8, 168.4, 161.7, 161.0, 141.7, 139.5, 137.6, 137.5, 134.4, 131.5, 129.4, 128.2, 127.4, 127.1, 126.0, 124.0, 122.7, 122.1, 121.6, 121.6, 118.8, 114.2, 113.8, 110.2, 109.5, 55.4. The other characterization was in accordance with the reported data(3).

3-(3,5-Dibromo-4-hydroxy-benzylidene)-1,3-dihydro-indol-2-one (**G5**): Indolin-2-one (302 mg, 2.27 mmol), 3,5-dibromo-4-hydroxybenzaldehyde (636 mg, 2.27 mmol), p-toluenesulfonic acid monohydrate (51 mg, 0.269 mmol), and ethanol (15 mL) were heated to at reflux. The reaction proceeded for 16 h. [(E:Z ratio) = 1:1.1] **^{1}H NMR** (300MHz ,DMSO-d_6) δ ppm = 10.66 -10.60 (broad singlets, 3.54), 8.79 (s, 2.19), 7.90 (s, 2.00), 7.69 (s, 1.10), 7.63 (d, *J* = 7.5 Hz, 1.16), 7.51 - 7.45 (m, 2.16), 7.27 - 7.16 (m, 2.26), 6.98 (t, *J* = 7.6 Hz, 1.15), 6.92 - 6.79 (m, 3.21), 3.63 (br s, 1.63). **^{13}C NMR** (75MHz ,DMSO-d_6) δ ppm = 168.5, 167.3, 152.5, 151.9, 143.1, 140.7, 136.1, 133.9, 133.2, 133.1, 130.3, 128.9, 128.7, 128.2, 127.6, 126.1, 125.6, 124.9, 122.0, 121.2, 120.7, 119.6, 111.8, 111.1, 110.3, 109.5. These compounds were synthesized and verified with the reported characterization(3).

(*Z*)-3-(3,5-dibromo-4-methoxybenzylidene)-5-iodoindolin-2-one (**G6**): Piperidine (0.091 mmol, 7.7 mg), 3,5-dibromo-4-methoxybenzaldehyde: (0.49 mmol, 0.145 mg), 5-iodoindolin-2-one (0.45 mmol, 0.118 mg), and ethanol (10 mL) were charged to a flask with a condenser. The mixture was heated to a reflux for 16 h. The orange solid was collected and washed repeatedly with petroleum ether to afford 0.1509 g (62%) of the desired product. (**R_f** =0.73, 1% MeOH/DCM) **[(**E:Z ratio) = 1:2.8] (compound isomerizes in DMSO over time.) [**m.p.** 273-275 °C] (Z isomer NMR data) **^{1}H NMR** (500MHz ,DMSO-d_6) δ ppm = 10.83 (s, 1 H), 8.78 (s, 2 H), 8.04 (d, *J* = 1.7 Hz, 1 H), 7.85 (s, 1 H), 7.55 (dd, *J* = 1.7, 8.1 Hz, 1 H), 6.68 (d, *J* = 8.1 Hz, 1 H), 3.86 (s, 3 H). **^{13}C NMR** (126MHz ,DMSO-d_6) δ ppm = 166.5, 154.8, 140.5, 137.5, 136.2, 134.5, 132.9, 128.4, 127.1, 126.8, 117.1, 112.0, 84.2, 60.7. **IR:** 3149.8 (m), 3080.3 (w), 3024.4 (w), 2866.2 (w), 1701.2 (s), 1604.8 (s), 1525.7 (m), 1475.5 (m), 1465.9 (m), 1361.7 (m), 1273.0 (s), 1201.7 (s), 985.6 (s), 804.3 (s) cm^{-1}. **HRMS (ESI)** Calc. 532.8123 Obs. 532.8118.

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