

Small Molecule Modulators of Endogenous and Co-chaperone-stimulated Hsp70 ATPase Activity*

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The molecular chaperone and cytoprotective activities of the Hsp70 and Hsp40 chaperones represent therapeutic targets for human diseases such as cancer and those that arise from defects in protein folding; however, very few Hsp70 and no Hsp40 modulators have been described. Using an assay for ATP hydrolysis, we identified and screened small molecules with structural similarity to 15-deoxyspergualin and NSC 630668-R/1 for their effects on endogenous and Hsp40-stimulated Hsp70 ATPase activity. Several of these compounds modulated Hsp70 ATPase activity, consistent with the action of NSC 630668-R/1 observed previously (Fewell, S. W., Day, B. W., and Brodsky, J. L. (2001) *J. Biol. Chem.* 276, 910–914). In contrast, three compounds inhibited the ability of Hsp40 to stimulate Hsp70 ATPase activity but did not affect the endogenous activity of Hsp70. Two of these agents also compromised the Hsp70/Hsp40-mediated post-translational translocation of a secreted pre-protein *in vitro*. Together, these data indicate the potential for continued screening of small molecule Hsp70 effectors and that specific modulators of Hsp70-Hsp40 interaction can be obtained, potentially for future therapeutic use.

The constitutively expressed and stress-inducible 70-kDa heat shock proteins Hsc70 and Hsp70, respectively, are ubiquitous molecular chaperones that bind and release polypeptides in an ATP-dependent cycle. These chaperones contain three interdependent domains, which are a highly conserved 44-kDa N-terminal ATPase domain, an 18-kDa peptide binding domain, and a 10-kDa C-terminal helical lid domain (1–5). The lid domain gates the polypeptide binding pocket in an ATP-dependent fashion such that when ATP is bound in the N-terminal domain, the peptide binding channel is exposed and Hsp70s exhibit fast on and off rates for substrate binding. Transient interactions with peptide can stimulate ATP hydrolysis, triggering a conformational change in the chaperone (6, 7). This increases the affinity of Hsp70s for peptides by closing the lid domain on the peptide binding pocket and trapping bound

substrates (8). Ultimately, peptide substrates are released concomitant with the exchange of ADP for ATP.

Hsp70 function is regulated by and often dependent upon Hsp40 co-chaperones, which are defined by their homology to the DnaJ chaperone in *Escherichia coli*. Hsp40s stimulate the ATPase activity of Hsp70s and, thus, stabilize the Hsp70-peptide complex. An ~70-amino acid “J domain” defines the Hsp40 family and interacts with the ATPase domain of Hsp70 (for review, see Ref. 9). Other regions in Hsp40 chaperones might interact with Hsp70 near its peptide binding domain (10–12). Hsp40s can orchestrate substrate delivery to the peptide binding domain of Hsp70 (13–18) and have been shown to expand the diversity of Hsp70s substrates (8). Because not all Hsp40 J domains are interchangeable (19–22), Hsp40s may also dictate the specificity of Hsp70 function.

Because they are involved in protein folding and quality control, Hsp70 and Hsp40 co-chaperone functions represent a therapeutic target for human diseases caused by protein folding defects, such as cystic fibrosis (23, 24). The folding and subsequent trafficking beyond the endoplasmic reticulum (ER)¹ of an unstable, mutated form of the cystic fibrosis transmembrane conductance regulator (Δ F508-CFTR) is enhanced in cells exposed to chemical chaperones (e.g. glycerol, trimethylamine oxide (TMAO), inositol, taurine, or sorbitol (25–27), or compounds that modulate Hsp70 activity or levels (e.g. 15-deoxyspergualin or phenylbutyrate (28, 29)). Combined with modulators that enhance the activity of Δ F508-CFTR at the plasma membrane (30), it is hoped that analogous compounds might ultimately ameliorate the adverse phenotypes of this disease.

Hsp70 and Hsp40 also contribute to tumorigenesis (31, 32). Several unique tumor types exhibit elevated expression of these chaperones (33–36), and overexpression of Hsp70 alone can lead to cellular transformation (37) and tumorigenesis (38). Consistent with these observations, lowering the level of Hsp70 using antisense technology inhibits the proliferation of breast cancer cells by inducing apoptosis (39). In addition, some viral oncogenes recruit cellular Hsp70 to inactivate growth control checkpoints (40). The large T antigen (TAg) of the DNA tumor virus, simian virus 40 (SV40), contains a J domain that stimulates Hsp70 to rearrange multiprotein complexes involved in cell cycle regulation (41). Thus, modulators of Hsp70 and Hsp40 activity might also serve as anti-cancer drugs.

Three compounds that modulate the ATPase activity of Hsp70 have been described. One compound, 15-deoxyspergual-

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¹ The abbreviations used are: ER, endoplasmic reticulum; BiP, IgG heavy chain-binding protein; CFTR, cystic fibrosis transmembrane conductance regulator; DSG, 15-deoxyspergualin; R/1, NSC 630368-R/1; SV40, simian virus 40; TAg, simian virus 40 large T antigen; ppaF, prepro- α -factor.

lin (DSG), binds Hsp70 ($K_D = 4 \mu\text{M}$) and stimulates its steady-state ATPase activity by 20–40% (42, 43). DSG is currently being used in clinical trials to combat the rejection of transplanted kidneys (44) and has been shown *in vitro* to modestly facilitate the trafficking of $\Delta\text{F508-CFTR}$ to the plasma membrane (28). A second compound that bears structural similarity to DSG was identified, NSC-630668-R/1 (designated R/1), which inhibits the endogenous and Hsp40-stimulated ATPase activity of Hsp70 by 48 and 51% (45). R/1 also prevents the translocation of a pre-protein into yeast ER-derived vesicles, a process that requires cytosolic and luminal Hsp70 and Hsp40 co-chaperones (45). Finally, 3'-sulfogalactolipids bind the ATPase domain of Hsp70 (46) and inhibit the endogenous and Hsp40-stimulated steady-state ATPase cycle (47). Given the importance of Hsp70-Hsp40 function on cellular physiology, it is imperative that additional modulators with unique properties be discovered. To this end we identified and then biochemically screened structural analogs of DSG and R/1 for their effects on the endogenous and Hsp40-stimulated ATPase activity of a yeast Hsp70. We employed a sensitive assay to measure the rate of Hsp70 ATP hydrolysis in the presence or absence of the different compounds and for the first time obtained unique classes of chaperone modulators.

MATERIALS AND METHODS

Identification and Synthesis of DSG/NSC 630668-R/1 Analogs—After computational comparison of the DSG and R/1 structures (Fig. 1 (45)), analogs were identified by sub-structural analogy searches against libraries of compounds resident in the Developmental Therapeutics Program at the National Cancer Institute and in the University of Pittsburgh Center for Chemical Methodologies and Library Development.

The following compounds were obtained from the Developmental Therapeutics Program: NSC 624392, NSC 624393, NSC 624903, NSC 624904, NSC 624905, NSC 624906, NSC 624907, NSC 624908, NSC 625194, NSC 625195, NSC 625512, NSC 625513, NSC 632006, and NSC 655302. The synthesis of SC- $\alpha\alpha\delta\gamma$ was previously described (48). The ML2 and MAL3 series of compounds were prepared by Ugi and Biginelli reactions. The one-pot cyclocondensation of β -ketoesters **1**, aromatic aldehydes **2**, and urea **3** was performed in tetrahydrofuran at room temperature in the presence of catalytic amounts of HCl (49) or by heating in *N,N*-dimethylformamide to give heterocycle **4** (Fig. 2A). Although yields depended strongly on the substitution pattern and ranged from 33 to 84% for **4a–4e**, purities determined by liquid chromatography-mass spectrometry analysis (TIC MSD) were excellent and uniformly exceeded 90%. Subsequently, the *N*-1-substituted Biginelli dihydropyrimidinones **4a–4e** were subjected to a second multicomponent reaction, the Ugi condensation (50, 51). Although standard thermal conditions provided disappointing conversions in this reaction, microwave conditions using the CEM DiscoverTM Microwave reactor were more successful. A stirred solution of **4a–e** and amine in methanol was treated with aldehyde and *n*-butyl or isocyanide at room temperature. The mixture was then heated twice at 70 °C for 20–30 min in the microwave reactor. The MAL3 Ugi-Biginelli products were purified by column chromatography and analyzed by liquid chromatography-mass spectrometry. Finally, samples were dissolved in dimethyl sulfoxide (Me_2SO) and stored at 4 °C.

Structure-Activity Analysis—A computational conformational analysis was performed on R/1 using the Boltzman jump stochastic search method in Cerius² (v.4.5 Accelrys, Inc.). Because R/1 is a very flexible molecule with many rotatable bonds, we decomposed the molecular model of R/1 into fragments containing 4–7 rotatable bonds and assumed that the minimum energy structure obtained for each was a good local approximation of the structure in the corresponding region of the whole molecule. Conformational energies were computed using Merck Molecular Force Field (MMFF94) with no cutoffs. The resulting minimum energy conformer obtained for R/1 suggested that the presence of two carbamic ester moieties equally distributed between the two uracil rings (separated by six methylene groups) allows for the possibility of hydrogen bonding between the carbamic ester moieties and the hydrogen donors/acceptors from the uracil substituents and also hydrophobic interactions between the hydrocarbon chains that separate the two carbamic ester moieties from each other and from the uracil groups. These hydrophobic interactions may promote the packing of the hydro-

carbon chain into a low energy conformer.

Molecular mechanics-minimized models of the 31 test compounds were aligned with the hypothetical structure of R/1 described above using Cerius² (for example, see Fig. 2B). These aligned structures and the activity data were then used to define descriptors for the dataset, including steric, electrostatic and hydrogen donor/acceptor fields along with dipole moment and ClogP descriptors.

ATPase Measurements—Yeast Hsp70 (Ssa1p, Ydj1p, and Ssv40 large Tag were purified as described previously (22, 52–54). Assays that approximate single turnover measurements of endogenous Hsp70 ATPase activity were performed at 30 °C on pre-formed [$\alpha^{32}\text{P}$]ATP-Hsp70 complexes according to published methods (21, 45, 55, 56). Briefly, 25 μg of Hsp70 was incubated with 100 μCi of [$\alpha^{32}\text{P}$]ATP (PerkinElmer Life Sciences; 3000 Ci/mmol) on ice for 30 min in Complex buffer (100 mM Hepes-KOH (pH 7.5), 300 mM KCl, 80 mM magnesium acetate) and 25 μM ATP in a final volume of 100 μl (Step 1, Fig. 3A). After this incubation, the [$\alpha^{32}\text{P}$]ATP-Hsp70 complex was purified from free [$\alpha^{32}\text{P}$]ATP on a NICK G-50 column (Amersham Biosciences) at 4 °C (Step 2, Fig. 3A). Glycerol was added to a final concentration of 10%, and 25- μg aliquots ($\sim 0.6 \mu\text{M}$ Hsp70) were frozen in liquid nitrogen and stored at -80°C no longer than 3 weeks. To assay ATP hydrolysis, individual aliquots were rapidly thawed and added to an equal volume of Complex buffer containing test compounds or Me_2SO pre-equilibrated to 30 °C (Step 3, Fig. 3A). The final concentration of Hsp70 was $\sim 0.2\text{--}0.3 \mu\text{M}$ in these reactions. At the specified time points, 6- μl aliquots were removed and added to 2 μl of stop solution (2 M LiCl, 4 M formic acid, 36 mM ATP) on ice to quench ATP hydrolysis. Aliquots of each reaction time point were spotted onto thin layer chromatography (TLC) plates to determine the percentage of ATP hydrolyzed to ADP-P_i (21, 45). Data were obtained using different preparations of Hsp70. ATP turnover rates were calculated by using Kaleidagraph software (version 3.0.4) to fit the data to the single exponential equation,

$$\% \text{ Product} = A \times (1 - \exp(-kt)) + C \quad (\text{Eq. 1})$$

where the sum of the amplitude (*A*) represents the % of enzyme active sites that hydrolyze ATP to ADP-P_i, *k* is the rate of ATP hydrolysis in s^{-1} , *t* is time in seconds, and the constant term (*C*) estimates the % of enzyme active sites that hydrolyze ATP to ADP-P_i at time 0 of the assay but also takes into account any contaminating ADP from the purchased isotope. Because ATP hydrolysis is rate-limiting for Hsp70s (7), this experiment provides an approximation of the first-order rate constant for ATP hydrolysis.

In assays to which co-chaperones were added, 1 μg of Tag or 0.5 μg of Ydj1p (final concentrations of $\sim 0.2 \mu\text{M}$) was incubated with the [$\alpha^{32}\text{P}$]ATP-Hsp70 complex for 60 s at 30 °C before the addition of drug or Me_2SO unless indicated otherwise. ATP hydrolysis rates were calculated using the equation described above from data obtained at the 60-s time point and beyond. However, it should be noted that because Ydj1p has been shown to increase the dissociation of ATP from purified ATP-Hsp70 complexes by ~ 10 -fold (55), a decrease in the amount of ATP hydrolysis in these reactions could result from inhibition of γ -phosphate cleavage and/or ATP rebinding.

Tag ATPase Assays—Steady-state measurements of Tag ATPase activity were performed at 30 °C in 20- μl reactions containing 1 μg of Tag (0.56 μM) and 0.1 μCi of [$\alpha^{32}\text{P}$]ATP in 25 mM Hepes (pH 7.0), 5 mM MgCl_2 , 0.1 mM EDTA, 0.05% Nonidet P-40, 1 mM dithiothreitol, 25 μM ATP, and 2.5% (v/v) Me_2SO or 0.3 mM MAL3-90 or MAL3-101. Aliquots were quenched and analyzed as described above for Hsp70 ATPase assays. Purified T antigen mutant 5031 was a kind gift from J. Pipas (University of Pittsburgh).

In Vitro Translocation Assays—The synthesis and translocation of wild type and an unglycosylated form of yeast prepro- α -factor (ppaF and $\Delta\text{G-ppaF}$, respectively) into yeast ER-derived vesicles were performed using published methods (45, 57). Urea-denatured wild type substrate was synthesized and isolated as described (58). Translocation reactions were preincubated with the indicated compounds or Me_2SO for 10 min on ice before addition of an ATP-regenerating system. Translocation efficiency was determined by calculating the percent of trypsin-resistant, signal sequence-cleaved (and for wild type, glycosylated) ppaF relative to the total amount of ppaF and ppaF in reactions lacking trypsin. In Me_2SO -containing control reactions, 20–30% of the input ppaF is typically converted to ppaF. The relative translocation efficiencies were determined by dividing the translocation efficiency of the MAL3-39- and MAL3-101-containing reactions by the efficiency in the Me_2SO -containing control reactions.

TABLE I
Effects of DSG and R/1 analogs on the rate of Hsp70 ATP hydrolysis

ATPase assays were carried out as described under "Materials and Methods" with the indicated concentrations (0.1, 0.3, and 0.6 mM) of each compound. The rates of ATP hydrolysis were calculated using the formula $\% \text{ product} = A \times (1 - \exp(-kt)) + C$, where the sum of the amplitude (A) represents the percentage of enzyme sites that hydrolyze ATP, k is the rate constant in s^{-1} , t is time in seconds, and the constant term (C) estimates the percentage of enzyme sites that hydrolyze ATP at time 0. Rates shown are $\times 10^3$ and are given as the percent ATP hydrolyzed to ADP-P_i/s from at least two independent experiments $\pm$ the range or S.D. ND, not determined. The relative "Fold change" in the calculated rate of ATP hydrolysis in the presence of each compound compared to the rate of ATP hydrolysis in solvent (Me₂SO) is indicated.

Drug	0.1 mM		0.3 mM		0.6 mM	
	Rate ATP hydrolysis	-Fold change	Rate ATP hydrolysis	-Fold change	Rate ATP hydrolysis	-Fold change
Me ₂ SO	2.0 $\pm$ 0.1					
R/1			11.6 $\pm$ 1.0	5.8		
624392	ND	ND	1.6 $\pm$ 0.1	-1.3	1.4 $\pm$ 0.2	-1.4
642393	ND	ND	1.2 $\pm$ 0.3	-1.7	ND	ND
624903	ND	ND	0.9 $\pm$ 0.3	-2.2	ND	ND
624904	ND	ND	1.4 $\pm$ 0.1	-1.4	ND	ND
624905	ND	ND	1.7 $\pm$ 0.2	1.2	ND	ND
624906	ND	ND	1.1 $\pm$ 0.2	-1.8	1.8 $\pm$ 0.7	-1.1
624907	ND	ND	2.4 $\pm$ 0.5	1.2	ND	ND
624908	ND	ND	1.8 $\pm$ 0.1	-1.1	ND	ND
625194	2.0 $\pm$ 0.2	1	3.6 $\pm$ 0.2	1.8	2.7 $\pm$ 0.4	1.4
625195	6.1 $\pm$ 0.5	3	3.4 $\pm$ 0.3	1.7	2.5 $\pm$ 0.3	1.3
625512	2.3 $\pm$ 0.2	1.2	6.2 $\pm$ 0.7	3.1	6.0 $\pm$ 0.5	3
625513	2.2 $\pm$ 0.2	1.1	3.9 $\pm$ 0.3	2	4.9 $\pm$ 0.8	2.5
632006	ND	ND	2.8 $\pm$ 0.3	1.4	1.0 $\pm$ 0.2	2
655302	ND	ND	3.8 $\pm$ 0.5	1.9	1.5 $\pm$ 0.3	-1.3
aad9	ND	ND	1.2 $\pm$ 0.2	-1.7	ND	ND
ML2-193	3.2 $\pm$ 0.4	1.6	6.2 $\pm$ 0.5	3.1	4.5 $\pm$ 0.5	2.3
ML2-194	2.5 $\pm$ 0.3	1.3	4.1 $\pm$ 0.7	2.1	2.7 $\pm$ 0.1	1.4
ML2-214	1.8 $\pm$ 0.2	-1.1	3.4 $\pm$ 0.5	1.7	1.6 $\pm$ 0.3	1.3
ML2-215	2.3 $\pm$ 0.1	1.2	4.3 $\pm$ 0.4	2.2	4.2 $\pm$ 1.4	2.1
MAL3-38	3.5 $\pm$ 0.2	1.8	6.1 $\pm$ 0.8	3.1	4.1 $\pm$ 0.6	2
MAL3-39	ND	ND	2.1 $\pm$ 0.4	1	1.4 $\pm$ 0.7	-1.4
MAL3-40	3.5 $\pm$ 0.3	1.8	5.4 $\pm$ 0.5	2.7	6.0 $\pm$ 0.7	3
MAL3-51	ND	ND	1.7 $\pm$ 0.3	-1.2	1.6 $\pm$ 0.2	-1.3
MAL3-53	3.7 $\pm$ 0.2	1.9	4.9 $\pm$ 0.6	2.5	9.1 $\pm$ 1.0	4.6
MAL3-54	ND	ND	2.2 $\pm$ 0.3	1.1	2.5 $\pm$ 0.2	1.3
MAL3-55	1.3 $\pm$ 0.1	-1.5	2.8 $\pm$ 0.2	1.4	5.6 $\pm$ 0.9	2.8
MAL3-87	3.8 $\pm$ 0.3	1.9	4.5 $\pm$ 0.6	2.3	5.2 $\pm$ 0.7	2.6
MAL3-88	2.9 $\pm$ 0.3	1.5	3.1 $\pm$ 0.4	1.3	4.7 $\pm$ 0.7	2.4
MAL3-90	4.1 $\pm$ 0.6	2	10.3 $\pm$ 1.7	5.2	13.3 $\pm$ 1.6	6.7
MAL3-91	3.6 $\pm$ 0.3	1.8	5.8 $\pm$ 1.2	2.9	9.9 $\pm$ 1.3	5
MAL3-101	ND	ND	2.1 $\pm$ 0.3	1	2.4 $\pm$ 0.2	1.2

RESULTS

Identification of Novel Modulators of Hsp70 ATPase Activity—Based upon their structural similarity to R/1 and DSG, we identified and obtained 31 small molecules (molecular weight range 314–1040; see Table I). Compound R/1 is comprised of two uracil (2,4-pyrimidinedione) ring systems connected by a linker containing two *N*-substituted carbamic ester moieties, each holding two hydrogen bond acceptors and one hydrogen bond donor (Fig. 1). In addition, the substitutions at position 5 on the uracil rings also contain three hydrogen bond acceptors and one donor. Therefore, we sought compounds that would follow similar but non-identical ring, linker, and H-bonding capacities. Several closely related compounds were found by sub-structural searching of the Developmental Therapeutics Program data base at the National Cancer Institute: NSC 625512, NSC 625513, NSC 625194, NSC 625195, NSC 624908, NSC 624905, NSC 624904, NSC 624393, NSC 624906, and NSC 624903. The NSC compounds selected have one or two uracil rings with substituents bearing hydrogen bond donors or acceptors. Compounds with the ML and MAL prefix were obtained from the University of Pittsburgh Center for Chemical Methodologies and Library Development and have 2-dihydropyrimidinone rings with differing substituents (see "Materials and Methods" for synthesis; Fig. 2A). These substituents contain amide links instead of carbamic ester links found in R/1. The amide systems also contain hydrogen bond donor or acceptor features. The effect of SC- $\alpha\alpha\delta$ 9, a Cdc25 phosphatase in-

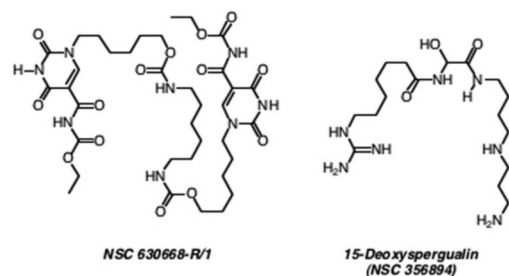


FIG. 1. Chemical structures of NSC 630668-R/1 and DSG. Structures were drawn to maximize structural similarity.

hibitor (59), was also examined, although there was no significant structural similarity with R/1 and DSG.

All 31 compounds described above were tested initially for their ability to modulate the endogenous ATPase activity of purified Ssa1p, a yeast Hsp70. Complexes of Hsp70 and [α -³²P]ATP were formed on ice and purified from free [α -³²P]ATP by gel filtration at 4 °C. The resulting [α -³²P]ATP-Hsp70 complexes were then incubated at 30 °C in the presence of the desired compound or solvent (Me₂SO), and the conversion of Hsp70-bound ATP to ADP-P_i was monitored by thin layer chromatography (Fig. 3A). Because the radiolabeled ATP-Hsp70 complexes were purified from unbound nucleotide, this procedure estimates the rate of ATP hydrolysis and is more specific and sensitive than steady-state experiments that measure the full cycle of ATP binding, hydrolysis, and nucleo-

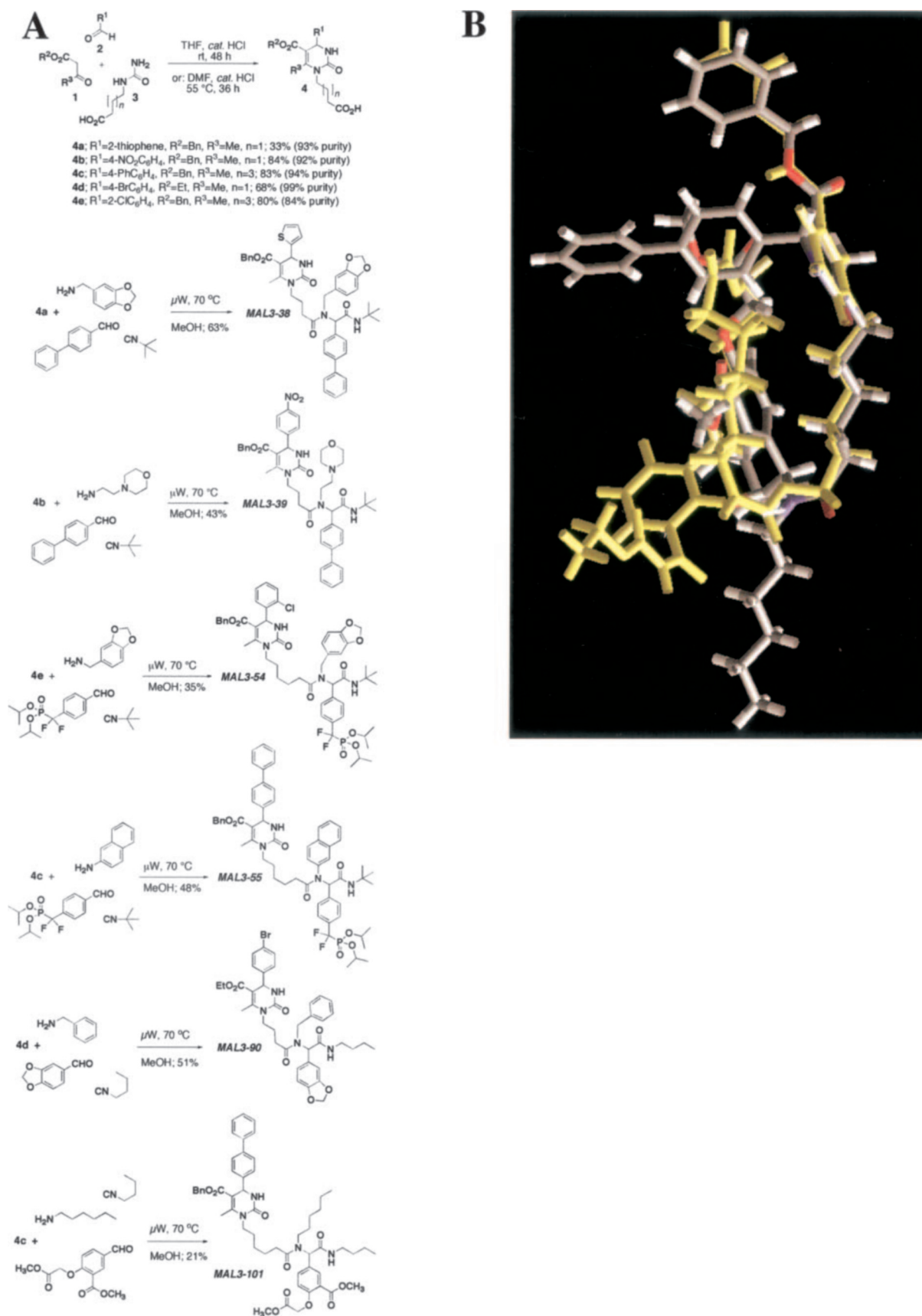


FIG. 2. **Synthesis of MAL3 compounds.** A, the first reaction depicts the synthesis of Biginelli dihydropyrimidinones, which were then subjected to Ugi condensation. The subsequent synthesis and structures of MAL3-38, MAL3-39, MAL3-54, MAL3-55, MAL3-90, and MAL3-101 are shown (see “Materials and Methods”). THF, tetrahydrofuran; DMF, *N,N*-dimethylformamide; cat., catalyst; rt, room temperature. B, molecular models of NSC 630668-R/1 (yellow) and MAL3-101 (atom-specific colors) are shown. Minimum energy conformations were calculated with MMFF94 in Cerius² (see “Materials and Methods”). Superimposition was performed by atom type matching and flexible fitting of MAL3-101 to a rigidly held model of NSC 630668-R/1, with subsequent molecular mechanics minimization of the MAL3-101 model. μW , microwave.

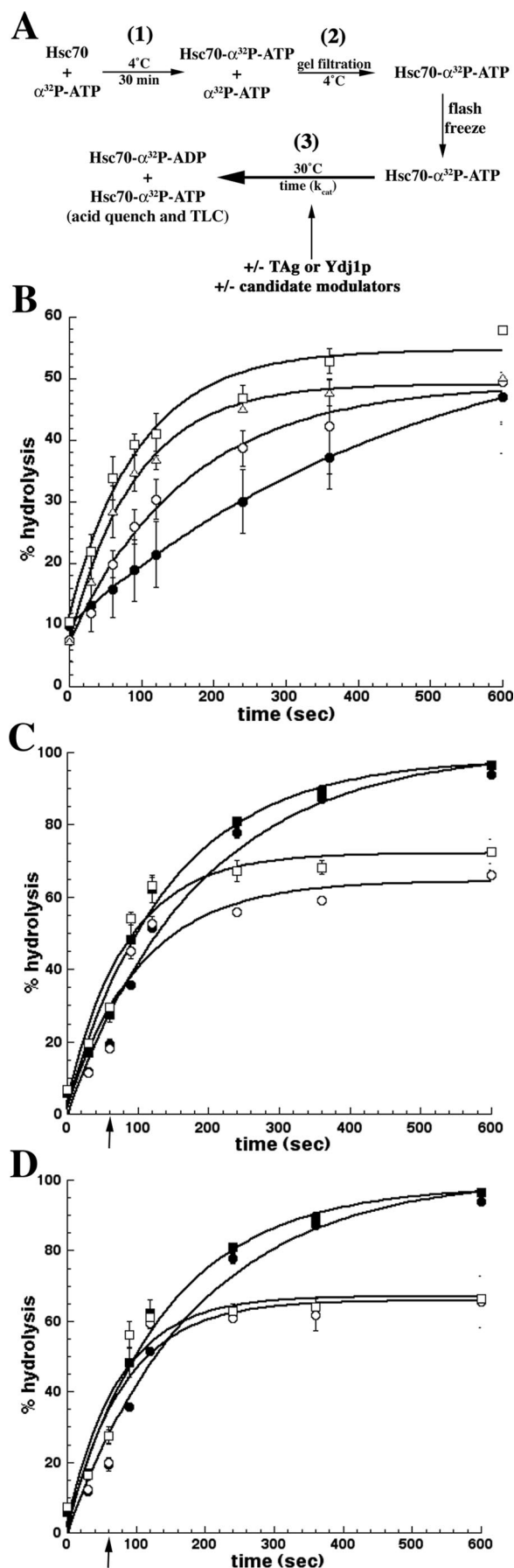


FIG. 3. MAL3-38 and MAL3-90 enhance the endogenous but inhibit the Hsp40-stimulated ATPase activity of Hsp70. A, outline of ATP turnover procedure, including 1) formation of the $[\alpha^{32}\text{P}]$ ATP-

tide exchange; however, the reader is referred to other studies in which these parameters were measured (see for example Refs. 7, 55, and 60). A similar procedure has been employed to examine the effect of cellular Hsp70 modulators (Hsp40s, GrpE-like nucleotide exchange factors, and peptide substrates) on the ATPase cycle of bacterial and yeast Hsp70s (55, 56, 61). In the absence of cellular modulators, ATP remains stably bound to Hsp70 after purification with an apparent half-life of 32–48 min with respect to ATP dissociation (55). In agreement with this published value, the apparent half-life of our Hsp70-ATP complex was ~ 44 min with respect to ATP dissociation (data not shown). Thus, this experiment approximates single turnover conditions and provides an estimate of the rate constant for ATP hydrolysis.

As described previously, R/1 induces a 5–6-fold increase in the ATP turnover rate of Hsp70 compared with the solvent control when present at a final concentration of 0.3 mM (Ref. 45; Table I; Fig. 3B). Of the 31 additional compounds tested, 7 increased the rate of Hsp70 ATP hydrolysis by ≥ 2.5 -fold at 0.3 mM (Table I). For example, MAL3-38 and MAL3-90 enhanced the ATP turnover rate by ~ 3 - and 5-fold, respectively, and like R/1, the rate of ATP hydrolysis slowed after 4 min in the presence of either compound (Table I, Fig. 3B). Some compounds, notably MAL3-55, exhibited concentration-dependent effects on Hsc70-mediated ATP hydrolysis in this range. Most of the compounds tested had little (< 2 -fold) or no effect on ATP hydrolysis by Hsp70 (Table I).

MAL3-39 and MAL3-101 Specifically Inhibit J-chaperone-stimulated Hsp70 ATPase Activity—TAG and Ydj1p harbor J domains that stimulate Hsp70 steady-state ATP hydrolysis by 6–8-fold when present at an equimolar concentration with Hsp70 (22, 52, 62). To examine the abilities of purified TAG and Ydj1p to enhance the hydrolysis of ATP by Hsp70, equimolar amounts of Ydj1p and TAG were incubated with approximately equimolar amounts of ATP-Hsp70 complex, and the extent of ATP hydrolysis was quantified over time. As shown in Fig. 3, C–D (closed symbols), Hsp70 ATPase activity was enhanced > 6 -fold by both proteins (compare with the closed circles in Fig. 3B and the Me₂SO controls in Tables I and II). Under similar conditions, the rate of ATP hydrolysis by Ssc1p, a yeast mitochondrial Hsp70, was stimulated ~ 3.5 -fold by Mdj1p (63). Importantly, nearly 100% of the Hsp70-bound ATP was hydrolyzed in the presence of Ydj1p or TAG within 6 min (Fig. 3, C–D), indicating that the purified yeast Hsp70 was fully competent for Hsp40-mediated activation.

Hsp70 complex, 2) purification of $[\alpha^{32}\text{P}]$ ATP-Hsp70 complex from free $[\alpha^{32}\text{P}]$ ATP and 3) measurement of ATP hydrolysis (see “Materials and Methods”). Because ATP turnover is rate-limiting for Hsp70, this assay approximates single turnover conditions. Therefore, the experimental rate observed estimates the rate constant for ATP hydrolysis (see “Materials and Methods” for exception). B, measurements of Hsp70 ATPase activity were performed in reactions containing equal volumes of Me₂SO (filled circles), MAL3-38 (open circles), MAL3-90 (open squares), and R/1 (open triangles). MAL3-38, MAL3-90 and R/1 were added to a final concentration of 0.3 mM. The first time point ($t = 0$) represents the first measurement of ATP hydrolysis, and the 4–8% of the ATP hydrolyzed at this point results from both background hydrolysis and the minimal ATP turnover by Hsp70 that occurs during purification of the $[\alpha^{32}\text{P}]$ ATP-Hsp70 complex. C, the ability of equimolar amounts (~ 0.2 μM) of TAG (circles) or Ydj1p (squares) to stimulate Hsp70 ATPase activity was assessed in the presence of MAL3-38 (open symbols) or Me₂SO (closed symbols). In this experiment the drug was added 60 s (arrow) after the Hsp40 homologue was added to the reaction. Although data were fit in this graph at $t = 0$, rates for the reactions in the presence and absence of MAL3-38 or Me₂SO (Table II) were calculated using data starting at 60 s (arrow). D, same as C except that MAL3-90 (open symbols) or Me₂SO (closed symbols) was added to TAG (circles) or Ydj1p (squares). All data represent the means of three independent experiments $\pm$ S.D., where the final concentration of Hsp70 was ~ 0.3 μM .

TABLE II
Effect of DSG and R/1 analogs on *T* antigen-stimulated
Hsp70 ATP hydrolysis

ATPase reactions supplemented with TAG were incubated for 60 s before the addition of each compound to a final concentration of 0.3 mM. Shown are the rates of ATP hydrolysis calculated from the 60-s time points using Equation 1 and determined from at least two independent experiments $\pm$ the range or S.D., and the relative “Fold change” of each compound compared to the rate of ATP hydrolysis in the presence of Me₂SO is indicated. Rates shown are $\times 10^3$ and are given as % ATP hydrolyzed to ADP/s.

Drug	Rate ATP hydrolysis	-Fold change
Me ₂ SO	12.6 $\pm$ 0.3	
R/1	8.3 $\pm$ 0.8	-1.5
MAL3-38	25.5 $\pm$ 8.7	2
MAL3-39	4.5 $\pm$ 1.2	-2.8
MAL3-40	18.9 $\pm$ 9.4	1.5
MAL3-51	11.1 $\pm$ 0.2	-1.1
MAL3-53	15.5 $\pm$ 3.1	1.2
MAL3-54	2.5 $\pm$ 1.7	-5.1
MAL3-55	6.8 $\pm$ 1.6	-1.8
MAL3-87	22.8 $\pm$ 9.4	1.8
MAL3-88	19.0 $\pm$ 5.6	1.5
MAL3-90	52.7 $\pm$ 14.3	4.2
MAL3-91	18.0 $\pm$ 6.0	1.4
MAL3-101	3.1 $\pm$ 0.3	-4.0

We next repeated the Hsp70 ATPase assay with TAG but added each of the indicated compounds 60 s after the reaction began. These compounds had distinct effects on the observed rate of TAG-stimulated ATP hydrolysis with five compounds altering the rate by ≥ 2 -fold (Table II). For example, MAL3-38 and MAL3-90 increased the initial rate of TAG-stimulated ATP turnover by 2-fold ($p = 0.02$) and ~ 4 -fold ($p = 0.03$), respectively, but reduced the amplitude of the reaction in the presence of TAG; this effect, also observed when Ydj1p was examined (Table II and Fig. 3, C–D), could result from inhibition of γ -phosphate cleavage and/or rebinding of dissociated ATP, as described above. MAL3-51 had no effect on either the endogenous or the TAG-stimulated rate of ATP hydrolysis, and MAL3-53 significantly affected the endogenous Hsp70 ATPase activity (~ 2.5 -fold) but had little impact on co-chaperone-mediated stimulation (Table I and II). Three compounds, MAL3-39, 3-54, and 3-101, selectively inhibited the ability of TAG to enhance Hsp70-ATP hydrolysis but did not significantly affect the endogenous rate of ATP turnover (Tables I and II). Interestingly, MAL3-39 and MAL3-101 inhibited TAG-stimulated Hsp70 ATPase activity by 2.8-fold ($p = 0.004$) and 4-fold ($p < 0.001$), respectively (Table II and Fig. 4, A and B); however, they slightly augmented Ydj1p-mediated stimulation of Hsp70 ATPase activity (Fig. 4C), suggesting that some compounds exhibit unique effects on the abilities of different Hsp40 homologues to activate Hsp70 ATPase activity. Although it is premature to speculate on the mechanisms by which these DSG and R/1 analogs affect Hsp70 and Hsp40 function (also see “Discussion”), it is striking that different classes of small molecule, chaperone modulators were uncovered from this relatively small scale analysis.

The Inhibitory Effect of MAL3-101 Is Concentration-dependent—We were particularly intrigued by the action of MAL3-39 and MAL3-101 because they did not affect endogenous Hsp70 activity. Instead, they compromised TAG J domain-dependent stimulation by 2.8 and 4.0-fold, respectively (Table I and II) but had no effect on Ydj1p-dependent stimulation (Fig. 4C). These results suggested that the compounds might block the ability of a specific Hsp40 co-chaperone to interact with and/or stimulate Hsp70. In principle, then, the ability of the compound to block interaction should be overcome by increasing the concentration of the co-chaperone, and the ability of the co-chaperone to stimulate Hsp70 ATPase activity should be lessened by in-

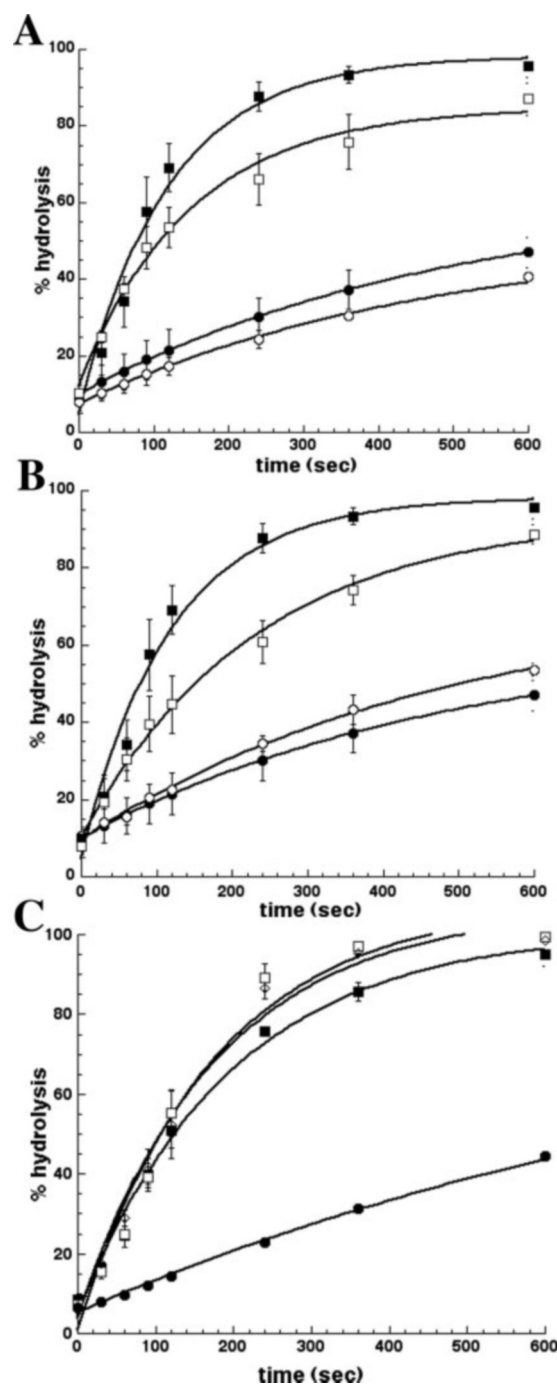


FIG. 4. MAL3-39 and MAL3-101 inhibit the ability of TAG to continually stimulate Hsp70 ATPase activity. A, the hydrolysis of Hsp70-bound ATP was measured in the presence of Me₂SO (filled circles), 0.3 mM MAL3-39 (open circles), TAG and Me₂SO (filled squares), or TAG and 0.3 mM MAL3-39 (open squares). B, reactions containing Me₂SO (filled circles) and TAG and Me₂SO (filled squares) are the same as in A. The effects of 0.3 mM MAL3-101 on endogenous (open circles), and TAG-stimulated (open squares) Hsp70 ATPase activity was also assessed. Data represent the means of three independent experiments $\pm$ S.D. C, reactions measuring Hsp70 ATPase activity contained Me₂SO (filled circles), Ydj1p and Me₂SO (filled squares), Ydj1p and 0.3 mM MAL3-39 (open squares), or MAL3-101 (open diamonds). Data represent the means of duplicate reactions. The final concentration of [³²P]ATP-Hsp70 in all assays was $\sim 0.3 \mu\text{M}$.

creasing concentrations of the compound. We, therefore, titrated the levels of MAL3-101 and TAG with respect to one another in the ATP turnover assays. When examined at lower concentrations (0.1 and 0.03 mM) MAL3-101 had little or no effect on TAG ability to stimulate Hsp70 ATPase activity (Fig.

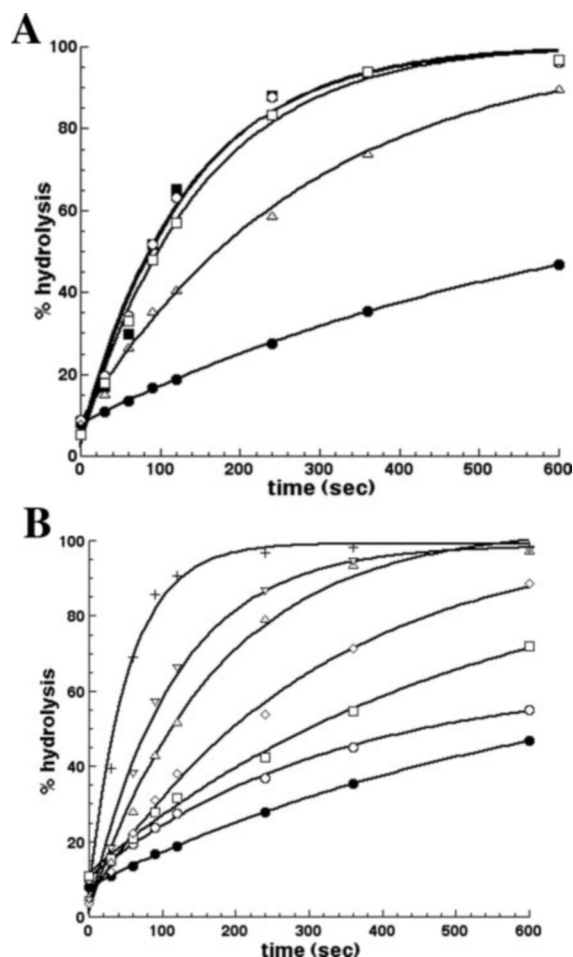


FIG. 5. Concentration dependence of MAL3-101 effect on TAG-stimulated Hsp70 ATPase activity. A, Hsp70 ATPase activity was assayed in the presence of Me_2SO (filled circles), $0.2 \mu\text{M}$ TAG and Me_2SO (filled squares), or $0.2 \mu\text{M}$ TAG with increasing concentrations of MAL3-101 (open circles, 0.03 mM ; open squares, 0.1 mM ; open triangles, 0.3 mM). B, the concentration of TAG was titrated in the presence of 0.3 mM MAL3-101. Open circles, $0.02 \mu\text{M}$ TAG; open squares, $0.05 \mu\text{M}$ TAG; open diamonds, $0.11 \mu\text{M}$ TAG; open triangles, $0.22 \mu\text{M}$ TAG; open, inverted triangles, $0.67 \mu\text{M}$ TAG; crosses, $1.3 \mu\text{M}$ TAG. The endogenous ATPase activity of Hsp70 in Me_2SO is shown as in A for comparison (filled circles). The final concentration of $[^{32}\text{P}]\text{ATP}$ -Hsp70 in all assays was $\sim 0.3 \mu\text{M}$.

5A), and as anticipated, increasing the concentration of TAG could overcome the effect of MAL3-101-mediated inhibition (Fig. 5B).

MAL3-90 and MAL3-101 Do Not Affect the ATPase Activity of TAG—To assess the specificity of these compounds for their effects on the Hsp70 ATPase cycle and to address the possibility that the observed dose-dependent inhibition of TAG co-chaperone function by MAL3-101 resulted from the inactivation of TAG rather than a direct effect on Hsp70, we conducted steady-state measurements of TAG ATPase activity. TAG is a multifunctional protein that orchestrates many aspects of SV40 viral infection through its numerous biochemical activities, several of which are regulated by ATP binding (Fig. 6A; Ref. 66). For example, the ability of TAG to bind and hydrolyze ATP is required for its function as a DNA helicase during viral DNA replication (67). Thus, if MAL3-101 interacts with the ATPase domain of Hsp70, it might also have an affinity for the TAG ATPase domain. However, we found that the rate of ATP turnover by TAG was similar in the absence (7.6 pmol of ATP hydrolyzed/min) and presence (8.7 pmol of ATP hydrolyzed/min) of MAL3-101 at 0.3 mM (Fig. 6B). In addition, the presence of MAL3-90, a compound that increased the endogenous rate of

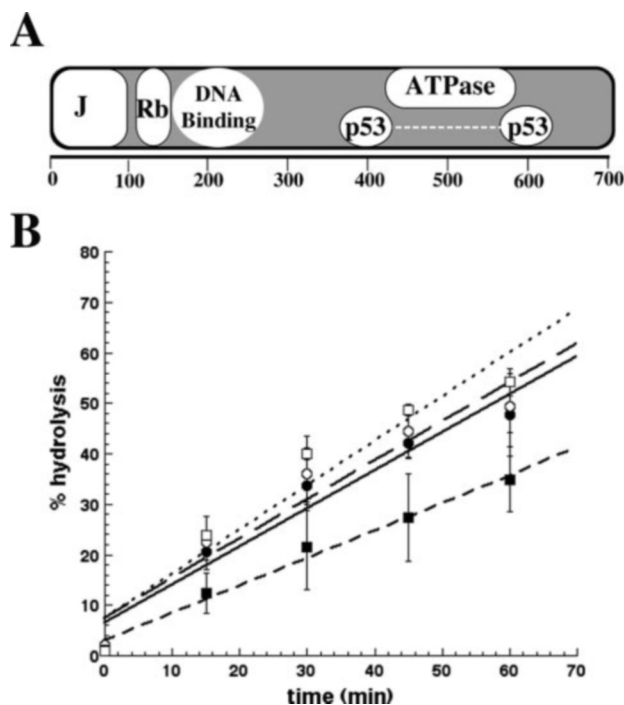


FIG. 6. MAL3-90 and MAL3-101 do not alter the rate of TAG ATPase activity. A, map of SV40 large TAG illustrating select functional domains (adapted from Srinivasan *et al.* (62)). B, steady-state measurements of TAG ATPase activity were conducted in the presence of 0.3 mM MAL3-90 (open squares), MAL3-101 (open circles), or an equal volume of Me_2SO (closed circles). The ATPase activity of TAG mutant 5031 (closed squares, D402N, V404M, V413M) was also measured. The final concentrations of wild type and mutant TAGs was $\sim 0.56 \mu\text{M}$.

Hsp70 ATPase activity by 10-fold and the TAG-stimulated ATPase activity by 4-fold (Table I–II; Fig. 3B), had no effect on TAG ATPase activity (7.8 pmol of ATP hydrolyzed/min) (Fig. 6B). In contrast, TAG mutant 5031, which harbors three point mutations within its ATPase domain (D402N, V404M, V413M), exhibited an $\sim 30\%$ slower rate of ATP hydrolysis (5.5 pmol of ATP hydrolyzed/min) than the wild type protein, which is consistent with previously published results (68). Taken together, these data indicate that MAL3-101 and MAL3-90 do not affect the endogenous ATPase activity of TAG.

MAL3-39 and MAL3-101 Inhibit Post-translational Translocation *In Vitro*—The post-translational translocation of secreted, signal sequence-containing pre-proteins into the yeast ER requires interaction between the cytosolic Hsp70-Hsp40 co-chaperones Ssa1p and Ydj1p, which maintain pre-proteins in a soluble, translocation-competent state, and the interaction between the luminal Hsp70/Hsp40 chaperones BiP and Sec63p, which ratchet or pull pre-proteins through the translocation pore (for review, see Ref. 69). We previously found that R1 inhibits the post-translational translocation of a pre-protein into ER-derived microsomes *in vitro* (45), but because this compound affects both innate and Hsp40-stimulated ATPase activity (Table I and II), we cannot discern the mechanism of inhibition. MAL3-39 and MAL3-101, on the other hand, inhibit only TAG-mediated stimulation of Hsp70 ATP hydrolysis. We, therefore, assessed the impact of these compounds on the translocation efficiency of the yeast pre-pheromone, pre-pro- α -factor (pp α F), into microsomes and found that MAL3-39 and MAL3-101 inhibited the translocation of pp α F by ~ 45 and 30% at 0.3 mM , respectively (Fig. 7, A and B). Intriguingly, MAL3-39 did not affect the ability of Ydj1p to promote release of a peptide substrate by Ssa1p² and, as mentioned previously, only

² S. W. Fewell and J. L. Brodsky, unpublished results.

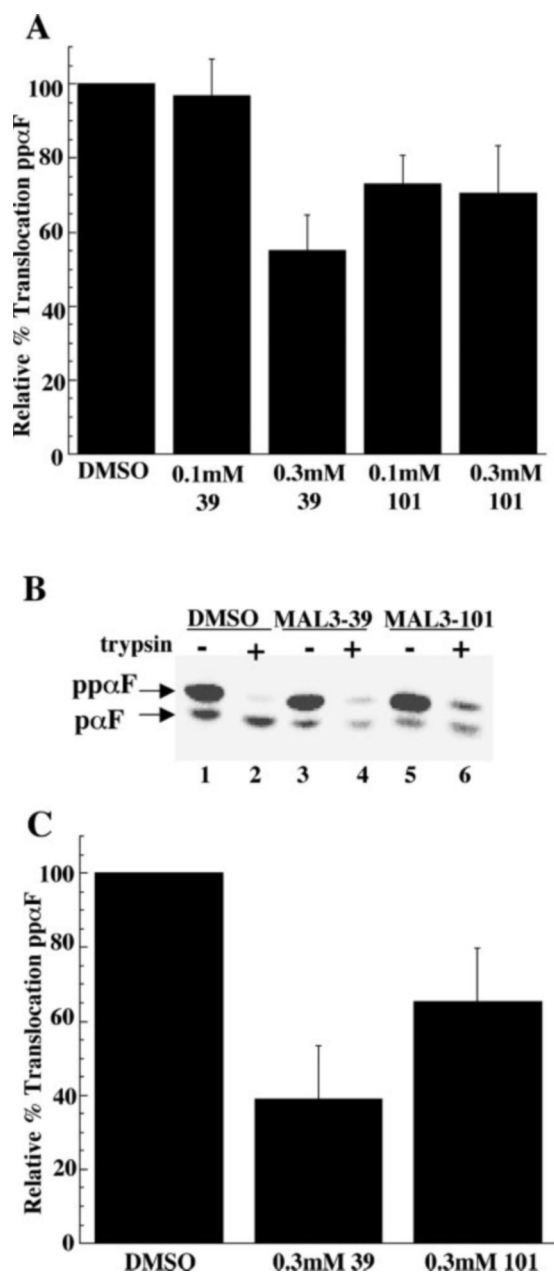


FIG. 7. MAL3-39 and MAL3-101 reduce the efficiency of post-translational protein translocation into yeast ER-derived microsomes. **A**, translocation reactions with *in vitro* translated ^{35}S -labeled ppαF were performed in the presence of Me_2SO (DMSO), MAL3-39, or MAL3-101 at the indicated concentrations. **B**, after the translocation reaction, one-half of the assay was precipitated with trichloroacetic acid (lanes 1, 3, and 5), whereas the other half was treated with trypsin before trichloroacetic acid precipitation (lanes 2, 4, and 6). Translocation efficiency was determined as described under "Materials and Methods." **C**, translocation reactions were performed as in **A** and **B**, except that the radiolabeled, wild type ppαF was denatured in 8 M urea and purified before incubation in the translocation reactions.

slightly increased the Ydj1p-stimulated rate of Ssa1p ATP hydrolysis (Fig. 4C), suggesting that the inhibition of post-translational translocation might be mediated through modulation of BiP function.

The requirement for cytosolic chaperones during *in vitro* post-translational translocation can be obviated if the ppαF is first denatured in 8 M urea and purified free of cytosolic chaperones (70, 71). We reasoned that if MAL3-39 and MAL3-101 modulate the interaction between BiP and Sec63p, then these compounds should inhibit the translocation of urea-denatured,

purified ppαF. Indeed, the translocation efficiency was reduced by ~66% the presence of 0.3 mM MAL3-39 and 55% in the presence of 0.3 mM MAL3-101 (Fig. 7C) (by comparison, translocation efficiency is reduced by 40–60% in microsomes obtained from BiP (*kar2*) mutants and by 90% in *sec63* mutants (72)). This result implies that MAL3-39 and MAL3-101 traversed the microsomal membrane and may have inhibited the ability of BiP and Sec63p to mediate protein translocation.

DISCUSSION

The ubiquitous Hsp70 and Hsp40 families of molecular chaperones have been implicated in a growing number of essential, cellular processes, including protein folding, synthesis, transport, and degradation. Members of these families also activate multiprotein enzymatic processes such as DNA replication, transcription, cell division, and apoptosis. As might be anticipated then, Hsp70 and Hsp40 chaperone function has been linked to a large number of human diseases, including cancer, cystic fibrosis, heart disease, and the neurodegenerative syndromes that arise from the accumulation of protein aggregates in the cell. Although potent inhibitors of the Hsp90 chaperones have been known for some time and are currently used clinically (73), very few Hsp70 modulators have been identified.

To this end, we sought novel modulators of Hsp70 function. ATP turnover experiments were employed to screen DSG and R/1 structural analogs for their influence on the endogenous and Hsp40-stimulated rate of Hsp70 ATP hydrolysis. Based on the previously described impact of DSG and R/1 on Hsp70 ATP hydrolysis (43, 45, 74), it was anticipated that additional compounds could be found with similar effects. As presented in Table I, we identified 10 agents that enhance Hsp70 ATPase activity by >2-fold. More intriguing was the identification of three compounds that had little impact on the endogenous rate of ATP hydrolysis but reduced the ability of a J domain-containing protein to stimulate Hsp70 ATPase activity. Two of these compounds, MAL3-39 and MAL3-101, also inhibited post-translational translocation *in vitro*, a known Hsp40-Hsp70-dependent phenomenon, and the extent of inhibition was magnified when a denatured pre-protein was used to eliminate the requirement for cytosolic chaperones. From these data, we suggest that MAL3-39 and MAL3-101 represent a new class of membrane-permeable compounds that can be used to modulate Hsp70-Hsp40-dependent cellular processes and/or to determine whether a cellular process is chaperone-dependent.

As a first step toward elucidating the mechanism of action of these compounds, we are currently attempting to map the interaction domain on Hsp70 and possibly Hsp40 to which the compounds bind. We envision several possibilities for the mechanism by which these modulators may alter Hsp70 activity. First, the analogs might bind to the conserved C-terminal EEVD motif in Hsp70. The EEVD motif is implicated in the regulation of Hsp70 ATPase activity, substrate binding, and Hsp40 interaction (75), and DSG can be cross-linked to the EEVD motif in bovine Hsc70 (76). Second, some of the modulators might recognize a binding site for a 3'-sulfogalactolipid, which was recently mapped to the ATPase domain of Hsp70 (46). Incubation of 3'-sulfogalactolipids or novel water-soluble 3'-sulfogalactolipids analogs with purified Hsp70 inhibited endogenous and Hdj1p-stimulated steady-state Hsp70 ATPase activity; in addition, the affinity between the lipid and Hsp70 was estimated to be between 100 and 300 μM (47). Third, because Ydj1p was previously shown to destabilize the Ssa1p-ATP complex (55), it is possible that the compounds that inhibit Hsp40-mediated stimulation of Hsp70 do so by altering the re-association of Hsp40-released ATP and/or by inhibiting Hsp70-mediated ATP hydrolysis. However, we do not favor this hypothesis because MAL3-38 and MAL3-90 accelerated the

endogenous rate of ATP hydrolysis and, thus, did directly impact catalysis in the absence of Hsp40. In addition, MAL3-39 and MAL3-101 selectively inhibited Tag- and not Ydj1p-mediated stimulation of Hsp70 and do not appear to generally inhibit ATP re-association. Finally, existing genetic and biochemical data suggest that Hsp40s bind to both the ATPase and peptide binding domains of Hsp70s (see the Introduction), and therefore, some of our modulators may block access to either of these sites. It is intriguing to speculate that the ability of MAL3-39 and MAL3-101 to selectively inhibit Tag- and not Ydj1p-mediated stimulation of Hsp70 could result from as yet uncharacterized differences in the interactions of these two Hsp40 with the yeast Hsp70. Future studies will explore these possibilities.

Our identification of unique classes of modulators suggests that distinct mechanisms of action may exist. This is consistent with our inability to obtain quantitative structure-activity relationships for our entire dataset (see "Materials and Methods"). Nonetheless, our computational analyses did suggest that the length of the R/1 hydrocarbon chains that separate the two carbamic ester moieties from each other and from the uracil groups may be important for packing the hydrocarbon chain in a lowest energy state. Therefore, disruption of the any of the features that maintain packing may result in a loss of activity. For example, linker shortening resulted in a decrease in activity (*e.g.* R/1 versus NSC 625195) and replacement of the carbamic ester moieties with peptide-like bonds together with disruption of the distance between the hydrogen-bonding elements (as in the case of NSC 625513) lead to decreased potency. In the case of NSC 625512, the distance between the carbamic ester moieties is preserved, but the distance between the uracil groups is decreased by one methylene. This resulted in a less pronounced decrease in activity compared with NSC 625513, which is consistent with the hypothesis that the length of the linker separating the carbamic ester moieties is important for maximum activity.

One use for modulators of Hsp70-Hsp40 activity or innate Hsp70 activity lies in the treatment of specific viral infections and in cancer. For example, cellular Hsp70-Hsp40 facilitates hepatitis B reverse transcriptase activity and envelope protein biosynthesis (77, 78), *Papillomavirus* DNA helicase activity (79), herpes simplex virus replication initiation (80), and papovavirus-mediated transcriptional activation (81). In addition, SV40 and related human polyomaviruses, BK and JC virus, encode J domain-containing TAG proteins (82). The importance of TAG J domain function is underscored by the fact that J domain mutations in TAG that abrogate Hsp70 ATPase activation also compromise SV40 growth and TAG-mediated tumorigenesis (21, 62, 83). The distinct effects of MAL3-39 and MAL3-101 on TAG and Ydj1p and the fact that the TAG J domain is only ~30% similar to the J domains in most Hsp40s, including Ydj1p (83, 84), suggest that TAG-specific modulators can be isolated. This undertaking is vital as SV40 was introduced into the human population as a polio vaccine contaminant and because the related human polyomaviruses can be lethal in immunocompromised patients (64, 65).

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