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Synthesis and Evaluation of Esterified Hsp70 Agonists in Cellular Models of Protein Aggregation and Folding

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Abstract

Over-expression of the Hsp70 molecular chaperone prevents protein aggregation and ameliorates neurodegenerative disease phenotypes in model systems. We identified an Hsp70 activator, MAL1–271, that reduces α -synuclein aggregation in a Parkinson's Disease model. We now report that MAL1–271 directly increases the ATPase activity of a eukaryotic Hsp70. Next, twelve MAL1–271 derivatives were synthesized and examined in a refined α -synuclein aggregation model as well as in an assay that monitors maturation of a disease-causing Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) mutant, which is also linked to Hsp70 function. Compared to the control, MAL1–271 significantly increased the number of cells lacking α -synuclein inclusions and increased the steady-state levels of the CFTR mutant. We also found that a nitrile-containing MAL1–271 analog exhibited similar effects in both assays. None of the derivatives exhibited cellular toxicity at concentrations up to 100 μ M, nor were cellular stress response pathways induced. These data serve as a gateway for the continued development of a new class of Hsp70 agonists with efficacy in these and potentially other disease models.

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Graphic abstract: **Keywords**

Molecular chaperone; proteostasis; Parkinson's Disease; Cystic Fibrosis, α -synuclein, CFTR

1. Introduction

Rapidly growing cells and cells with high metabolic rates require high levels of protein synthesis to survive. The folding, post-translational modification, assembly, and transport of these newly synthesized proteins must proceed efficiently or else misfolded proteins—and consequently protein aggregates—may accumulate. Moreover, all cells are continuously bombarded with stress-inducing agents, which can also lead to protein misfolding and aggregation. To offset the cellular stresses associated with these toxic species, all cells harbor molecular chaperones which reduce the misfolded protein burden.^{1, 2} Most molecular chaperones, such as Hsp70, bind linear arrays of amino acids with overall hydrophobic character, thereby limiting access of aggregation-prone motifs to the aqueous environment in the cell.^{3–5} The binding of protein clients to Hsp70 is driven by cycles of ATP binding and hydrolysis: Hsp70 in the ATP bound conformation exhibits a high on- and off-rate for protein binding, but hydrolysis and conversion to the ADP-bound conformation favors protein binding because a C-terminal “lid” domain closes over the substrate binding pocket.⁶ The associated substrate is subsequently released by the action of a nucleotide exchange factor, which exchanges bound ADP for ATP. In turn, Hsp40 co-chaperones deliver substrates to Hsp70 and catalyze ATP hydrolysis, trapping the client in the chaperone before they are released.^{7, 8}

If a protein client is preferentially bound to Hsp70, then components of specific cellular degradation pathways can associate with the chaperone-substrate complex.⁹ However, molecular chaperones may be overwhelmed under conditions of extreme stress, as occurs after a sudden increase in temperature or protein synthesis. In this case, cellular stress response pathways are induced which increase the synthesis of Hsp70 and other chaperones. This resulting “heat shock response” buffers cells from proteotoxic stress, but prolonged growth under stressful conditions can trigger apoptosis.¹⁰ Nevertheless, some cell types thrive even when confronted with chronic stress, such as tumor cells. Tumor cells rapidly divide when challenged with an acidic pH, accumulation of reactive oxygen species, low nutrient levels, and high protein production. Not surprisingly, increased levels of Hsp70 are evident in many tumors, but genetic silencing or knock-down of the chaperone induces apoptosis.^{11–13} Thus, chemical Hsp70 inhibitors have proven effective in preventing the growth of a variety of cancer cells.^{14–16}

In contrast to the situation in tumor cells, several neurodegenerative diseases arise from an accumulation of protein aggregates. In theory, the toxicity of these aggregates should be offset by an increase in the levels of Hsp70 or other chaperones that prevent protein aggregation. Indeed, Hsp70 over-expression by genetic manipulations or after incubation with compounds that increase the heat shock response temper the toxic effects of disease-causing protein aggregates, such as the Parkinson's Disease-associated α -synuclein protein.^{17–19} In this case, compounds that directly activate the biochemical activity (but not necessarily the levels) of Hsp70 should similarly prove effective in neurodegenerative disease models.

We previously reported on the synthesis and characterization of pyrimidinone-peptoid hybrid molecules that inhibit Hsp70 ATPase activity in vitro and exhibit potent effects in a variety of disease models, including cancer.^{20–25} We also reported that substitution of a bi-phenyl substituent with a chlorinated benzene converted the most effective inhibitor, MAL3–101, into an Hsp70 activator that accelerated the ATPase activity of a bacterial Hsp70, DnaK, in the presence of an Hsp40 co-chaperone.²⁶ NMR studies uncovered the binding site on DnaK, which is adjacent to the Hsp40 binding site. To determine whether the Hsp70 activator might prove effective in a neurodegenerative disease model, we next examined the effects of the compound, known as 115–7c or MAL1–271, in cells that express an aggregation-prone variant of α -synuclein. As hypothesized, the percentage of cells displaying α -synuclein inclusions decreased after MAL1–271 treatment, and this effect was potentiated when Hsp70 synthesis was also induced after application of a stress-generating compound.²⁷ These data suggest that specifically increasing the activity of Hsp70, as well as the levels of the chaperone, might offset the catastrophic consequences of Parkinson's Disease.

In this study, we sought to improve upon the efficacy of MAL1–271 and—for the first time—develop a structure-activity relationship (SAR) for an Hsp70 activator. We therefore designed and synthesized twelve chemical analogues of MAL1–271 and measured their cellular effects in a more quantitative α -synuclein aggregation assay. We also extended our analysis to include another cellular disease model in which enhanced Hsp70 activity would prove beneficial. Our combined results demonstrate the feasibility of synthesizing and screening diverse Hsp70 agonist libraries, confirm the ameliorative effects of increased Hsp70 activity in a neurodegenerative disease model, and begin to define the chemical features required to support Hsp70 agonist activity.

2. Materials and Methods

2.1 Chemical Synthesis

The Hsp70 inhibitor, MAL3–101, and the Hsp70 activator, MAL1–271 (1, 115–7c), were prepared as previously described.^{20, 26} For the synthesis of MAL1–271 derivatives, the following procedures were used.

All glassware was dried in an oven at 140 °C for at least 2 h prior to use. All air and moisture-sensitive reactions were performed under a dry N₂ atmosphere. Reactions carried out at 0 °C employed an ice bath and reactions carried out at –78 °C employed a dry ice/

acetone bath. THF and Et₂O were distilled over sodium/benzophenone ketyl, Et₃N, CH₂Cl₂ and toluene were distilled from CaH₂. All other materials were obtained from commercial sources and used as received. Infrared spectra were obtained from neat solids or oils on ATR FT-IR spectrometers. Melting points were determined in open capillary tubes and are uncorrected. High-resolution mass spectra were obtained on a Q-TOF MS or a Thermo Scientific Exactive Orbitrap LC-MS. ¹H and ¹³C spectra were obtained at 300 MHz, 400 MHz, 500, or 600 NMR in CDCl₃ or DMSO-d₆, as indicated. ¹³C NMR spectra were obtained at 100, 125, or 150 MHz using a proton-decoupled pulse observed peak. Chemical shifts (δ) are reported in parts per million with the residual solvent peak used as an internal standard δ ¹H/¹³C (Solvent) and are tabulated as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), and coupling constant(s), number of protons. Reactions were monitored by thin layer chromatography analysis (pre-coated silica gel 60 F254 plates, 250 μm layer thickness) and visualization was accomplished with a KMnO₄ solution (1.50 g of KMnO₄, 10 g of K₂CO₃, and 1.25 mL of 10% NaOH in 200 mL of H₂O) when needed. Chromatography was performed under flash conditions using SiO₂ (40–63 μm). All final compounds passed RP-LC-MS/UV/ELSD analysis with >95% purity.

AMT-628–27 (2).—A solution of **MAL1–271** (0.600 g, 0.105 mmol) in toluene (12.6 mL) and MeOH (5 mL) was treated at 0 °C with a 2 M solution of TMSCHN₂ (1.26 mL, 2.51 mmol) in hexanes and stirred at room temperature for 2 h. The reaction mixture was evaporated to dryness under reduced pressure and the resulting oil was purified by gradient chromatography on SiO₂ (hexanes/EtOAc) to provide AMT-628–27 (2, 0.396 g, 0.799 mmol, 64%) as a colorless solid: IR 1681, 1620, 1390, 1160, 1109, 730 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.35 (d, 1 H, J = 2.0 Hz), 7.26–7.20 (m, 3 H), 7.15–6.98 (m, 4 H), 5.75 (s, 2 H), 5.07 (d, 1 H, J = 12.8 Hz), 4.95 (d, 1 H, J = 12.8 Hz), 3.96–3.85 (m, 1 H), 3.70–3.60 (m, 1 H), 3.68 (s, 3 H), 2.69 (s, 3 H), 2.39–2.22 (m, 2 H), 1.98–1.85 (m, 1 H), 1.85–1.70 (m, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 173.4, 165.2, 152.6, 151.6, 137.9, 135.8, 134.5, 133.6, 129.9, 128.5, 128.4, 127.8, 101.4, 66.0, 51.8, 50.7, 42.0, 30.9, 24.9, 16.1; HRMS [ESI+] calcd for C₂₄H₂₅N₂O₅Cl₂ (M+H) 491.1135, found 491.1135.

DWN-723–39 (3).—A solution of **MAL1–271** (0.147 g, 0.308 mmol), 4-dimethylaminopyridine (4 mg, 0.03 mmol), and cyclopropane methanol (0.03 mL, 0.03 g, 0.4 mmol) in CH₂Cl₂ (10 mL) was treated at room temperature with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.107 g, 0.555 mmol) and stirred for 14 h. The mixture was evaporated to dryness under reduced pressure. The resulting residue was dissolved in CH₂Cl₂, treated with celite (200 mg), and solvent was removed under reduced pressure. Chromatography on SiO₂ of the crude product adsorbed on celite using gradient elution (hexanes/EtOAc) provided pure DWN-723–39 (3, 0.126 g, 0.238 mmol, 77%): IR ν 1681, 1390, 1105, 696 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.36 (d, 1 H, J = 2.0 Hz), 7.30–7.00 (m, 7 H), 5.98 (d, 1 H, J = 3.2 Hz), 5.78 (d, 1 H, J = 3.2 Hz), 5.10 (d, 1 H, J = 12.4 Hz), 4.97 (d, 1 H, J = 12.4 Hz), 3.98–3.88 (m, 1 H), 3.94 (d, 2 H, J = 7.2 Hz), 3.72–3.62 (m, 1 H), 2.71 (d, 3 H, J = 0.4 Hz), 2.40–2.30 (m, 2 H), 2.03–1.90 (m, 1 H), 1.90–1.77 (m, 1 H), 1.20–1.10 (m, 1 H), 0.62–0.58 (m, 2 H), 0.32–0.28 (m, 2 H); ¹³C NMR (100 MHz, CDCl₃) 173.0, 165.1, 152.6, 151.6, 137.9, 135.8, 134.4, 133.6, 129.8, 128.5,

128.4, 128.0, 127.7, 101.3, 69.5, 65.9, 50.6, 42.0, 31.2, 24.9, 16.0, 9.8, 3.3; HRMS [ESI+] calcd for C₂₇H₂₉N₂O₅Cl₂ (M+H) 531.1448, found 531.1447.

DWN-723-38 (4).—A solution of MAL1-271 (0.150 g, 0.315 mmol) in MeOH (3 mL) was treated at room temperature with cesium carbonate (0.104 g, 0.318 mmol) and stirred for 10 min. The solution was evaporated to dryness under reduced pressure. Dry acetonitrile (2.0 mL) was added and the solution was charged with crotyl bromide (0.08 mL, 0.7 mmol), and stirred for 81 h. The mixture was then evaporated to dryness under reduced pressure. The resulting residue was dissolved in CH₂Cl₂, treated with Celite (200 mg), and solvent was removed under reduced pressure. Chromatography on SiO₂ of the crude product adsorbed on celite using gradient elution (hexanes/EtOAc) provided pure **DWN-723-38 (4)**, 0.121 g, 0.228 mmol, 72%): IR 2981, 1685, 1625, 1390, 1150 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.38 (d, 1 H, J = 2.0 Hz), 7.30–7.00 (m, 7 H), 5.90–5.70 (m, 2 H), 5.70–5.55 (m, 2 H), 5.11 (d, 1 H, J = 12.4 Hz), 4.99 (d, 1 H, J = 12.4 Hz), 4.54 (d, 2 H, J = 6.8 Hz), 4.00–3.90 (m, 1 H), 3.75–3.65 (m, 1 H), 2.72 (s, 3 H), 2.40–2.30 (m, 2 H), 2.05–1.90 (m, 1 H), 1.90–1.75 (m, 1 H), 1.74 (d, 3 H, J = 7.6 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 172.6, 165.1, 152.5, 151.6, 137.8, 135.8, 134.4, 133.6, 131.9, 129.8, 128.4, 128.0, 127.7, 124.9, 124.0, 101.3, 66.0, 65.4, 50.6, 42.0, 31.1, 24.8, 17.8, 16.0; HRMS [ESI+] calcd for C₂₇H₂₉N₂O₅Cl₂ (M+H) 531.1448, found 531.1441.

CSM-724-05 (5).—A suspension of 5-(aminocarbonylamino)pentanoic acid (1.00 g, 6.25 mmol) in THF (30 mL), 2,4-dichlorobenzaldehyde (2.80 g, 15.8 mmol) and benzyl acetoacetate (3.11 g, 15.7 mmol) was stirred for 5 min at room temperature, treated with concentrated HCl (6 drops) and stirred for 5 days. The precipitate was filtered, washed (3:1, hexanes/acetone), and dried under vacuum to yield **CSM-724-05 (5)**, 0.790 g, 1.61 mmol, 26%) as a colorless solid: Mp 181.1–182.5 °C; IR 1713, 1696, 1655, 1621, 1160, 695 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) 12.0 (bs, 1 H), 7.96 (d, 1 H, J = 3.2 Hz), 7.52 (d, 1 H, J = 2.4 Hz), 7.38–7.20 (m, 5 H), 7.01–6.99 (m, 2 H), 5.60 (d, 1 H, J = 3.2 Hz), 5.03 (d, 1 H, J = 12.8 Hz), 4.93 (d, 1 H, J = 12.4 Hz), 3.90–3.80 (m, 1 H), 3.62–3.52 (m, 1 H), 2.57 (s, 3 H), 2.24 (t, 2 H, J = 7.0 Hz), 1.75–1.40 (m, 4 H); ¹³C NMR (100 MHz, DMSO-d₆) δ 174.4, 164.9, 151.8, 151.6, 139.9, 136.3, 133.0, 132.9, 129.8, 129.1, 128.2, 127.9, 127.8, 127.5, 100.5, 65.1, 50.6, 41.9, 33.3, 28.9, 21.9, 15.7; HRMS [ESI+] calcd for C₂₄H₂₅N₂O₅Cl₂ (M+H) 491.1135, found 491.1138.

CSM-724-06 (6).—A solution of **CSM-724-05 (5)**, 1.03 g, 2.10 mmol) in MeOH (15 mL) was treated at room temperature with cesium carbonate (0.660 g, 2.03 mmol) and stirred for 10 min. The solution was evaporated to dryness under reduced pressure. Dry DMF (10.0 mL) was added and the solution was charged with iodomethane (0.128 mL, 2.04 mmol), and stirred for 6 h. The mixture was extracted with EtOAc (20 mL) and water (10 mL), and the aqueous layer was washed with EtOAc (5x). The combined organic layers were dried (Na₂SO₄) and concentrated, and the resulting oil was purified by chromatography on SiO₂ (6% MeOH/CH₂Cl₂) to provide pure **CSM-724-06 (6)**, 0.728 g, 1.44 mmol, 69%) as a light yellow solid: Mp 117.5–125.5 °C; IR 1728, 1707, 1677, 1623, 1390, 1159, 698 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ 7.96 (d, 1 H, J = 3.6 Hz), 7.52 (d, 1 H, J = 2.0 Hz), 7.35–7.32 (m, 1 H), 7.25–7.20 (m, 4 H), 7.00–6.98 (m, 2 H), 5.58 (d, 1 H, J = 3.6 Hz), 5.03 (d, 1

H, $J = 12.8$ Hz), 4.93 (d, 1 H, $J = 12.8$ Hz), 3.88–3.78 (m, 1 H), 3.60–3.50 (m, 1 H), 3.57 (s, 3 H), 2.56 (s, 3 H), 2.33 (t, 2 H, $J = 7.0$ Hz), 1.60–1.40 (m, 4 H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 173.2, 164.8, 151.7, 151.6, 139.7, 136.2, 133.0, 132.9, 129.8, 129.0, 128.2, 127.8, 127.7, 127.4, 100.4, 65.0, 51.3, 41.9, 32.8, 30.7, 28.7, 21.8, 15.6; HRMS [ESI+] calcd for $\text{C}_{25}\text{H}_{27}\text{N}_2\text{O}_5\text{Cl}_2$ (M+H) 505.1292, found 505.1277.

DWN-723–23 (7).—A solution of **MAL1–271** (0.150 g, 0.314 mmol) in MeOH (3 mL) was treated at room temperature with cesium carbonate (0.102 g, 0.314 mmol) and stirred for 10 min. The solution was evaporated to dryness under reduced pressure. Dry acetonitrile (2.0 mL) was added and the solution was charged with bromoacetonitrile (0.045 mL, 0.63 mmol), and stirred for 14 h. The mixture was then evaporated to dryness under reduced pressure. The resulting residue was dissolved in CH_2Cl_2 , treated with celite (200 mg), and solvent was removed under reduced pressure. Chromatography on SiO_2 of the crude product adsorbed on celite (95:5, $\text{CH}_2\text{Cl}_2/\text{EtOAc}$) provided pure **DWN-723–23 (7)**, 0.057 g, 0.111 mmol, 35%: IR ν 1753, 1622, 1391, 1162, 1110 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 7.39 (d, 1 H, $J = 2.0$ Hz), 7.30–7.00 (m, 7 H), 5.79 (d, 1 H, $J = 2.8$ Hz), 5.62–5.53 (m, 1 H), 5.11 (d, 1 H, $J = 12.8$ Hz), 4.99 (d, 1 H, $J = 12.8$ Hz), 4.75 (s, 2 H), 3.98–3.88 (m, 1 H), 3.78–3.68 (m, 1 H), 2.71 (s, 3 H), 2.50–2.40 (m, 2 H), 2.05–1.92 (m, 1 H), 1.92–1.80 (m, 1 H); ^{13}C NMR (100 MHz, CDCl_3) δ 171.3, 165.1, 152.5, 151.2, 137.7, 135.7, 134.6, 129.9, 128.4, 128.0, 127.7, 114.2, 101.4, 66.1, 50.7, 48.4, 41.6, 30.4, 24.6, 16.0; HRMS [ESI+] calcd for $\text{C}_{25}\text{H}_{24}\text{N}_3\text{O}_5\text{Cl}_2$ (M+H) 516.1088, found 516.1087.

DWN-723–36 (8).—A solution of **MAL1–271** (0.150 g, 0.315 mmol), 4-dimethylaminopyridine (3 mg, 0.02 mmol), and 2-methoxyethanol (0.025 mL, 0.024 g, 0.31 mmol) in CH_2Cl_2 (10 mL) was treated at room temperature with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.110 g, 0.556 mmol) and stirred for 5 h. The mixture was evaporated to dryness under reduced pressure. Chromatography on SiO_2 of the crude product using gradient elution (hexanes/EtOAc) provided pure **DWN-723–36 (8)**, 0.138 g, 0.257 mmol, 82%: IR 1681, 1391, 1162, 906 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 7.37 (d, 1 H, $J = 2.0$ Hz), 7.30–7.01 (m, 7 H), 5.91 (d, 1 H, $J = 3.2$ Hz), 5.78 (d, 1 H, $J = 3.2$ Hz), 5.10 (d, 1 H, $J = 12.8$ Hz), 4.97 (d, 1 H, $J = 12.4$ Hz), 4.30–4.22 (m, 2 H), 3.95–3.88 (m, 1 H), 3.72–3.58 (m, 1 H), 3.30 (s, 3 H), 2.61 (s, 3 H), 2.45–2.35 (m, 2 H), 2.03–1.90 (m, 1 H), 1.90–1.77 (m, 1 H); ^{13}C NMR (100 MHz, CDCl_3) δ 172.8, 165.1, 152.5, 151.6, 137.9, 135.8, 134.4, 133.6, 129.8, 128.5, 128.4, 128.0, 127.7, 101.3, 70.4, 65.9, 63.6, 59.0, 53.5, 50.6, 41.9, 31.0, 24.8, 16.0; HRMS [ESI+] calcd for $\text{C}_{26}\text{H}_{29}\text{N}_2\text{O}_6\text{Cl}_2$ (M+H) 535.1397, found 531.1398.

DWN-723–35 (9).—A solution of **MAL1–271** (0.153 g, 0.321 mmol) in MeOH (3 mL) was treated at room temperature with cesium carbonate (0.103 g, 0.316 mmol) and stirred for 10 min. The solution was evaporated to dryness under reduced pressure. Dry acetonitrile (2.0 mL) and additional cesium carbonate (0.210 g, 0.645 mmol) were added, and the solution was charged with 4-(2-chloroethyl)morpholine hydrochloride (0.124 g, 0.658 mmol), and stirred for 14 h. The mixture was then evaporated to dryness under reduced pressure. The resulting residue was dissolved in CH_2Cl_2 , the insoluble white precipitate was filtered off, and the filtrate was concentrated and resuspended (95:5, $\text{CH}_2\text{Cl}_2/\text{EtOAc}$).

Chromatography on SiO₂ using gradient elution (CH₂Cl₂/EtOAc) provided pure **DWN-723-35 (9)** (0.052 g, 0.089 mmol, 28%): IR 1700, 1621, 1391, 1162, 1112 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) 7.29 (d, 1 H, J = 2.4 Hz), 6.72–6.90 (m, 7 H), 5.69 (d, 1 H, J = 4.0 Hz), 5.59 (bs, 1 H), 5.01 (d, 1 H, J = 12.8 Hz), 4.88 (d, 1 H, J = 12.4 Hz), 4.15 (t, 2 H, J = 6.0 Hz), 3.90–3.80 (m, 1 H), 3.70–3.50 (m, 5 H), 2.62 (s, 3 H), 2.55 (t, 2 H, J = 6.0 Hz), 2.45–2.38 (m, 4 H), 2.30–2.20 (m, 2 H), 1.94–1.80 (m, 1 H), 1.80–1.65 (m, 1 H); ¹³C NMR (100 MHz, CDCl₃) 172.7, 165.1, 152.5, 151.6, 137.8, 135.7, 134.4, 133.6, 129.8, 128.5, 128.4, 128.0, 127.7, 101.3, 66.9, 66.0, 61.6, 57.1, 53.8, 50.7, 41.9, 31.1, 24.8, 16.0; HRMS [ESI+] calcd for C₂₉H₃₄N₃O₆Cl₂ (M+H) 590.1819, found 590.1820.

DWN-723-12 (10).—A solution of **MAL1-271** (0.090 g, 0.19 mmol) in MeOH (2 mL) was treated at room temperature with cesium carbonate (0.064 g, 0.20 mmol) and stirred for 10 min. The solution was evaporated to dryness under reduced pressure. Dry DMF (1.0 mL) was added and the solution was charged with benzyl bromide (0.020 mL, 0.19 mmol), and stirred for 45 h. The solution was then extracted with water (20 mL) and CH₂Cl₂ (3×20 mL). The combined organic layers were purified by chromatography on SiO₂ (1:1, hexanes/EtOAc) to provide **DWN-723-12 (10)** (0.090 g, 0.16 mmol, 85%) as a colorless solid: Mp 91.1–95.0 °C; IR 3050 (b), 1700 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) 7.30–6.90 (m, 13 H), 5.68 (d, 1 H, J = 1.5 Hz), 5.55 (d, 1 H, J = 1.5 Hz), 5.05 (s, 2 H), 5.00 (d, 1 H, J = 12.0 Hz), 4.88 (d, 1 H, J = 12.5 Hz), 3.88–3.78 (m, 1 H), 3.62–3.52 (m, 1 H), 2.56 (s, 3 H), 2.35–2.20 (m, 2 H), 1.90–1.80 (m, 1 H), 1.80–1.70 (m, 1 H); ¹³C NMR (125 MHz, CDCl₃) 172.6, 165.1, 152.5, 151.6, 137.8, 135.8, 134.4, 133.6, 129.8, 128.8, 128.7, 128.6, 128.5, 128.4, 128.0, 127.7, 127.0, 101.3, 66.5, 66.0, 50.6, 41.9, 31.2, 24.8, 16.0; HRMS [ESI+] calcd for C₃₀H₂₉N₂O₅Cl₂ (M+H) 567.1448, found 567.1452.

DWN-723-24 (11).—A solution of **MAL1-271** (0.158 g, 0.332 mmol) in MeOH (3 mL) was treated at room temperature with cesium carbonate (0.108 g, 0.329 mmol) and stirred for 10 min. The solution was evaporated to dryness under reduced pressure. Dry acetonitrile (2.0 mL) was added and the solution was charged with 2-fluorobenzyl chloride (0.080 mL, 0.66 mmol), and stirred for 14 h. The mixture was then evaporated to dryness under reduced pressure. The resulting residue was dissolved in CH₂Cl₂, treated with celite (200 mg), and solvent was removed under reduced pressure. Chromatography on SiO₂ of the crude product adsorbed on celite (97:3, CH₂Cl₂/EtOAc) provided pure **DWN-723-24 (11)** (0.074 g, 0.13 mmol, 38%) as a colorless solid: Mp 114.2–116.0 °C; IR 3337, 1739, 1702, 1676, 1235, 1160, 1106 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) 7.30–6.90 (m, 12 H), 5.69 (d, 1 H, J = 2.8 Hz), 5.45 (d, 1 H, J = 2.8 Hz), 5.12 (s, 2 H), 5.01 (d, 1 H, J = 12.8 Hz), 4.89 (d, 1 H, J = 12.4 Hz), 3.90–3.80 (m, 1 H), 3.62–3.52 (m, 1 H), 2.58 (s, 3 H), 2.35–2.25 (m, 2 H), 1.95–1.80 (m, 1 H), 1.80–1.70 (m, 1 H); ¹³C NMR (125 MHz, CDCl₃) 172.5, 165.1, 152.5, 151.6, 137.8, 137.0, 135.7, 134.5, 133.5, 130.8, 130.4, 129.8, 128.4, 128.0, 127.7, 124.2, 115.7, 115.5, 101.3, 66.0, 60.5, 50.7, 41.9, 31.1, 24.8, 16.0; HRMS [ESI+] calcd for C₃₀H₂₈N₂O₅FCl₂ (M+H) 585.1354, found 585.1356.

DWN-723-28 (12).—A solution of **MAL1-271** (0.149 g, 0.313 mmol) in MeOH (3 mL) was treated at room temperature with cesium carbonate (0.108 g, 0.331 mmol) and stirred for 10 min. The solution was evaporated to dryness under reduced pressure. Dry acetonitrile

(2.0 mL) was added and the solution was charged with additional cesium carbonate (0.209 g, 0.640 mmol) and 2-(bromomethyl) pyridine hydrobromide (0.172 g, 0.668 mmol), and stirred for 14 h. The mixture was then evaporated to dryness under reduced pressure. Chromatography on SiO₂ of the crude product (99:1, CH₂Cl₂/EtOAc) provided pure **DWN-723-28 (12)**, 0.148 g, 0.261 mmol, 84%) as a colorless solid: Mp 112.8–113.6 °C; IR ν 1700, 1389, 1106, 1076, 749 cm⁻¹; ¹H NMR (600 MHz, CDCl₃) δ 8.62 (d, 1 H, J = 4.2 Hz), 7.74–7.72 (m, 1 H), 7.37–7.34 (m, 1 H), 7.30–7.00 (m, 9 H), 5.78 (d, 1 H, J = 3.0 Hz), 5.72–5.64 (m, 1 H), 5.27 (s, 2 H), 5.09 (d, 1 H, J = 12.6 Hz), 4.97 (d, 1 H, J = 12.6 Hz), 3.98–3.90 (m, 1 H), 3.75–3.65 (m, 1 H), 2.68 (s, 3 H), 2.50–2.40 (m, 2 H), 2.05–1.95 (m, 1 H), 1.90–1.82 (m, 1 H); ¹³C NMR (151 MHz, CDCl₃) δ 172.5, 165.1, 155.4, 152.5, 151.6, 149.4, 137.8, 137.0, 135.7, 134.4, 133.5, 129.8, 128.4, 128.0, 127.7, 123.1, 122.0, 101.2, 66.9, 66.0, 50.6, 41.9, 31.0, 24.8, 16.0; HRMS [ESI+] calcd for C₂₉H₂₈N₃O₅Cl₂ (M+H) 568.1401, found 568.1401.

DWN-723-40 (13).—A solution of **MAL1-271** (0.153 g, 0.321 mmol) in MeOH (3 mL) was treated at room temperature with cesium carbonate (0.103 g, 0.316 mmol) and stirred for 10 min. The solution was evaporated to dryness under reduced pressure. Dry acetonitrile (2.0 mL) was added and the solution was charged with additional cesium carbonate (0.203 g, 0.623 mmol) and 2-(chloromethyl)quinoline hydrochloride (0.134 g, 0.622 mmol), and stirred for 14 h. The mixture was then evaporated to dryness under reduced pressure. The resulting residue was dissolved in CH₂Cl₂, treated with Celite (200 mg), and solvent was removed under reduced pressure. Chromatography on SiO₂ of the crude product adsorbed on celite using gradient elution (hexanes/EtOAc) provided pure **DWN-723-40 (13)**, 0.087 g, 0.140 mmol, 44%): IR 1681, 1161, 1108, 697 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.19 (d, 1 H, J = 8.4 Hz), 8.08 (d, 1 H, J = 8.4 Hz), 7.83 (d, 1 H, J = 8.0 Hz), 7.74–7.72 (m, 1 H), 7.58–7.55 (m, 1 H), 7.44 (d, 1 H, J = 8.8 Hz), 7.34 (d, 1 H, J = 2.0 Hz), 7.26–7.21 (m, 4 H), 7.24–7.23 (m, 1 H), 7.22–6.97 (m, 3 H), 5.76 (d, 1 H, J = 3.2 Hz), 5.69 (d, 1 H, J = 2.8 Hz), 5.42 (s, 2 H), 5.07 (d, 1 H, J = 12.4 Hz), 4.95 (d, 1 H, J = 12.4 Hz), 3.98–3.88 (m, 1 H), 3.75–3.65 (m, 1 H), 2.66 (s, 3 H), 2.55–2.42 (m, 2 H), 2.05–1.95 (m, 1 H), 1.95–1.82 (m, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 172.5, 165.1, 155.9, 152.6, 151.6, 147.6, 137.8, 137.2, 135.7, 134.5, 133.6, 130.0, 129.8, 129.1, 128.4, 128.0, 127.7, 127.6, 126.8, 119.5, 101.3, 67.5, 66.0, 50.6, 42.0, 31.1, 24.9, 16.0; HRMS [ESI+] calcd for C₃₃H₃₀N₃O₅Cl₂ (M+H) 618.1557, found 618.1553.

2.2 Biochemical Assays

The rate of ATP hydrolysis was measured using an in vitro steady state ATPase assay with modifications.^{28, 29} Yeast Hsp70 (Ssa1) was purified from recombinant yeast cells expressing a hexa-histidine-tagged form of the chaperone, as described,³⁰ and 4 pmol of Hsp70 was preincubated on ice for 5 min in 50 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM DTT, 2 mM MgCl₂ with either the DMSO vehicle or in the presence of the compound. One pmol yeast Hsp40 (Ydj1) was purified from a bacterial expression strain using DEAE and hydroxyapatite as described.³¹ Where indicated, the co-chaperone was then added and the incubation was continued for 10 min followed by a 5 min incubation at 30°C. ATP hydrolysis was initiated by the addition of 4 μ Ci of α -³²P ATP (Perkin Elmer) and 250 μ M ATP (Sigma). Aliquots of the reaction were taken at 15 min intervals for 1 h, and samples

were subjected to thin layer chromatography run in a LiCl/formic acid solution. Dried plates were imaged on a Typhoon FLA 7000 phosphorimager (GE) and results were quantified using ImageJ (<https://imagej.nih.gov/ij/>). Data represent the means of 15 independent experiments $\pm$ SD, and statistical analysis was performed using one-way ANOVA to compare percent ATP hydrolysis in the presence of Hsp40 and in the presence or absence of compound **1** to DMSO at 60 min.

ATPase activity was also measured in a single-turnover assay, in which a complex between Ssa1 and α -³²P ATP was formed and then hydrolysis was directly measured over time in the presence or absence of compounds and/or the unfolded model substrate for Hsp70, CMLA.^{32, 33}

2.3 Quantitation of α -synuclein aggregation in a mammalian cell model

Human neuroglioma (H4) cells were maintained in OptiMEM 1x with Glutamax medium (Gibco, Carlsbad, Germany) with 10% FCS and 1% penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. Twenty-four hours prior to transfection, approximately 100,000 H4 cells were plated per well in a 12-well plate (Costar, Corning, New York, USA). Six hours prior to transfection the medium was replaced with fresh one. 5 μ L of a 2.5 M CaCl₂ solution was added dropwise to 45 μ L of a solution containing equal amounts of the plasmids encoding α -synuclein (SynT) and Synphilin-1 as we previously described.³⁴ 1xHBS calcium phosphate buffer pH 7.1 (50 mM N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) pH 7.05, 280 mM NaCl, 1.5 mM Na₂PO₄ $\times$ 2 H₂O) was added dropwise to the solution and vigorously mixed. Following 20 min of incubation, the mixture was added dropwise to the cells and the plate was gently rocked.

Sixteen hours after transfection, H4 cells were fed with fresh medium and treated either with the Hsp70 agonists DWN-72–323 (compound **7**) or MAL1–271 (compound **1**) at final concentrations of 1, 10 and 100 μ M. Additionally, some plates were treated with 10 μ L of culture-grade DMSO as it was used as solvent for both Hsp70 agonists. Twenty-four hours after treatment, cells were washed with PBS and fixed with 4% PFA for 15 minutes at room temperature (RT), followed by a permeabilization step with 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) for 20 minutes at RT. After blocking in 1.5% BSA (NZYTECH in PBS for 1 h, cells were incubated with primary antibody. Primary antibodies used were: rabbit anti-ASYN (1:2000, Santa Cruz Biotechnology) and mouse anti-V5 (1:2000, Life Technologies-Invitrogen) overnight at 4 °C, followed by rinsing with PBS and incubation for 2 h at RT with the following secondary antibodies: Alexa fluor 488 donkey anti-rabbit (1:2000, Life Technologies-Invitrogen) and Alexa Fluor 555 donkey anti-mouse (1:2000, Life Technologies-Invitrogen). Finally, cells were stained with DAPI (1:5000 in PBS) for 5 min and maintained in PBS for epifluorescence microscopy.

Transfected cells were detected and scored based on the α -synuclein inclusions pattern and classified into four groups; cells without inclusions, cells with less than six inclusions, cells between six and ten inclusions and cells with more than ten inclusions. Results were expressed as the percentage of the total number of transfected cells obtained from three independent experiments. All data were obtained from three independent experiments and expressed as mean $\pm$ SEM. Two-group comparisons were performed with Student's t test

and multiple comparisons with one-way ANOVA. Differences were considered as statistically significant at $p < 0.05$. Statistical analyses were carried out with GraphPad Prism 5.

2.4 Triton X-100 solubility assay to measure α -synuclein aggregation

Human neuroglioma (H4) cells were maintained in OptiMEM 1x with Glutamax medium (Gibco, Carlsbad, Germany) with 10% FCS and 1% penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. Twenty-four hr prior to transfection, ~150,000 cells were plated per well in a 6-well plate (Costar, Corning, New York, USA). Transfection was performed by adding 10 μ L of 2.5 M CaCl₂ dropwise to 90 μ L of a solution containing equal amounts of the plasmids encoding SynT and Synphilin-1 as well as 2xHBS calcium phosphate buffer, pH 7.1, 50 mM N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid, pH 7.05, 280 mM NaCl, and 1.5 mM Na₂PO₄ $\times$ 2 H₂O, and was vigorously mixed. After 20 min of incubation, the mixture was added dropwise to the cells and the plate was gently rocked. Sixteen hr after transfection, cells were fed with fresh medium and treated either with compounds **1** and **7** at a concentration of 100 μ M. Cells treated with 10 μ L of culture-grade DMSO were used as a control.

Twenty-four hr after treatment cells were trypsinized, washed with PBS, and resuspended in PBS with protease inhibitors (Roche), and sonicated 3 times at 10% intensity for 30 sec. Next, the protein concentration was determined and Triton X-100 was added to a final concentration of 1% to solutions containing 150 μ g of protein. The Triton X-100 mixture was incubated on ice for 30 min, followed by centrifugation (15000g for 60 min at 4°C). The resulting pellet was dissolved in 40 μ L 2% SDS-containing lysis buffer and sonicated once at 10% intensity for 10 sec. After analysis on a 12% Bis-Tris polyacrylamide gel, proteins were transferred to PVDF membranes using an iBlot system (Invitrogen) and blocked with 5% BSA. Membranes were incubated overnight with 1:1000 anti-aSyn antibody (BD Transduction laboratory, New Jersey, USA) and for 2 hr with 1:6000 HRP conjugated anti-mouse IgG at (ECL GE Healthcare). Detection was performed using Immobilon Western HRP substrate and visualized with a chemiluminescence detection system (Fusion FX Vilber Loumat). Protein levels were quantified using ImageJ and the ratio insoluble aSyn/total aSyn was determined. Comparisons were performed with one-way ANOVA followed by Dunnett's test using Microsoft excel with the Total Statistics add-in.

2.5 Effects of MAL1–271 Analogs on Maturation of the F508del Cystic Fibrosis Transmembrane Conductance Regulator

HEK293 cells were grown in DMEM (Sigma) supplemented with 10% fetal bovine serum (VWR) in a humidified atmosphere at 37 °C, 5% CO₂. Cells were plated at a density of 5–6 $\times$ 10⁵ cells per well in 6 well plates. After 24 h of growth, the cells were transiently transfected with pCDNA3.1-F508del-CFTR³⁵ using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, the cells were treated with the DMSO vehicle, 10 μ M VX809 (Selleck), and/or 30 or 100 M of the examined compound. After another 24 h, the cells were lysed in 50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail (Roche) and then cleared by centrifugation at 13,000 rpm for 15 min at 4 °C in a microfuge. Cleared lysates were used to determine

protein concentrations (Pierce BCA protein assay kit) and for subsequent immunoblot analyses. Primary antibodies for immunoblot analyses included anti-CFTR (217, Cystic Fibrosis Foundation Therapeutics), anti-actin (Cell Signaling), and anti-Hsp70 (Cell Signaling). Secondary antibodies were anti-mouse or anti-rabbit HRP conjugates (Cell Signaling). Blots were developed using SuperSignalTM West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and imaged on a BioRad Chemidoc XRS.

2.6 Cell Toxicity Assays

Growth inhibition 50% values (GI_{50} s) for all compounds were determined in HEK293 and MCF7 cells by using a CellTiter-Glo[®] Luminescent Cell Viability Assay kit (Promega). Cells were grown as described above and were plated at a density of 1×10^4 cells/well in 96 well plates and allowed to grow for 5.5 h. After this pre-incubation period, 33 μ L of the DMSO vehicle, Thapsigargin (Sigma) or compounds **1–13** at final concentrations of 0.1, 1.0, 3.0, 10, 30, and 100 μ M were added and the cells were incubated for an additional 24 h. A total of 67 μ L of the assay reagent was added to each well, and the wells were incubated at room temperature for 30 min and then imaged using a BioRad Chemidoc XRS. Quantification was performed using ImageJ and data were analyzed with SigmaPlot (version 11.0) to determine GI_{50} values.

2.7 Quantification of message levels by RT-PCR

Cells were seeded into 6 well plates, and after 24 hr were treated with 10 μ M of VX809, 30 or 100 μ M of compound **1** or **7**, or an equivalent volume of DMSO for 6 hrs. After washing and collection of non-adherent cells, adherent cells were treated with TrypLE (ThermoFisher) for ~5 min, the cell fractions were combined, and TrypLE was quenched with HyClone RPMI-1640 Medium (GE Life Sciences: + 10% FBS). The cells were collected by centrifugation and each cell pellet was washed in Phosphate Buffer Saline (PBS: Biorad). Total RNA was extracted from cell pellets using the RNeasy Kit (Qiagen) with additional DNase treatment, as described in product manual. RNA concentration and purity were determined by measuring the $A_{260/280}$. To synthesize cDNA, 0.5 μ g of each sample was combined with oligo dT (Thermo Scientific: 25 ng/ μ L) and incubated at 80°C for 3 minutes. Next, 50 U of Murine Leukemia Virus reverse transcriptase (ThermoFischer), 20 Units of RNase inhibitor (Qiagen), and 2 mM deoxynucleotides were added to each sample, followed by incubation at 42°C for 1 hr and then at 92°C for 10 min, based on the RETROscript RT kit protocol (ThermoFisher). cDNAs were diluted 1:40 and PCR was carried out on a StepOnePlus with gene-specific primers and the SYBR green reagent using the following parameters: 10 min at 95°C, then 40 cycles of 15 min at 95°C and 1 min at 60°C. Fold induction was calculated using the CT with following equation: fold change = $(E^{GOI} \wedge (GOI_{cmpd} - GOI_{DMSO})) / ((E^{ACTIN} \wedge (ACTIN_{cmpd} - ACTIN_{DMSO})))$, where “E” is a factor controlling for primer efficiency, “GOI” is the target gene of interest, and “cmpd” is compound treatment.

3. Results

3.1 MAL1–271 stimulates the ATPase activity of a eukaryotic Hsp70 in the presence of Hsp40

The MAL1–271 Hsp70 agonist was previously shown to activate the ATPase activity of a bacterial Hsp70 in the presence of an Hsp40 co-chaperone, and the binding site in Hsp70 was mapped by NMR.²⁶ To verify that the compound would also activate the ATPase activity of a eukaryotic Hsp70, we assayed the rate of ATP hydrolysis under steady-state conditions in the presence of yeast Hsp70 (Ssa1) and Hsp40 (Ydj1), which are highly homologous to the human chaperones, can be isolated to greater purity, and have been used extensively to isolate compounds that proved efficacious in mammalian cells.^{20, 23, 29, 36–38} Results obtained with the yeast chaperone/co-chaperone system have invariably been recapitulated with the human homologs. As shown in Fig. 1, addition of MAL1–271 (compound **1**) potentiated the effect of Hsp40 when incubated with Hsp70. The magnitude of this effect mirrored the effect on the bacterial chaperones²⁶, and the data are consistent with the ability of MAL1–271 to suppress the formation of α -synuclein aggregates in a neuroglioma cell model.³⁴ We also note that the turnover rate measured in these experiments (0.08 min⁻¹) is consistent with published values for this chaperone³² and that none of the pyrimidinone-peptoid hybrid molecules acts on Hsp90, consistent with their specific binding to the co-chaperone binding pocket in Hsp70.²⁶

We next examined whether the compound affected the peptide-stimulated or endogenous activity of the Hsp70 ATPase. For these experiments, we performed single-turnover studies since the effects of peptide stimulation are magnified. As shown in Fig. 2, the compounds failed to impact the peptide-stimulated activity of Hsp70 ATP hydrolysis. However, the unfolded peptide, CMLA, robustly activated Hsp70 ATP hydrolysis. Thus, the effect of the agonists is specific to the Hsp40 (Ydj1)-mediated activation of the chaperone. These data are again consistent with the agonists acting at the co-chaperone binding site in Hsp70.²⁶

3.2 Synthesis of MAL1–271 analogs

The specific chemical characteristics of MAL1–271 required to suppress α -synuclein aggregation have not been explored. To this end, we began with modifications of the free acid and synthesized twelve MAL1–271 derivatives (Fig. 3). The compounds were generated in 28–85% yield by alkylation of the cesium salt of MAL1–271, by treatment of the acid with TMS-diazomethane, or by coupling of the acid with alcohols in the presence of -(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (Scheme 1). The homologous acid CSM-724–05 (**5**) was obtained in modest yield by a standard Biginelli reaction, and was converted via the cesium salt by alkylation with methyl iodide in 69% yield to the methyl ester CSM-724–06 (**6**). Cellular analyses of these derivatives will allow us to begin to develop structure-activity relationships (SAR) for further compound refinements.

3.3 A MAL1–271 analog shows efficacy in two cellular models

To screen the activity of the MAL1–271 analogs in a cellular disease model, we employed a quantitative assay in which α -synuclein aggregation could be monitored.³⁴ In contrast to other assays in which α -synuclein aggregation is monitored, the model we used (SynT/

synphillin-1) enables quantification of the percentage of cells displaying inclusions. Therefore, compounds **1–13** were initially examined in this assay at a final concentration of 100 μ M and the percentage of cells lacking (Fig. 4A) or containing 1–5 α -synuclein inclusions (Fig. 4B) was quantified relative to the DMSO control. In this preliminary screen, MAL1–271 (compound **1**) appeared to be the most efficacious, but several of other compounds (e.g., compounds **5**, **6**, and **7**) both increased the percentage of cells lacking α -synuclein inclusions and decreased the percentage of cells displaying ^{1–5} inclusions. The effect of compound **1** on aggregation is consistent with prior data demonstrating suppression of a 104 poly-Q repeat protein in yeast in the presence of this compound.³⁹

To confirm and compare the effect of compounds **1** and **7** on the amelioration of substrate aggregation, we next performed a biochemical supernatant-pellet assay with Triton X- 100-solubilized cells. As shown in Fig. 5, cells treated with MAL1–271 (compound **1**) showed a significant (~50%) decrease in Triton X-100-insoluble α -synuclein in comparison with control cells (treated with DMSO). In cells treated with compound **7**, we observed a somewhat reduced (~30%) level of reduced aggregation. Because the two compounds exhibited similar effects when cellular puncta were examined (Fig. 4), these data suggest that the nature of the aggregate/cellular inclusion might differ when cells are treated with compound **1** versus compound **7**: Both may appear as puncta visually but the relative biochemical properties (e.g., size of the aggregate or nature of associated partners) modestly differ.

The most common disease-causing mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), F508del, leads to protein misfolding and degradation in the endoplasmic reticulum (ER). However, protein folding “corrector” compounds have been FDA-approved to treat individuals who express F508del-CFTR.⁴⁰ One such compound is VX809, which stabilizes intra-domain contacts in the protein during folding.^{41, 42} Hsp70 also plays an important role during CFTR folding and as well as degradation.^{43–46} Once wild type CFTR or the corrected mutant protein completely folds in the ER, it is transported to the Golgi apparatus, resulting in a transition from the ER glycosylated form (the “B” band) to the Golgi glycosylated form (the “C” band), which can be resolved by SDS-PAGE. Therefore, we used an established immunoblot assay (see Materials and Methods) in which the levels of band B and band C can be quantified and examined the effects of MAL1–271 in the presence or absence of VX809. As shown in Fig. 6A in which equal amounts of protein were loaded (compare lanes 1 and 2), VX809 increased the amounts of band B and C F508del-CFTR, as anticipated. We also found that MAL1–271 at a final concentration of 30 μ M (compound **1**, compare “B” band, lanes 1 and 5) increased the amount of band B (~2.1 fold) to an even greater extent than VX809 (~1.5 fold; lane 2), perhaps reflecting the fact that a portion of the immature protein (i.e., band B) had folded into a proteasome-resistant state, yet there was no increase in band C. Because the complete folding and trafficking of CFTR requires the concerted action of many chaperones,⁴⁷ and we have only targeted the activity of Hsp70, we believe that F508del-CFTR has only partially been rescued, which increases the amount of band B without a concomitant increase in band C. Combined addition of compound **1** and VX809 (lane 6) also increased the amount of band C relative to the DMSO control (lane 1). In contrast, an Hsp70 inhibitor, MAL3–101, had only a modest

effect on band B and inhibited conversion of F508del-CFTR to the C band when combined with VX809. These data suggest the potential for Hsp70 agonist efficacy in Cystic Fibrosis and provide us with an independent assay to monitor the effects of Hsp70 agonists in a second disease model.

Based on these results, we next assayed compounds **1–13** in triplicate at 30 and 100 μ M in the F508del-CFTR maturation assay (Fig. 6B). The amount of band B was quantified and a range of effects were observed. Notably, compound **7**, which was one of the three most effective derivatives in the α -synuclein assay (Fig. 4), was the most effective new compound at increasing the levels of band B at 30 μ M and to a lesser extent at 100 μ M. Specifically, compound **7** increased band B levels by $\sim$ 1.7-fold. Based on these results, we re-assayed α -synuclein aggregation in the presence or absence of compounds **1** and **7** in at least 60 cells that were treated for 24 h. As shown in Fig. 7, in cells expressing α -synuclein, the number of inclusions was lower when the cells were treated with the compounds in comparison to vehicle (DMSO). When this experiment was repeated 3 times, the quantified data confirmed that compounds **1** and **7** decrease α -synuclein aggregation and indicate that compound **1** is somewhat more potent than **7**. This result is consistent with their relative activities in the F508del-CFTR assay (Fig. 6).

Finally, we examined whether the compounds are toxic. While Hsp70 inhibitors, such as MAL3–101, are cytotoxic—especially in cancer cell lines—enhanced Hsp70 activity is predicted to be less toxic and even protective.⁴⁸ Preliminary data support this hypothesis. Indeed, neither MAL1–271 (compound **1**) nor its analogs exhibited GI₅₀ values that were <100 μ M (Table 1). Some toxicity was noted at this highest concentration, which may explain the reduced effect at 100 μ M compared to 30 μ M when compound **7** was examined (Fig. 6B). In addition, no loss of cell viability was noted in the α -synuclein aggregation assays (data not shown); these data are also consistent with the lack of induction of the heat shock response or the unfolded protein response as measured by the relative levels of Hsp70 or an Hsp40 (Fig. 8) or the ER luminal Hsp70 homolog, BiP (data not shown), in cells treated with 30 μ M of compound **7** and 100 μ M of compound **1**. Although further optimization of the medicinal chemical properties of pyrimidinone-peptoid Hsp70 agonists is imperative, and further examination of the potential toxicities of these compounds is in order, our results warrant additional development and SAR analyses of this new compound class.

4. Discussion and Conclusions

In this paper, we report the first attempt to develop an SAR for an Hsp70 activator, MAL1–271, which showed efficacy in a cellular model for Parkinson's Disease. To this end, 12 derivatives of the activator were synthesized, a more quantitative and refined α -synuclein aggregation assay was employed, and a new Hsp70-dependent assay was developed in which the stability of the disease-causing F508del-CFTR protein was monitored. We discovered that a derivative containing a nitrile instead of a free acid at the end of a flexible hydrocarbon chain (compound **7**) was almost equally as active as MAL1–271 in these assays, indicating that further modification of this substituent is in order. Interestingly, we also note that select esters can be converted to the free acid in the time frame of hours, and

not minutes (data not shown). Therefore, we suggest that the cell based assays to indeed reflect the activity of the parent esters, and not the rate of hydrolysis to the acid, and that the binding site has a preference for polar substituents at that site, as reflected by the modeling studies.

We had previously established that a 2,4-dichlorophenyl moiety was optimal for agonistic activity at the 4-position of the pyrimidinone. It is noteworthy, however, that the cyanomethyl ester **7** shows activity levels similar to the acid **1**, potentially suggesting that **7** was a prodrug of **1** that was cleaved in the assay. However, LC-MS studies of the stability of **7** under assay conditions demonstrated a lack of conversion and thus disputed this hypothesis. The cyanomethyl ester moiety in **7** is expected to convey superior druglike properties vs the carboxylate in **1**, in particular with regard to its biological absorption potential.⁴⁹ The presence of the polar nitrile group in the side chain of **7** also suggest the importance of a hydrogen bond acceptor at that position. In support of this SAR hypothesis, hydrophobic esters such as **2**, **3**, **4**, **8**, **10**, and **11**, as well as the under physiological conditions positively charged pyridine **12** and morpholine **9**, were generally ineffective. The moderate activity of a weakly basic, potential hydrogen acceptor, quinoline **13**, is also in agreement with the side chain H-acceptor SAR hypothesis. Extension of the carbon linker chain from 3 methylene groups (acid **1** and methyl ester **2**) to 4 (acid **5** and methyl ester **6**) also diminished efficacy while maintaining the relatively higher potency of the free acid, suggesting a need for a precise positioning of the putative hydrogen bond acceptor group in the side chain binding site. Figure 9 shows docking models for compounds **1** and **7** that are in agreement with this binding hypothesis. While the flexible side chain folds toward different residues that interact with carboxylate (in **1**) and cyanomethyl ester (in **7**), both polar end groups interact with polar side chains (E218 and R155) in Hsp70. The remainder of the two ligands is nested in an identical pose into the J-domain binding cleft, and the dichlorobenzene substituent makes contact with key residue R171. The urea substructures in the heterocycles are mainly solvent exposed. The slightly different binding modes in **1** and **7** will support the design of next-generation analogs that can take advantage of the opportunity to further tailor the side chain interactions with both binding pockets defined by E218, F217, and R155, possibly through branching points or heterocyclic substituents, and thus enhance affinity.

In the immediate future, our goals will also include the replacement of substituents at other sites of the pyrimidinone, in particular the benzyl ester moiety and the C-6 methyl group. Our longer term goals are to further develop this novel class of Hsp70 agonists and establish pre-clinical candidates that can be optimized to treat diseases in which increased Hsp70 activity would prove beneficial.

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Scheme 1.

Overview of the synthesis of MAL1-271 analogs.

Figure 1.

An esterified pyrimidone increases Hsp40-stimulated Hsp70 ATP hydrolysis. The relative activity of Hsp70 in the presence of the DMSO vehicle, Hsp40 and DMSO vehicle, or Hsp40 and 100 M MAL1-271 compound **1** was measured in a steady state ATPase assay. Statistical analysis was performed by comparing results from 15 independent reactions with one-way ANOVA; * indicates $p < 0.01$.

Figure 2. Hsp70 agonists do affect neither the endogenous nor peptide-stimulated turnover of ATP.

Single-turnover ATPase assays were performed as described in the Materials and Methods with the yeast Hsp70, Ssa1, and in the presence or absence of X M compound and/or Y M CMLA, a permanently unfolded peptide substrate for Hsp70.

Figure 3. Structures of an Hsp70 agonist, MAL1-271 (1), and newly synthesized derivatives (2-13).

Figure 4.

Effect of the compounds on α -synuclein inclusion formation. Cells expressing α -synuclein were treated for 24 h in the presence of 100 μ M of the indicated compound or the DMSO vehicle and cells with either (A) 0 or (B) 1–5 inclusions were quantified. Data represent the average of two experiments and are presented as a percentage of the total number of cells $\pm$ the range of the data. The number of cells examined under each condition ranged from 41–125.

Figure 5.

Biochemical evaluation of the compounds on α -synuclein aggregation. Cells expressing α -synuclein were treated with compound 1 (grey) or compound 7 (orange) at a final concentration of 100 μ M or DMSO (blue) and lysed in a Triton X-100-containing buffer. The amount of α -synuclein was detected by immunoblot analysis and quantified. Data are standardized to the amount of insoluble material in DMSO-treated cells, represent the means from three independent experiments, and are expressed as mean value $\pm$ SD. * denotes $P < 0.05$.

Figure 6.

Analysis of putative Hsp70 agonists on the levels of band B and band C F508del-CFTR. (A) MAL1–271 (**1**) increases the steady state protein levels of immature F508del-CFTR. HEK293 cells transiently transfected with a F508del-CFTR expression vector were treated with a final concentration of 30 μ M of either MAL3–101 (an Hsp70 antagonist) or MAL1–271 (**1**) in the presence or absence of 10 μ M VX809. Protein extracts were quantified to ensure equal loading and a total of 25 μ g of cleared lysate was examined by SDS-PAGE using 5% polyacrylamide gels. The protein concentrations in each samples were determined to ensure equal loading in this experiment. Blots were probed with anti-CFTR antibody. Data represent the average of 5–10 independent experiments, $\pm$ SD, and $p < 0.0001$ for the comparison between DMSO (lane 1) and compound **1** in the presence or absence of VX809

(lanes 5 and 6, respectively). Errors were calculated using Graphpad t-test calculator (2018 Graphpad software). The position of immature (band B) CFTR is indicated. (B) Compound **7** increases the steady state protein levels of band B F508del-CFTR. HEK293 cells transiently transfected with an F508del-CFTR expression vector were treated with 30 or 100 M of compounds **2** – **13** as indicated. Cleared lysates from 3 independent experiments were examined as described above, and the means of the data are provided relative to the DMSO control. Actin serves as a loading control in this panel. Standard deviations for this experiment were 0.02 – 0.30; p = .004 for the comparison between DMSO and compound **7** in the presence of 30 or 100 M compound. Statistical analyses were calculated as described in part A.

Figure 7.

Effect of compounds **1** (MAL1-271) and **7** on α -synuclein inclusion formation. (A) Cells were treated with 100 M (compound **1**) and 100 M (compound **7**) for 24 h and were imaged in a Leica DMI 6000B microscope. Arrows denote select aggregates. (B) The number of cells lacking (top) inclusions and containing 1–5 inclusions (bottom) was calculated based on the averages of 3 independent experiments, $\pm$ SD, in which 60–90 cells were imaged. *denotes $P < 0.002$ and ** denotes $P < 0.02$.

Figure 8.

Neither compounds 1 nor 7 activate a heat shock response. The levels of the HSPA1A (Hsp70, open bars) and DNAJB1 (Hsp40, shaded bars) message levels were obtained by RT-PCR relative to 18S RNA as a reference control after cells were treated for 24 hr with the indicated compound. Data represent the means of four independent experiments $\pm$ SD.

Figure 9.

Docking studies with **1** (MAL1-271) and **7** (UPMCLD40ADWN07232) on the crystal structure of human Hsp70 NBD (green) in the ADP-, Mg ion-, and K ion-bound state (pdb code 3AY950) using the MCule, Inc., docking algorithm.⁵¹ Only the poses with the highest docking scores are shown. **Panel A:** Compound **1** docked into the J domain interaction site cleft next to R171 (olive colored). Residues making contact with **1** are colored in orange. **Panel B:** Residue interaction map for **1**. R171 and I216 contact the dichlorobenzene and phenyl moieties, respectively, in **1**. The urea segment of the pyrimidinone core is solvent exposed, and the ester, methyl-enamide, and propyl chain are nested against T177, N174, and V219. The carboxylic acid group is juxtaposed to F217 and E218. **Panel C:** Compound **7** docked into the J domain interaction site cleft next to R171 (olive colored). Residues making contact with **1** are colored in orange. **Panel D:** Residue interaction map for **7**. R171 and Q376 contact the dichlorobenzene and phenyl moieties, respectively, in **7**. The urea

segment of the pyrimidinone core is solvent exposed, and the benzyl ester, methyl-enamide, and propyl chain are nested against I173, N174, F217, and V219. The cyanomethyl ester group makes contacts with E218 and R155.

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Table 1.

Compounds **1–13** lack toxicity. GI₅₀ values were calculated for HEK293 and MCF7 cells treated for 24 h with a range of concentrations of Thapsigargin or compounds **1–13**. The percent viable cells at 100 μ M of compound was calculated relative to the DMSO control. Data were quantified using SigmaPlot (version 11.0).

Compound	HEK293H GI ₅₀	HEK293H % viable at 100 μ M (relative to DMSO)	MCF7 GI ₅₀	MCF7 % viable at 100 μ M (relative to DMSO)
1	>300 μ M	87	>300 μ M	110
2	>300 μ M	82	>300 μ M	90
3	>100 μ M	75	>100 μ M	85
4	>100 μ M	70	>100 μ M	83
5	>100 μ M	109	>100 μ M	113
6	>100 μ M	81	>100 μ M	82
7	>100 μ M	69	>100 μ M	98
8	>100 μ M	65	>100 μ M	89
9	>100 μ M	56	>100 μ M	72
10	>100 μ M	85	>100 μ M	94
11	>100 μ M	108	>100 μ M	106
12	>100 μ M	73	>100 μ M	60
13	>100 μ M	93	>100 μ M	99
Thapsigargin	24 μ M	0	5 μ M	0