Members of the hsc70 family of molecular chaperones are critical players in the folding and quality control of cellular proteins. Because several human diseases arise from defects in protein folding, the activity of hsc70 chaperones is a potential therapeutic target for these disorders. By using a known hsc70 modulator, 15-deoxyspergualin, as a seed, we identified a novel inhibitor of hsc70 activity. This compound, R/1, inhibits the endogenous and DnaJ-stimulated ATPase activity of hsc70 by 48 and 51%, respectively, and blocks the hsc70-mediated translocation of a preprotein into yeast endoplasmic reticulum-derived microsomal vesicles. Biochemical studies demonstrate that R/1 most likely exerts these effects by altering the oligomeric state of hsc70.

The ubiquitous hsc70 molecular chaperones bind polypeptides in an ATP-dependent cycle and facilitate diverse cellular functions including protein folding, sorting, and translocation (transport) across organelar membranes (reviewed in Ref. 1). Specifically, by binding exposed hydrophobic patches in newly translated proteins, hsc70s maintain secreted proteins in a translocation-competent state and facilitate proper protein folding by preventing aberrant interactions. hsc70s also participate in the quality control process that identifies mis-folded proteins and targets them for degradation (reviewed in Refs. 2 and 3). In addition, hsc70s interact with specific native proteins to modulate their oligomeric state or their interaction with various cofactors (reviewed in Refs. 1, 4, and 5).

Binding and release of polypeptide substrates by hsc70s is governed by the coordinated actions of their nucleotide- and substrate-binding domains. Polypeptides interact transiently with the substrate-binding domain of ATP-bound hsc70. This interaction stimulates ATP hydrolysis, which results in a conformational change in the substrate-binding domain that increases its affinity for peptide (6, 7). Concomitant with the exchange of ADP for ATP on hsc70, bound peptides are released and the cycle repeats. Members of the DnaJ family of molecular co-chaperones stimulate ATP hydrolysis and promote peptide binding, in some cases by delivering specific substrates to hsc70 (8, 9). Recent structural and genetic studies indicate that DnaJ chaperones interact with the ATPase domain at a site near or coincident with the peptide-binding domain of hsc70 (10–12).

Because they are involved in protein folding and because several human diseases arise from protein folding defects (2, 13), the modulation of hsc70 and DnaJ chaperone activity may be used to combat these disorders. In addition, cellular hsc70 is co-opted by viral oncopgenes to induce cellular transformation (14–16) and is implicated in the development of certain tumors (17). Thus, identifying novel hsc70-regulating compounds could prove beneficial.

One hsc70-interacting compound is 15-deoxyspergualin (DSG; see Fig. 1). DSG binds hsc70 ($K_D = 4 \mu M$) and stimulates its ATPase activity (18). We recently found that DSG stimulates the steady-state ATPase activity of bovine and yeast cytosolic hsc70s by 42 and 22%, respectively, but has no effect on BiP/Kar2p, an hsc70 that resides in the lumen of the yeast endoplasmic reticulum (ER) (19). In addition, DSG prevents neither the DnaJ-mediated stimulation of hsc70 ATPase activity nor the ability of hsc70 to bind (and release) peptide substrates in vitro, even though DSG is hydrophobic and resembles a peptide (19–21).

DSG is currently being examined as an immunosuppressive agent. The ability of DSG to reduce tissue rejection in transplant patients may occur through the inhibition of macrophage function and induction of cytolytic T and B cells (22). Because the immunosuppressive effects of some DSG analogs correlate with their binding affinity for hsc70 (21, 23), these activities are thought to be mediated at least in part by the ability of DSG to modulate the activity of hsc70 (18). In addition, a recent study suggests that DSG could be the prototype of a new class of drugs for the treatment of cystic fibrosis. Addition of DSG to cells expressing a mutant form of the cystic fibrosis transmembrane conductance regulator (DF508-CFTR) enhances the plasma membrane cAMP-stimulated chloride current (24). It is possible that DSG dissolves a fraction of the hsc70-DF508-CFTR complexes that are trapped in the ER. Similarly, Rubenstein and Zeitlin (25) reported that phenylbutyrate decreases the cellular concentration of hsc70-DF508-CFTR complexes, resulting in a rescue of the DF508-CFTR defect.

To identify new and potentially more reactive chemical entities with hsc70-perturbing effects, we searched the Developmental Therapeutics Program data base at the National Cancer Institute for DSG-related compounds. In this study we report the analysis of a compound with some structural similarities to DSG. This compound, NSC 630668-R/1 (designated R/1), inhibits hsc70 ATPase activity and hsc70-mediated protein translocation in vitro.

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1 The abbreviations used are: DSG, 15-deoxyspergualin; ER, endoplasmic reticulum; CFTR, cystic fibrosis transmembrane conductance regulator; PAGE, polyacrylamide gel electrophoresis; BiP, IgG heavy chain binding protein.
Inhibition of hsc70 Molecular Chaperone Activity

MATERIALS AND METHODS

Identification of DSG Analog—The structure of DSG (7-guanidinoheptanoic acid [(4-3-aminopropylamino) butylcarbamoyl]hydroxymethyl amide) and its G_{H70} activities in the 60 cell line antitumor screen performed by the Developmental Therapeutics Program at the National Cancer Institute were used as seeds to probe for compounds with similar attributes using the web-based version of the COMPARE algorithm. In activity-activity comparisons, six compounds with pairwise correlation coefficients of >0.8 were found. NSC 630668-R/1 (15-(6-[(5-ethoxy carbamylamino carboxyl)2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl]-hexoxy carbamyl amino) hexyl) carboxylic acid 6-(5-ethoxycarbamyl aminocarboxyl)2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-hexoxy carbamyl amine) was selected for biochemical analyses.

R/1 was dissolved in dimethyl sulfoxide (MeSO) at a final concentration of 12.2 mM and was stable at 4°C for at least 1 month. A 3 mM dilution of the 12.2 mM stock in MeSO was prepared before each experiment to avoid precipitation of R/1 upon addition to aqueous solvents.

ATPase Measurements—Ssa1p, BiP, and Ydj1p were purified as described previously, and ATPase measurements were performed using published methods (26–29) except that steady-state reactions containing R/1 were preincubated 15 min on ice before the addition of radiolabeled ATP. For single turnover ATPase assays, R/1 was added to buffer before the addition of ATP-Ssa1p complex, and the reaction was incubated at 30 °C for the indicated times.

In Vitro Translocation Assays—The synthesis and import (translocation) into ER-derived vesicles of an unglycosylated form of yeast prepro-α-factor (ΔGppαF) was performed essentially as described (30). In brief, translocation reactions were assembled with ΔGppαF, yeast ER-derived microsomes, and R/1 or MeSO and preincubated on ice for 30 min before the addition of an ATP-regenerating system. Import reactions were performed at 20 °C for 40 min and split; half was treated with trypsin at a final concentration of 0.2 mg/ml for 30 min on ice to determine the extent of membrane-enclosed, signal sequence-cleaved substrate. All samples were treated with trichloroacetic acid, and the precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography.

The integrity of microsomal membranes in the presence of R/1 was assessed by trypsin accessibility to BiP and translocated yeast ΔGppαF. For BiP protection experiments, mock translocation reactions were assembled with equal volumes of R/1 (to a final concentration of 300 μM), MeSO, Triton X-100 (to a final concentration of 1%) or with Buffer 88 (20 mM HEPES (pH 6.8); 150 mM KOAc; 5 mM MgOAc; 250 mM sorbitol). Following a 45-min incubation at 20 °C, translocation reactions were split, and one-half was treated with trypsin at a final concentration of 0.35 mg/ml for 60 min on ice. All samples were trichloroacetic acid-precipitated and loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) and probed with anti-BiP antibody (31) followed by horseradish peroxidase-conjugated anti-rabbit anti-serum (Amersham Pharmacia Biotech) and the SuperSignal West Chemiluminescence system from Pierce. For analysis of translocated pre-pro-α-factor, translocation reactions were performed in the absence of R/1. Subsequently, the reactions were spun at 13,000 rpm in a Sorvall Bsofuge Pico microcentrifuge for 2 min at 4 °C, washed with 1 volume of Buffer 88, and spun again as above before being resuspended in the same volume of Buffer 88. The reactions were divided into thirds, and equal volumes of R/1 (to a final concentration 300 μM), MeSO, or Triton X-100 (to a final concentration of 1%) were added. Trypsin-treated and untreated reactions were trichloroacetic acid-precipitated and loaded onto an 18% urea/SDS-polyacrylamide gel (30). After electrophoresis, the gels were fixed, dried, and analyzed by PhosphorImager analysis using the MacBas software program (version 2.4) from Fuji Photo Film Inc.

Analysis of R/1-mediated Protein Precipitation—The indicated proteins were incubated for 15 min on ice in Buffer A (50 mM HEPES (pH 7.4); 50 mM NaCl; 100 mM dithiorethiol; 2 mM MgCl₂, 50 μM ATP) with equal volumes of MeSO or R/1 (at a final concentration 300 μM) in a total volume of 30 μl. The reactions were then centrifuged at 100,000 × g (40,000 rpm) for 1 h at 4 °C in a TLS-55 Beckman rotor. Supernatant and pellet fractions were separated and analyzed by SDS-PAGE followed by silver staining, BiP and Sae1p were purified in the Brodsky laboratory (28, 32). Malate synthase, lysozyme, bovine serum albumin, and citrate synthase were purchased from Sigma.

RESULTS

R/1 Inhibits the Endogenous and J-chaperone-stimulated ATPase Activity of Ssa1p and BiP—The therapeutic and biochemical properties of DSG prompted us to search for additional DSG-like compounds that could modulate hsc70 activity. One compound, R/1 (Fig. 1), gave a pairwise correlation coefficient of 0.801 using the COMPARE algorithm (see “Materials and Methods”). A sample of R/1 was consequently obtained from the Developmental Therapeutics Program at the National Cancer Institute.

To determine whether R/1, like DSG, could modulate hsc70 function, the steady-state ATPase activity of purified Ssa1p, a yeast cytosolic hsc70 chaperone, was measured in the presence and absence of R/1. At 300 μM, the concentration at which DSG maximally stimulated Ssa1p (19), R/1 caused a 48% decrease in the specific activity of Ssa1p, from 1.25 nmol·min⁻¹·mg⁻¹ in the absence of R/1 to 0.65 nmol·min⁻¹·mg⁻¹ in its presence (Fig. 2). Purified Ydj1p, the cytosolic DnaJ partner of Ssa1p (27, 33), increased the specific activity of Ssa1p 6.2-fold in this experiment; a result consistent with previously published data (14, 27, 28). Addition of R/1 decreased the Ydj1p-mediated stimulation of Ssa1p from 9 to 4.4 nmol·min⁻¹·mg⁻¹, a 51% reduction (Fig. 2). Thus, R/1 compromises the endogenous and DnaJ-stimulated ATPase activity of Ssa1p.

We next titrated the effect of R/1 on Ssa1p ATPase activity. In this assay, half-maximal levels of inhibition were achieved at ~100 μM R/1 (Fig. 3). R/1 also inhibited the ATPase activity of BiP in a dose-dependent manner, similar to its effect on Ssa1p (Fig. 3).

R/1 Inhibits Post-translational Translocation in Vitro—The post-translational translocation of preproteins into the ER is an ATP-dependent, chaperone-mediated process that can be recapitulated in vitro using yeast ER-derived microsomes. In yeast, two hsc70s are involved in this process; Ssa1p binds unfolded polypeptides to confer them transport function and to retain them in a soluble translocation-competent state (34, 35); and BiP, a lumenal ER protein, “ratchets” or drives translocating peptides through the ER translocation pore (36). Ssa1p and BiP interact with specific DnaJ homologs, Ydj1p and Sec63p, respectively, both of which are also required for post-translational translocation (26, 31, 33, 37).

To assess the effect of R/1 on this chaperone-mediated process, we examined the efficiency with which an in vitro trans-

Fig. 1. Chemical structures of NSC 630668-R/1 and DSG. Structures were drawn in a way to roughly depict potential structural superimposition. Conformational analysis and superimposition were performed using Cerius version 4.2MS software (MSI/Biosym). Consensus alignment was achieved employing flexible root mean square atoms fitting after finding a global conformational minimum energy structure of R/1 with the Adopted Basis Newton-Raphson coordinate minimization method and a systematic search of the 35 torsions in the molecule.
lated yeast pre-pheromone, prepro-α-factor (ppαF), was translocated into yeast ER-derived microsomes in the presence of increasing concentrations of R/1. Translocation of ppαF into microsomes was assessed by cleavage of the signal sequence of ppαF to form pro-α-factor (pαF) and by the subsequent resistance of pαF to trypsin digestion due to its membrane enclosure. As shown in Fig. 4, A and B, we found that translocation was almost completely blocked at 300 μM, and the half-maximal level of inhibition occurred at ~6 μM.

Because R/1 is relatively hydrophobic, it might have prevented translocation by solubilizing microsomal membranes. To exclude this possibility, we examined the trypsin accessibility of two luminal proteins, BiP and translocated pαF, after the addition of R/1 to microsomes. Both proteins remained resistant to trypsin after treatment with R/1 or Me2SO (Fig. 5A, lanes 4 and 6; Fig. 5B, lanes 4 and 5) but were degraded by trypsin when microsomes were permeabilized with detergent (Fig. 5A, lane 8, and 5B, lane 6). These results indicate that R/1 does not prevent translocation by destroying microsome integrity.

R/1 Oligomerizes hsc70—Steady-state ATPase assays measure the ability of a chaperone to bind, hydrolyze, and rebind ATP. Thus, to determine whether R/1 specifically affects ATP hydrolysis, we performed single turnover experiments on preformed ATP-Ssa1p complexes. This allows measurement of initial rates of hydrolysis and is therefore more sensitive than steady-state experiments. As shown previously (29), hydrolysis of Ssa1p-bound ATP is rapid (Fig. 6). Surprisingly, when R/1

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**Fig. 2.** R/1 inhibits the endogenous and co-chaperone-stimulated ATPase activity of Ssa1p. Steady-state measurements of Ssa1p ATPase activity were performed in reactions containing equal volumes of R/1 (at a final concentration of 300 μM) or Me2SO and in the presence or absence of Ydj1p. The molar ratio of Ssa1p to Ydj1p used in this experiment was 1:2. The mean specific activities from three independent experiments (±S.D.) are expressed in nanomoles of ATP hydrolyzed per min per mg of Ssa1p.

**Fig. 3.** Dose dependence of R/1-mediated inhibition of Ssa1p and BiP ATPase activities. Steady-state ATPase measurements of Ssa1p (open triangles) and BiP (open circles) were performed as in Fig. 2 with the indicated concentrations of R/1. Each data point represents the mean (±S.D.) of four independent measurements. The mean specific activities of Ssa1p and BiP in these experiments were 1.5 and 1.8 nmol·min⁻¹·mg⁻¹, respectively.

**Fig. 4.** R/1 inhibits posttranslational translocation. Translocation reactions with radiolabeled, in vitro translated ppαF were performed as described under “Materials and Methods” with the indicated concentrations of R/1. A, after translocation, one-half of the reaction was trichloroacetic acid-precipitated (lane 1), and the other half was treated with trypsin (lane 2) before precipitation. Trypsin-resistant ppαF accumulated in reactions containing higher concentrations of R/1 (see “Discussion”) (B). The percentage of mature pαF in translocation reactions containing the indicated concentrations of R/1 was assessed by quantification of trypsin-protected pαF in relation to the total input ppαF. Shown are the means from three independent experiments performed at each R/1 concentration, ±S.D.

**Fig. 5.** Microsomal membrane integrity is unaffected by R/1. A. microsomes were incubated in buffer, Me2SO, R/1, or Triton X-100 as indicated, and the susceptibility of BiP to protease was assessed by immunoblot analysis. B, PαF-loaded microsomes were incubated in Me2SO, R/1, or Triton X-100 and then treated with (lanes 1–3) or without trypsin (lanes 4–6) on ice for 20 min.
was included in the reaction, the initial rate of hydrolysis dramatically increased. At 300 μM R/1, the initial rate of ATP turnover was ~5-fold higher than that for the reaction without R/1, an increase comparable to that seen by addition of peptide substrates to other hsc70s (38). Although previous experiments performed in our laboratory demonstrated that 95% of the Ssa1p-ATP complex can be hydrolyzed (29), the rate of ATP hydrolysis leveled off after ~4 min in the presence of R/1, suggesting that a fraction of Ssa1p became inactivated by R/1. At lower R/1 concentrations where translocation was compromised (Fig. 4), the rate of ATP hydrolysis was again enhanced (2.5- and 4.5-fold at 10 μM and 30 μM R/1, respectively), but after 2 min, the rate of hydrolysis decreased (Fig. 6). These data indicate that R/1 compromises Ssa1p ATPase activity at concentrations as low as 10 μM, thus providing an explanation for the inhibition of in vitro translocation at this concentration (Fig. 4).

King et al. (39, 40) previously found that a rapid burst of ATP hydrolysis, mediated by the Ydj1p co-chaperone, induces the formation of metastable Ssa1p polymers. Because R/1 similarly affects Ssa1p activity (Fig. 4), one scenario for the R/1-mediated inhibition of Ssa1p ATP hydrolysis is that R/1 oligomerizes Ssa1p. To test this hypothesis, the amount of hsc70 that precipitates after centrifugation at 100,000 g was measured at 100,000 × g in the presence and absence of R/1 was examined. In control reactions, ~30% of Ssa1p and BiP precipitated, whereas in the presence of 300 μM R/1, 60–70% of Ssa1p and BiP were found in the pellet (Fig. 7). Interestingly, similar results were observed with yeast Sse1p, a member of the hsp110 family of molecular chaperones that is 27% identical to Ssa1p (41).

To determine whether the ability of R/1 to precipitate proteins was a general phenomenon, we examined several other proteins in this assay. In the presence of R/1 the amount of precipitable malate synthase was only enhanced ~15% (Fig. 7). Similarly, R/1 had a lesser effect on the amounts of lysozyme, bovine serum albumin, and citrate synthase than on the amounts of the Ssa1p, BiP, and Sse1p chaperones found in the pellet after centrifugation (Fig. 7).

To confirm the chaperone specificity of R/1, the ability of malate synthase to catalyze the formation of (2S)-malate from acetyl-CoA and glyoxylate in the presence and absence of R/1 was assayed as described (42). We found that the specific activity of malate synthase was 0.57 and 0.62 μmol·min⁻¹·mg⁻¹ in the presence of Me₂SO and 300 μM R/1, respectively.

**Fig. 6.** R/1 initially enhances but then inhibits single turnover ATP hydrolysis. Ssa1p-ATP complexes were incubated at 30 °C for the indicated times, and the percentages of ATP hydrolyzed over time in the absence (closed circles) or presence of 10 μM R/1 (open circles), 30 μM R/1 (open squares), and 300 μM R/1 (open triangles) were analyzed.

**Fig. 7.** R/1 precipitates molecular chaperones. The indicated proteins were incubated with either Me₂SO (DMSO) or R/1 and pellet (P) and supernatant (S) fractions after centrifugation of the reactions were analyzed by SDS-PAGE.

**DISCUSSION**

We report here on the identification of the first specific inhibitor of posttranslational protein translocation. Single turnover experiments show that hsc70 ATP hydrolysis is initially stimulated but inhibited at later time points, presumably by hsc70 precipitation and inactivation. This property is specific for the chaperones examined as R/1 failed to precipitate several other proteins to the same degree. The ability of R/1 to inhibit translocation does not arise from the increase in trypsin-resistant ppαF observed (Fig. 4A, lane 2) as ~26% of ppαF became resistant at 60 μM R/1, and translocation was inhibited by ~90% at this concentration. Therefore, we conclude that the abrogation of ppαF translocation is most likely mediated by the ability of R/1 to inhibit the activities of two hsc70s, Ssa1p and BiP, that are required to engineer preprotein transport into the yeast ER (31, 33–36, 43).

The simplest explanation for the effect of R/1 on hsc70 is that the length and hydrophobic nature of R/1 (Fig. 1) enable it to mimic a peptide substrate. This would explain the initial burst of ATP hydrolysis observed in the single turnover experiments (Fig. 6) and the inhibition of posttranslational translocation (Fig. 4) if R/1 competes effectively with ppαF for occupation of the substrate-binding site of Ssa1p and/or BiP. Alteration of the oligomeric state of hsc70 could result indirectly from precipitation of hsc70-bound R/1 molecules (Fig. 7). Alternatively, R/1 might promote the formation of hsc70 polymers by binding to a site on hsc70 distinct from that which interacts with peptides. Support for the latter hypothesis derives from the observation that DnaJ homologs can polymerize hsc70 in the absence of substrate (39, 40). This phenomenon may arise from hsc70 homopolymer formation. In the absence of a substrate, hsc70s may bind one another to form metastable polymers (40). In our experiments, the initial burst of ATP hydrolysis caused by R/1 could promote the formation of hsc70 polymers, which in turn would inhibit ATP hydrolysis of existing ATP-Ssa1p complexes.

Despite their structural similarity (Fig. 1), there are several differences in the activities of DSG and R/1. First, DSG stimulates the steady-state ATPase activity of Ssa1p (19), whereas R/1 inhibited Ssa1p steady-state activity. Second, DSG inhibits neither the ATPase activity of BiP (19) nor the posttranslational translocation, in contrast to R/1. Third, although R/1 may be a peptide mimic (see above), DSG does not appear to interact with the substrate-binding domain of hsc70 as it does not prevent Ssa1p or mammalian hsc70 from binding polypeptide substrates (19, 20). Finally, there is no evidence that DSG inactivates or alters the oligomeric state of hsc70.

*J. L. Brodsky, unpublished results.*
Inhibition of hsc70 Molecular Chaperone Activity

Thus, DSG and R/1 probably modulate hsc70 activity by distinct mechanisms.

Our discovery of a small molecule that is capable of inhibiting a hsc70-mediated, DNA-stimulated process is profound. Because molecular chaperones influence the biogenesis and degradation of many medically relevant protein substrates (2, 13), R/1 and R/1 analogs could prove to be therapeutically valuable. Future studies will be directed toward identifying and characterizing these compounds.

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Identification of an Inhibitor of hsc70-mediated Protein Translocation and ATP Hydrolysis

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