

High-throughput screen for small molecules that modulate the ATPase activity of the molecular chaperone DnaK

Lyra Chang^a, Eric B. Bertelsen^b, Susanne Wisén^a, Erik M. Larsen^a,
Erik R.P. Zuiderweg^b, Jason E. Gestwicki^{a,c,*}

^a Life Sciences Institute, University of Michigan, Ann Arbor, MI 48109, USA

^b Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA

^c Department of Pathology, University of Michigan, Ann Arbor, MI 48109, USA

Received 1 July 2007

Available online 22 August 2007

Abstract

DnaK is a molecular chaperone of *Escherichia coli* that belongs to a family of conserved 70-kDa heat shock proteins. The Hsp70 chaperones are well known for their crucial roles in regulating protein homeostasis, preventing protein aggregation, and directing sub-cellular traffic. Given the complexity of functions, a chemical method for controlling the activities of these chaperones might provide a useful experimental tool. However, there are only a handful of Hsp70-binding molecules known. To build this area, we developed a robust, colorimetric, high-throughput screening (HTS) method in 96-well plates that reports on the ATPase activity of DnaK. Using this approach, we screened a 204-member focused library of molecules that share a dihydropyrimidine core common to known Hsp70-binding leads and uncovered seven new inhibitors. Intriguingly, the candidates do not appear to bind the hydrophobic groove that normally interacts with peptide substrates. In sum, we have developed a reliable HTS method that will likely accelerate discovery of small molecules that modulate DnaK/Hsp70 function. Moreover, because this family of chaperones has been linked to numerous diseases, this platform might be used to generate new therapeutic leads.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Dihydropyrimidine; Heat shock protein; Hsp70; Malachite green; Stress response

DnaK is an extensively studied member of the family of 70-kDa heat shock proteins. These proteins are evolutionarily conserved at the amino acid level; for example, *Escherichia coli* DnaK and human Hsp70 are 46% identical and 65% similar [1]. Moreover, they are functionally conserved; like other Hsp70s, DnaK acts as a molecular chaperone that assists in protein folding, aids in trafficking of newly synthesized polypeptides, and helps dissolve protein aggregates [2–6]. In humans, Hsp70s have also been implicated in numerous diseases, including cancer, neurodegenerative disorders, and viral infections [7–12]. However, the complexity of their functions has made studying the specific roles of Hsp70 challenging. Even for the relatively well-

studied DnaK, numerous questions with regard to how this chaperone carries out its myriad cellular tasks remain unanswered.

DnaK is able to take part in a variety of pathways because nearly all proteins contain hydrophobic sequences that are suitable as substrates for chaperone binding. Despite the diversity of potential clients, binding affinity is tightly regulated. Specifically, DnaK (like all Hsp70s) consists of three domains: a 25-kDa substrate-binding domain (SBD),¹ a 45-kDa N-terminal nucleotide binding domain that harbors ATPase activity, and a 10-kDa helical

* Corresponding author. Fax: +1 734 764 1247.

E-mail address: gestwick@umich.edu (J.E. Gestwicki).

¹ Abbreviations used: SBD, substrate-binding domain; DSG, 15-deoxy-spergualin; SAR, structure-activity relationships; HTS, high-throughput screening; MG, malachite green; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; DMSO, dimethyl sulfoxide.

“lid” region [2,13]. Allosteric communication between these modules provides regulatory control. The ATP-bound form of DnaK has a “loose” configuration and poor affinity for peptide substrates [13,14]. Hydrolysis of the nucleotide causes conformational changes in the adjacent SBD that enhance the affinity for the peptide substrate [2,15]. Thus, ATP hydrolysis is one driving force behind chaperone structure and function. Importantly, DnaK’s intrinsic rate of nucleotide hydrolysis is slow ($\sim 0.14 \mu\text{mol ATP}/\mu\text{mol DnaK}/\text{min}$ *vide infra*) compared to other typical ATPases, such as porcine Na,K-ATPase ($\sim 75 \mu\text{mol ATP}/\mu\text{mol enzyme}/\text{min}$) [16]. This modest intrinsic rate permits tight regulation of the cycle by cochaperones. For example, the binding of the cochaperone DnaJ stimulates ATP hydrolysis (and, thus, enhances affinity for substrate), while the nucleotide exchange factor GrpE promotes ADP release to complete the catalytic cycle [17]. In addition to its role in ATP hydrolysis, DnaJ is thought to recruit DnaK into its various cellular functions and, consequently, there are typically more DnaJ-like proteins in a cell than core DnaK/Hsp70 chaperones [4–22]. For example, there are 6 DnaJ homologs in *E. coli*, 22 in *Saccharomyces cerevisiae*, and 41 putative family members in humans. In this way, cochaperones steer DnaK/Hsp70 into various combinatorial partnerships and likely coordinate different responses to cellular stress, signaling, or protein aggregation.

In contrast to the questions surrounding Hsp70 function, the roles of the related heat shock protein Hsp90 are becoming increasingly well characterized. This is especially true in relation to Hsp90’s prominent roles in cancer. In part, knowledge about Hsp90 has been accelerated by the availability of potent and selective chemical inhibitors, such as geldanamycin and radicicol [23]. In addition, these reagents have facilitated the development of anticancer drugs that are currently undergoing clinical trials [24–27]. This success suggests that a parallel “chemical genetic” approach might be used to dissect the complex Hsp70 network and, moreover, that this process might also lead to the discovery of new Hsp70-based therapeutics. Of particular interest are compounds that can selectively recognize specific DnaK–DnaJ combinations because these complexes are likely to be involved only in subsets of the total chaperone functions.

To date, only a handful of small molecules that bind DnaK or Hsp70 have been reported. Among the first partners identified was the polyamine 15-deoxyspergualin (DSG), which binds Hsp70 in pull-down assays and, later, was found to enhance its steady state ATPase activity by 20–40% [23–31]. Based on structural similarity to DSG, a small-scale (~ 40 compounds) search for new Hsp70 modulators led to the discovery of NSC 630668-R/1 (R/1), which inhibits ATPase activity and blocks Hsp70-mediated trafficking of polypeptides [32]. More recently, another collection of ~ 30 dihydropyrimidines related to R/1 was studied using single-turnover ATP hydrolysis reactions [33]. In that work, unique classes of chaperone modulators were uncovered: some directly inhibited ATPase activity whereas oth-

ers, such as MAL3-101, selectively blocked the ATPase-enhancing ability of specific J domain proteins [33]. Interestingly, despite their diverse activities, many known Hsp70-binding compounds share a central dihydropyrimidine core and vary only in their pendant functionality. However, this is not the only chemical scaffold that has affinity for Hsp70-class proteins. For example, a family of acylated benzamido derivatives was reported to bind DnaK’s SBD, inhibit its chaperone function, and thereby display antibacterial activity [34]. Thus, multiple chemical classes have been reported to modify Hsp70’s functions. Despite the potential uses of these reagents, structure–activity relationships (SAR) that govern potent effects on ATPase activity are not yet clear. One factor contributing to this lack of information is that only a small number of compounds have been screened in low-throughput formats. We hypothesized that high-throughput screening (HTS) would be a useful platform for identifying new, potent chemical modulators and discerning SAR between similar compounds.

Surprisingly, Hsp70 has not been subjected to extensive HTS. In contrast, several assays have been developed to uncover new inhibitors of Hsp90 [35–39]. One of these platforms [38] employs the inorganic phosphate chelator malachite green (MG) to monitor the ATPase activity of Hsp90. The MG assay is colorimetric and has the advantages of being robust, cost effective, and suitable for automated screening [40–42]. These advantages led us to explore whether this method could be employed to screen for compounds that alter the ATPase activity of DnaK. Because of DnaK’s relatively slow ATP turnover rate, this assay required modification to improve the signal and reduce the noise from spontaneous nucleotide hydrolysis. Importantly, we found that, using optimized conditions, the signal was linear for up to 4 h; therefore, an end-point measurement was sufficient to represent the steady state rate. Using this assay, we discovered seven new inhibitors of DnaK from a focused collection of 204 dihydropyrimidines. This result suggests that the MG assay is a useful platform for finding small-molecule modulators. These selected compounds, in turn, might be applied as chemical genetic tools to elucidate DnaK’s biology and might be used in future development of medicines that target Hsp70-related diseases.

Materials and methods

Protein expression and purification

DnaK, DnaJ, and GrpE proteins were expressed in *E. coli* BL21(DE3) using T7-based vectors. DnaK and GrpE were expressed at 37 °C, whereas DnaJ was expressed at 25 °C to increase the fraction of soluble protein. All purification steps were carried out at 4 °C. Protein concentration was estimated by Bradford assay, using bovine serum albumin as the standard. Following purification, proteins were frozen on liquid nitrogen and stored at -80 °C until use.

Purification of DnaK was accomplished by a modification of established procedures [43,44]. Briefly, cell pellets were suspended in buffer A (25 mM Tris, 10 mM KCl, 5 mM MgCl₂, pH 7.5) containing 0.01 mM PMSF and 1 mM DTT and disrupted using a microfluidizer (Microfluidics). Cleared extracts were applied to a Q-Sepharose fast-flow column (GE Healthcare), and the protein was eluted with a 10–500 mM gradient of KCl in buffer A. Fractions containing DnaK were pooled and applied to ATP-agarose (Sigma). After extensive washing with buffer A and buffer A containing 1 M KCl, the protein was eluted with buffer A containing 3 mM ATP. The pure protein was concentrated and exchanged to buffer A for storage.

DnaJ was purified using a streamlined version of established methodology [45,46]. Briefly, cell pellets were resuspended in buffer B (25 mM Tris, 2 M urea, 0.1% Brij-58, 2 mM DTT, pH 7.5) containing 0.01 mM PMSF and disrupted using a microfluidizer. Cleared extracts were applied to a Source SP (GE Healthcare) column, and the protein was eluted with a gradient of 0–350 mM KCl in buffer B. Fractions containing DnaJ were pooled and applied to a hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories) column that had been equilibrated with buffer C (buffer B containing 50 mM KCl and no detergent). After extensive washing with buffer C and buffer C containing 1.0 M KCl, the protein was eluted with a 0–350 mM gradient of potassium phosphate, pH 7.4. Fractions containing DnaJ were pooled, diluted twofold with 25 mM Tris, 50 mM KCl, 2 M urea, pH 9.0, and applied to a Q-Sepharose fast-flow column. Pure DnaJ was collected in the flow-through, concentrated, and exchanged into buffer A containing 150 mM KCl for storage.

GrpE was purified as follows: Cell pellets were resuspended in buffer D (25 mM Tris, pH 7.5) containing 0.01 mM PMSF and disrupted using a microfluidizer. Cleared extracts were applied to a Q-Sepharose fast-flow column, and protein was eluted with a 0–500 mM gradient of KCl in buffer D. Fractions containing GrpE were pooled, supplemented with 1 M ammonium sulfate, and applied to a phenyl-Sepharose high-performance 16/10 column (GE Healthcare). Protein was eluted with a 0–0.1 M gradient of ammonium sulfate in buffer D. Fractions containing GrpE were pooled and concentrated, and applied to a Superdex 200 26/60 column (GE Healthcare). Protein was eluted at 0.5 ml/min in buffer D. Fractions containing GrpE were pooled and concentrated, and the protein was exchanged into buffer A for storage. The protein was greater than 90% pure as judged by SDS-PAGE and Coomassie staining. Note that minor impurities with hydrolysis activity can be co-purified.

Colorimetric determination of ATPase activity

The assay procedure was adopted from previous reports [38] with modifications where indicated. Stock solutions of malachite green (0.081% w/v), polyvinyl alcohol (2.3% w/v), and ammonium heptamolybdate tetrahydrate (5.7%

w/v in 6 M HCl) were prepared, stored at 4 °C, and mixed with water in the ratio of 2:1:1:2 to prepare the malachite green reagent. This reagent was stable at 4 °C for at least 1 week. All components were from Sigma and used without further purification.

For compound screening, a master mix of DnaK:DnaJ:GrpE (1.0:1.7:1.6 μM) was prepared in assay buffer (0.017% Triton X-100, 100 mM Tris-HCl, 20 mM KCl, and 6 mM MgCl₂, pH 7.4). An aliquot (14 μL) of this mixture was added into each well of a 96-well plate. To this solution, 1 μL of either compound (5 mM) or DMSO was added and the plate was incubated for 30 min at 37 °C before adding 10 μL of 2.5 mM ATP to start the reaction. Thus, the final reaction volume was 25 μL and the conditions were 0.6 μM DnaK, 1.0 μM DnaJ, 0.9 μM GrpE, 4% DMSO, 0.01% Triton X-100, and 1 mM ATP. These concentrations were chosen because they provide intermediate signal and thereby encourage the possibility of discovering both inhibitors and (potentially) activators. After 1–3 h incubation at 37 °C, 80 μL of malachite green reagent was added into each well. Immediately following this step, 10 μL 34% sodium citrate was used to halt the nonenzymatic hydrolysis of ATP. The samples were mixed thoroughly and incubated at 37 °C for 15 min before measuring OD₆₂₀ on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). To control these results for intrinsic hydrolysis, the signal from ATP in identically treated buffer lacking chaperones was subtracted. To permit comparisons between screens performed at different times, a phosphate standard curve (using sodium phosphate) was generated each day (see Supplemental Fig. 3).

Chemical synthesis of a compound library

The dihydropyrimidine compounds used in this study were synthesized by Biginelli cyclocondensation reactions, utilizing methods modified from the independent work of Kappe and Wipf [28,47]. The collection of 204 compounds used in this work includes those generated in-house, those received from the University of Pittsburgh's Center for Chemical Methodologies and Library Development (UPCMLD), and those purchased from ChemBridge. A more detailed description of this collection will be reported elsewhere. Geranylgeraniol was obtained from Sigma and the peptide substrate (NRLLLTG) was provided by Syn-Pep [48]. None of the compounds had appreciable absorbance at 620 nm and, thus, did not interfere with the MG signal (data not shown).

Results

Influence of DnaK and ATP on MG signal

In an effort to adapt an existing MG-based method [38] for use in monitoring DnaK's ATPase activity, we first measured OD₆₂₀ while varying the concentration of DnaK and ATP. We found that, between 0.3 and 0.9 μM, the sig-

nal increased with DnaK levels (Fig. 1A). At constant DnaK (0.6 μM), ATP concentration was varied from 0.13 to 8.0 mM, and we found that, above 4 mM, the OD_{620} was independent of nucleotide (Fig. 1B). Based on these studies, we chose 0.6 μM DnaK and 1 mM ATP for screening conditions because these parameters provided good signal while minimizing reagent costs.

Influence of DnaJ and GrpE concentrations

During its normal physiological function, DnaK is assisted by the action of DnaJ and GrpE [2,17]. Moreover, certain small molecules that are known to modulate Hsp70's ATPase activity are active only in the presence of cochaperones [33]. Therefore, we sought to develop a screen protocol that includes these components. Our approach was to vary the levels of recombinant DnaJ or GrpE and monitor effects on ATP hydrolysis by DnaK. In these studies, we were interested in both the rate enhancement and the cochaperone concentration that yielded half-maximal stimulation ($K_{0.5}$). Rate enhancement is calculated by comparing the turnover rate of the cochaperone-stimulated system against the rate due to DnaK alone and the $K_{0.5}$ can be used to approximate the affinity for DnaK. Using the MG assay, we found that the $K_{0.5}$ of DnaJ was around 0.8 μM and that it provided up to ~ 11 -fold stimulation of ATPase activity (Fig. 2A). On the other hand, GrpE had better apparent affinity for DnaK ($K_{0.5} = 0.19 \mu\text{M}$) but stimulated ATPase activity only approximately 7-fold (Fig. 2B). These results are generally consistent with the findings of McCarty et al. [49] who used single-turnover ATP assays to determine that when DnaK was fixed at 0.7 μM , 1.4 μM DnaJ and 0.7 μM GrpE stimulate DnaK ATPase by 13-fold and 1.3-fold, respectively.

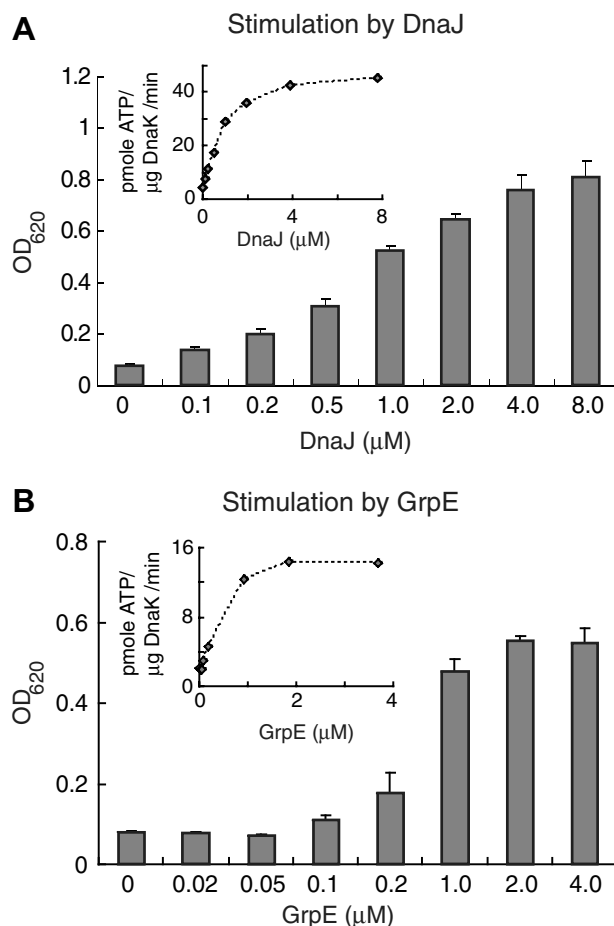


Fig. 2. Stimulatory effect of cochaperones DnaJ and GrpE. The ATPase activity of DnaK at different concentrations of (A) DnaJ and (B) GrpE was measured at 1 mM ATP and 0.6 μM DnaK. The OD_{620} value was determined after 3 h incubation at 37 $^{\circ}\text{C}$. In the insets, the OD_{620} values were converted into ATP hydrolysis rates based on a standard curve. These results are the average of triplicates and the error is standard deviation.

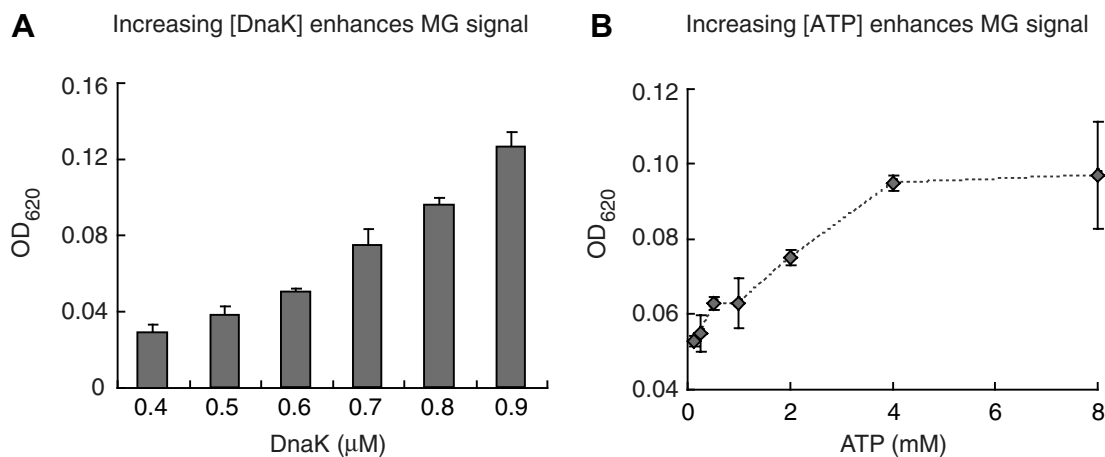


Fig. 1. Dependence of MG signal on the concentration of DnaK and ATP. (A) The concentration dependence of DnaK was observed at 1 mM ATP. (B) DnaK was fixed at 0.6 μM while changing the concentration of ATP. The OD_{620} value of the ATP control ($\text{OD}_{620} = \sim 0.25$) was subtracted in all figures shown. These results are the average of triplicates and the error is standard deviation.

We were interested also in finding a ternary combination that would provide the most robust signal and the broadest dynamic range because this information could be used to select optimal screening conditions. To explore this idea, we fixed the concentration of DnaK (0.6 μM) and either DnaJ (1.0 μM) or GrpE (0.9 μM) and changed the concentration of GrpE or DnaJ, respectively. We discovered that, at a saturating level of GrpE, the $K_{0.5}$ of DnaJ increased to around 3.1 μM and ATPase activity was stimulated an additional 15.5-fold compared to the DnaK:GrpE complex in the absence of DnaJ. This ternary combination led to a 130-fold stimulation over DnaK alone (Fig. 3A). Alternatively, when DnaJ and DnaK were held constant, the $K_{0.5}$ of GrpE did not change. Under these conditions, we observed a 3.5-fold stimulation over DnaJ alone and only a 30-fold total increase in ATP hydrolysis (Fig. 3B). Next, the rate of DnaK's ATPase activity over time was measured at different combinations of DnaK, DnaJ, and GrpE. A linear increase in all conditions up to 4 h was observed

(Fig. 4). Finally, we sought to ensure that this platform faithfully reproduces known kinetic parameters of the enzyme. The V_{max} and k_{cat} of each reaction condition were calculated and, as shown in Table 1, we found that these parameters were in good agreement with previous data [49,50]. Thus, we hypothesized that, despite the slow turnover rate of DnaK, these cochaperone-stimulated conditions appear to be sufficient for screening.

Screening a focused chemical library

Using the optimized reaction conditions, we screened a small chemical library for compounds that modulate DnaK activity (Fig. 5). This compound collection is composed of 204 dihydropyrimidines that are structurally similar to the lead candidate MAL3-101 [33]. These compounds were assembled from three sources: "cherry-picked" molecules from commercially available collections, those synthesized by the UPCMLD, and those generated by our group. Together, these compounds form a focused library from which we sought to identify potent modulators of DnaK.

Using the MG assay, we screened at 200 μM in 96-well plates. During our first screening attempt, we found that most (~90%) of the compounds displayed weak ATPase-stimulating ability (Supplemental Fig. 1). We suspected that this might result from nonspecific binding of the largely hydrophobic compounds to the SBD. It has been reported that detergents can remove promiscuous hits and improve the reliability of HTS methods [51]. Therefore, we repeated the screen in the presence of 0.01% Triton X-100. Intriguingly, the mild stimulation effect disappeared, whereas the activity of the inhibitors remained largely unchanged or became more pronounced. We defined compounds that decreased activity >20% as inhibitors and, by this definition, seven inhibitors were identified (3.5% of the library; Table 2). These results demonstrated that the MG assay can be used to readily screen a chemical collection.

Confirming "hits" from the screen

From the seven hits, we picked four inhibitors, 0116-2F, 0116-4G, 0116-7G, and 0116-9E, for further study (Fig. 6). To confirm these hits and generate IC_{50} values, we studied their effects (between ~50 and 400 μM) on DnaK's ATPase activity. Importantly, we found that the inhibitory effect was reproducible and the IC_{50} values were between 120 and 200 μM (Fig. 6). At saturating concentrations, 0116-4G, 0116-7G, and 0116-9E provide ~50% inhibition of DnaK ATPase activity, whereas the potency of 0116-2F is modest (80% original activity). The effects of the validated inhibitors were independent of detergent (Supplemental Fig. 4).

Characterization of the binding of candidate compounds to DnaK

The substrate-binding domain of DnaK has affinity for exposed hydrophobic regions on unfolded polypeptides.

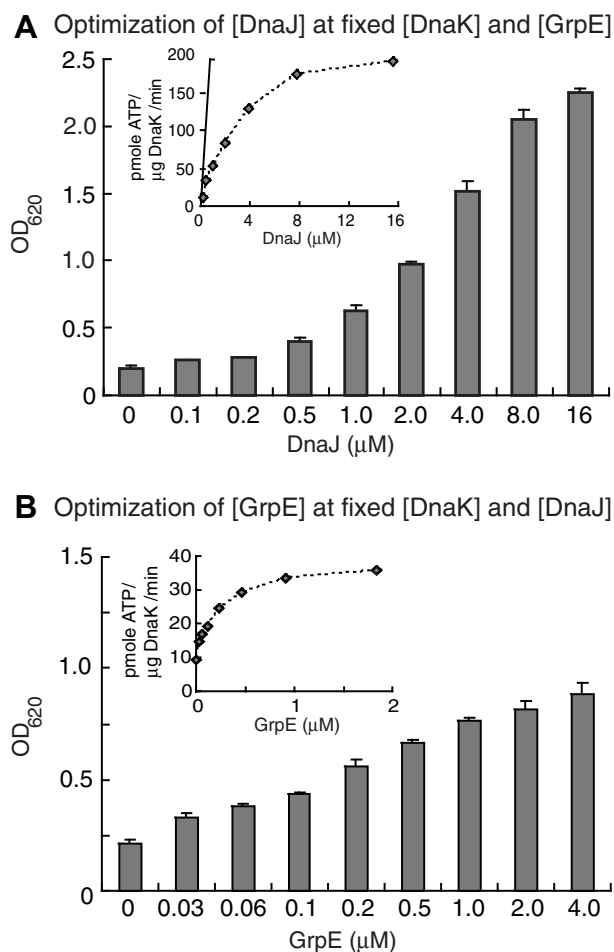


Fig. 3. Optimization of the concentrations of DnaJ and GrpE for screening. (A) DnaJ stimulates ATP hydrolysis at fixed DnaK (0.6 μM) and GrpE (0.9 μM). (B) GrpE promotes the ATPase activity of 0.6 μM DnaK and 1.0 μM DnaJ. In the insets, the OD₆₂₀ values were converted into ATP hydrolysis rates based on a standard curve. The OD₆₂₀ value was determined after 1.5 h incubation at 37 °C. These results are the average of triplicates and the error is standard deviation.

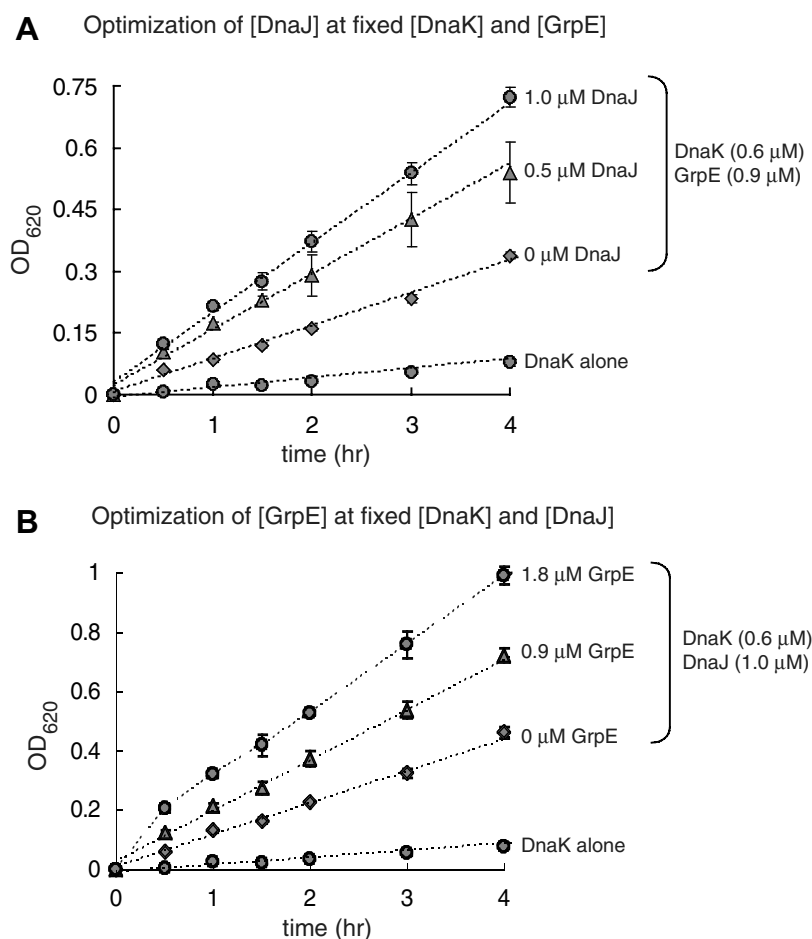


Fig. 4. ATPase activity over time at different combinations of cochaperones. (A) Variable concentrations of DnaJ were mixed with 0.6 μM DnaK and 0.9 μM of GrpE. (B) The DnaK (0.6 μM) and DnaJ (1.0 μM) concentrations were fixed, whereas different amounts of GrpE were added. At given times, the reaction was stopped and the OD₆₂₀ value measured. These results are the average of triplicates and the error is standard deviation.

Table 1
Calculated and reported V_{max} and K_{cat} values for the DnaK system

DnaK (μM)	DnaJ (μM)	GrpE (μM)	V_{max} (pmol ATP/ μg DnaK/min)	K_{cat} (min ⁻¹)	Ref.
0.58	0	0	1.9 ± 0.12	0.12 ± 0.01	This work
0.58	0	0.91	6.5 ± 0.14	0.45 ± 0.01	This work
0.58	0.48	0.91	11 ± 0.56	0.75 ± 0.04	This work
0.58	0.97	0.91	14 ± 0.59	0.94 ± 0.04	This work
0.58	0.97	0	8.6 ± 0.24	0.59 ± 0.02	This work
0.58	0.97	1.8	17 ± 0.72	1.1 ± 0.05	This work
0.69	0	0	0.65		[49]
0.93	0	0	2.29	0.16	[50]
0.69	0	0.69	0.87		[49]
0.69	1.40	0	8.25		[49]

Because all the selected inhibitors have hydrophobic regions, we assumed that they might bind to SBD. To test this hypothesis, we used a known SBD-binding peptide (NRLLLTG) to compete for the SBD binding site [48]. First, we confirmed that the peptide stimulates ATPase function as previously reported [52]. In these studies, we found that the peptide provides approx fivefold stimulation at concentrations above 100 μM (Fig. 7). Second, we

explored the activity of the candidate compounds, 0116-2F, 0116-4G, 0116-7G, and 0116-9E, in the presence of saturating levels of peptide. In these experiments, geranylgeraniol, with a structure similar to reported SBD-binding antibacterials [34], was also included as a control; we expected that peptide substrates would compete with this compound for binding to the SBD. Consistent with this idea, the ATPase-enhancing activity of geranylgeraniol was eliminated (Fig. 8). Intriguingly, for three of four selected compounds, inhibition remained largely unchanged in the presence of peptide (Fig. 8 and Supplemental Fig. 2). These experiments suggest that the dihydropyrimidines act via a site independent of the substrate-binding groove of the SBD to modulate ATPase activity.

Discussion

Hsp70 chaperones are central mediators of protein homeostasis and are involved in a variety of signaling pathways [2–9]. Therefore, small molecules that specifically modulate their activities are intriguing as research tools and, potentially, as therapeutic leads [23,53]. In this paper,

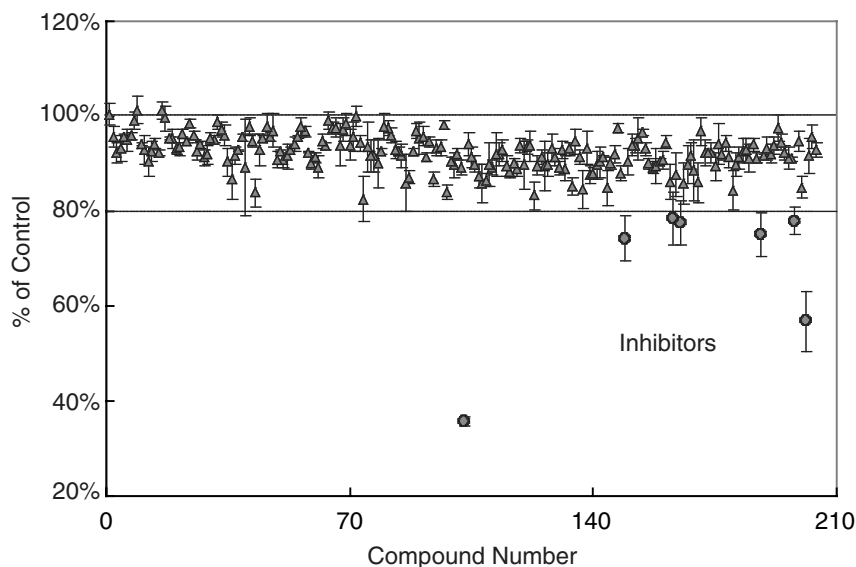


Fig. 5. Screening of a 204-compound focused library. Each spot represents the average of triplicate wells for a single compound and the error is standard deviation. Triplicates were performed to demonstrate that screening results are reproducible. All compounds were screened at 200 μM with 0.6 μM DnaK, 1.0 μM DnaJ, 0.9 μM GrpE, 0.01% Triton X-100, and 1 mM ATP. Compounds resulting in <80% original DnaK ATPase activity were defined as inhibitors. As a control, 200 μM each compound was incubated with 1 mM ATP and 0.01% Triton X-100 and the resultant OD_{620} value was subtracted from the measured signal at the presence of DnaK, DnaJ, and GrpE for the specific compound before calculating the percentage of control activity.

Table 2
Distribution of the screening results

Class	% Control	Hits	% Total compounds
Inhibitor	< 50	1	0.5
	50–80	6	3
Inactive	80–90	46	23
	90–100	149	73

we described a MG-based assay and the application of this method to uncover small molecules that inhibit the bacterial Hsp70, DnaK. This method is reliable ($Z \cong 0.7$; $S/N > 10$) [54], low cost, and potentially amenable to the 384-well plate format [55]. By carefully varying the levels of chaperone and stimulatory cochaperones, we were able to arrive at a ratio that provided good dynamic range and signal-to-noise. Moreover, because this system contains both chaperone and cochaperone, it provides the opportunity for uncovering compounds that modify either protein. Of course, this feature requires subsequent follow-up studies to isolate the binding site and mode of action. Finally, we observed a linear increase in OD_{620} signal for greater than 4 h, which is a feature that permits usage of simple endpoint measurements.

As a preliminary test of this method, we screened 204 compounds that share a dihydropyrimidine core similar to the previously reported lead, MAL3-101 [33]. In this relatively small collection, we successfully identified seven inhibitors (>20% inhibition). All selected inhibitors (4 compounds) were reproducible and their IC_{50} values ranged from approximately 120 to 200 μM . While these potencies are modest, the library size was not large and we would predict that activity might be improved following additional lead optimization and structural studies.

We designed this assay to report on compounds that modulate the function of the DnaK:DnaJ:GrpE chaperone machine. Thus, any “hits” from these screens might be expected to inhibit the complex by a number of distinct mechanisms, including direct binding to DnaK’s SBD or disrupting the interactions between DnaJ and DnaK. To illustrate the follow-up studies that we envision will be needed to deconvolute the binding site of hits, we performed exploratory experiments to identify the binding site for dihydropyrimidines on the chaperones. We considered it likely that these compounds bind to the SBD of DnaK and, therefore, modulate ATPase function. This mechanism would be similar to that thought to be invoked by other Hsp70-binding ligands, such as acidic glycolipids, phospholipids, and fatty-acylated benzamido derivatives. These compounds, with structures similar to geranylgeraniol, are reported to bind at the SBD [34,56], and the formation of an Hsp70-acidic lipid complex was hypothesized to play a role in chaperoning membrane proteins [56]. Surprisingly, we determined that, unlike geranylgeraniol, the activity of three selected dihydropyrimidines was independent of peptide competition or addition of detergent (Fig. 8). This finding suggests that dihydropyrimidines do not interact with the hydrophobic pocket of the SBD. However, we observed that adding peptide alters the compound’s IC_{50} . For example, the IC_{50} of 0116-7G drops two-fold (from 130 to 65 μM) in the presence of peptide substrate. This observation suggests that the compounds might have different affinity for the apo- and substrate-bound conformations of DnaK. Further structural studies will be required to elucidate the binding site on the chaperone, but these studies suggest that binding occurs outside the SBD.

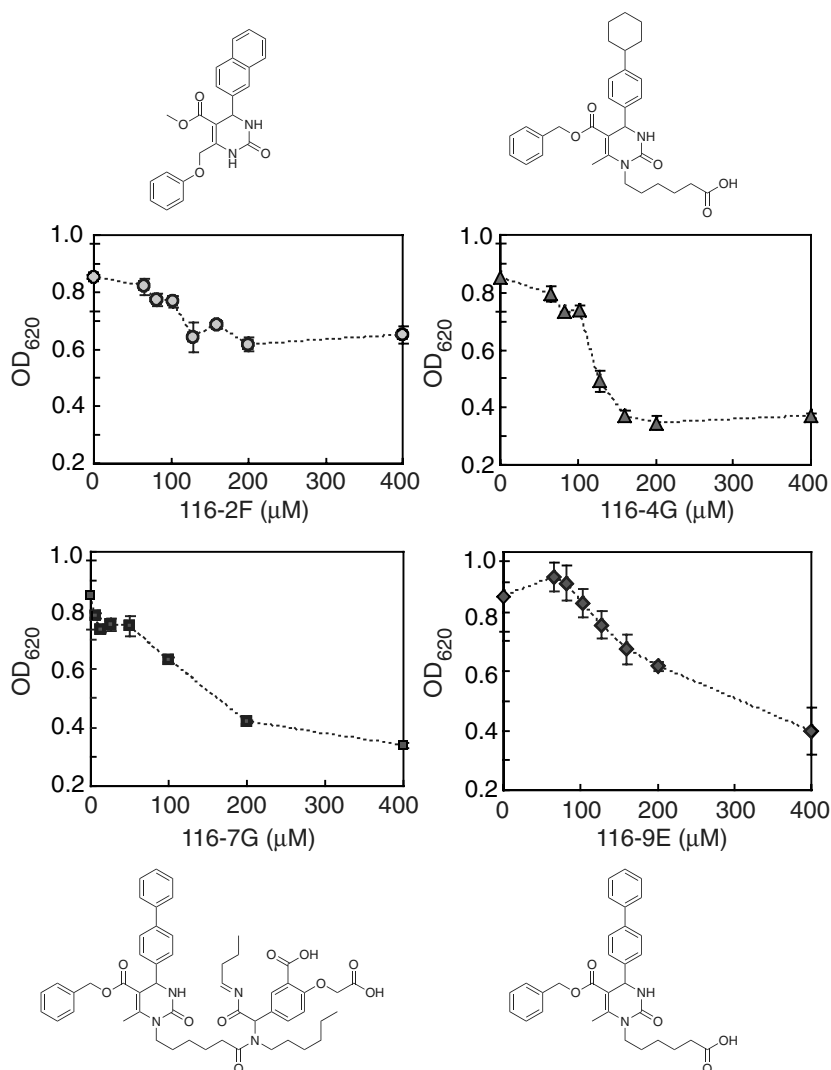


Fig. 6. Dose-dependence curves and IC₅₀ values for selected inhibitors. The curves were fitted in GraphPad Prism using the equation: $y = \text{Min} + (\text{Max} - \text{Min}) / (1 + 10^{((\text{LogIC}_{50} - x) * \text{HillSlope}))}$; ($x = \log[\text{compound}]$).

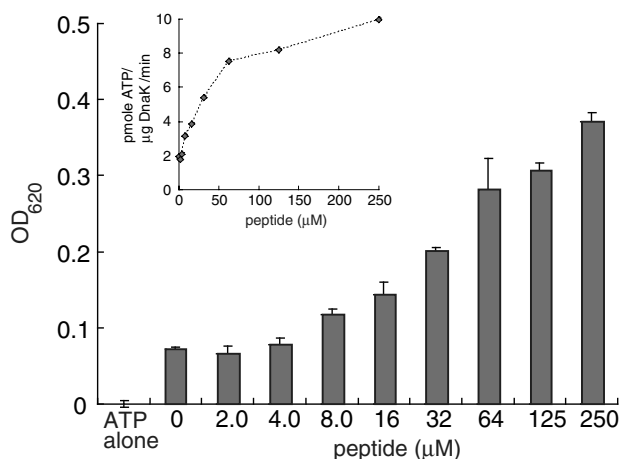


Fig. 7. Peptide substrate stimulates ATPase activity. The experiment was carried out at 0.6 μM DnaK, 1 mM ATP, and the OD₆₂₀ signal was measured after 3 h incubation at 37 °C. In the inset, the OD₆₂₀ values were converted into ATP hydrolysis rates based on a phosphate standard curve. These results are the average of triplicates and the error is standard deviation.

In conclusion, we have developed a HTS for the DnaK·DnaJ·GrpE system. This platform has potential for both uncovering new modifiers of DnaK and studying its steady state kinetics. It is worth noting that the identity of the chaperone can be varied to favor discovery of selective modulators. For example, we performed screens against rabbit Hsp70 and found a number of unique hits (L.C. and J.E.G., unpublished results). Likewise, the combination of chaperone and cochaperone might be varied to identify selective inhibitors of distinct pairs. Therefore, we expect that this assay could be used to discover compounds that block specific chaperone pathways. These chemical modulators might be used to study chaperone biology and may provide leads for developing Hsp70-targeting therapeutics and antibiotics.

Acknowledgments

The authors thank Stephan Warner, Peter Wipf, and the University of Pittsburgh's Center for Chemical Methodol-

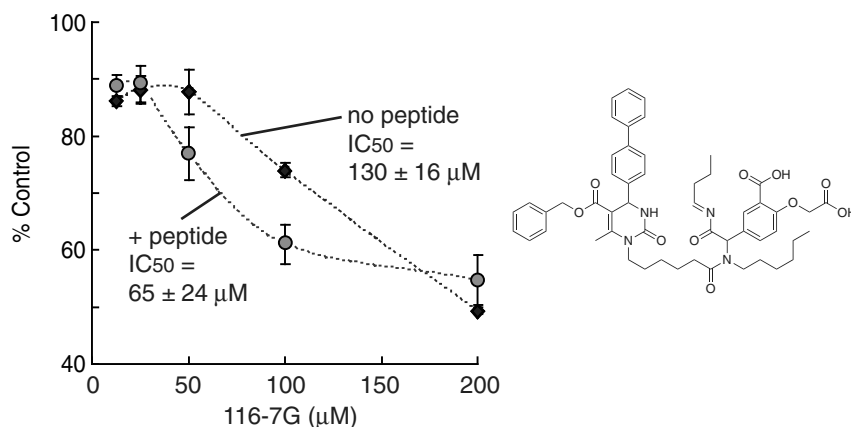
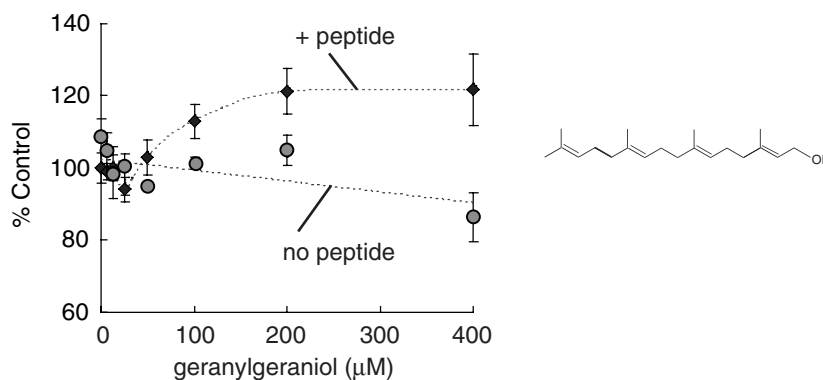
A Peptide shifted the IC₅₀ of 0116-7G but did not change its inhibitory activity**B** Peptide eliminated the stimulatory activity of geranylgeraniol

Fig. 8. Dose dependence of (A) inhibitor, 0116-7G, and (B) geranylgeraniol in the presence and absence of 50 µM SBD-binding peptide. These results are the average of triplicates and the error is standard deviation.

ogies and Library Development (funded through P50/GM067082 to P.W.) for the generous access to their chemical libraries. The authors thank Kate Carroll and the anonymous reviewers for helpful comments. Finally, we especially thank James Pankun for providing useful information. This work was supported by the University of Michigan's BioMedical Sciences Research Council, the McKnight Endowment Fund for Neuroscience, Rackham Graduate School, and an NIH grant to E.R.P.Z. (R01/GM63027).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ab.2007.08.020](https://doi.org/10.1016/j.ab.2007.08.020).

References

- [1] S.A. Rensing, U.G. Maier, Phylogenetic analysis of the stress-70 protein family, *J. Mol. Evol.* 39 (1994) 80–86.
- [2] M.P. Mayer, B. Bukau, Hsp70 chaperones: cellular functions and molecular mechanism, *Cell. Mol. Life. Sci.* 62 (2005) 670–684.
- [3] N. Morishima, Control of cell fate by Hsp70: more than an evanescent meeting, *J. Biochem. (Tokyo)* 137 (2005) 449–453.
- [4] B. Bukau, J. Weissman, A. Horwich, Molecular chaperones and protein quality control, *Cell* 125 (2006) 443–451.
- [5] M.E. Feder, G.E. Hofmann, Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology, *Annu. Rev. Physiol.* 61 (1999) 243–282.
- [6] J.C. Young, J.M. Barral, F. Ulrich Hartl, More than folding: localized functions of cytosolic chaperones, *Trends Biochem. Sci.* 28 (2003) 541–547.
- [7] A.J. Macario, E. Conway de Macario, Sick chaperones, cellular stress, and disease, *N. Engl. J. Med.* 353 (2005) 1489–1501.
- [8] P.J. Muchowski, J.L. Wacker, Modulation of neurodegeneration by molecular chaperones, *Nat. Rev. Neurosci.* 6 (2005) 11–22.
- [9] E.V. Pierpaoli, The role of Hsp70 in age-related diseases and the prevention of cancer, *Ann. N. Y. Acad. Sci.* 1057 (2005) 206–219.
- [10] S.D. Westerheide, R.I. Morimoto, Heat shock response modulators as therapeutic tools for diseases of protein conformation, *J. Biol. Chem.* 280 (2005) 33097–33100.
- [11] D.D. Mosser, R.I. Morimoto, Molecular chaperones and the stress of oncogenesis, *Oncogene* 23 (2004) 2907–2918.
- [12] J.M. Barral, S.A. Broadley, G. Schaffar, F.U. Hartl, Roles of molecular chaperones in protein misfolding diseases, *Semin. Cell Dev. Biol.* 15 (2004) 17–29.
- [13] J.F. Swain, E.G. Schulz, L.M. Gierasch, Direct comparison of a stable isolated Hsp70 substrate-binding domain in the empty and substrate-bound states, *J. Biol. Chem.* 281 (2006) 1605–1611.
- [14] J.F. Swain, G. Dinler, R. Sivendran, D.L. Montgomery, M. Stotz, L.M. Gierasch, Hsp70 chaperone ligands control domain association via an allosteric mechanism mediated by the interdomain linker, *Mol. Cell* 26 (2007) 27–39.

- [15] S.V. Slepnev, S.N. Witt, The unfolding story of the *Escherichia coli* Hsp70 DnaK: is DnaK a holdase or an unfoldase? *Mol. Microbiol.* 45 (2002) 1197–1206.
- [16] E. Cohen, R. Goldshleger, A. Shainskaya, D.M. Tal, C. Ebel, M. le Maire, S.J. Karlish, Purification of Na⁺,K⁺-ATPase expressed in *Pichia pastoris* reveals an essential role of phospholipid-protein interactions, *J. Biol. Chem.* 280 (2005) 16610–16618.
- [17] R.K. Siegenthaler, P. Christen, Tuning of DnaK chaperone action by nonnative protein sensor DnaJ and thermosensor GrpE, *J. Biol. Chem.* 281 (2006) 34448–34456.
- [18] C. Harrison, GrpE, a nucleotide exchange factor for DnaK, *Cell Stress Chaperones* 8 (2003) 218–224.
- [19] X.B. Qiu, Y.M. Shao, S. Miao, L. Wang, The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones, *Cell. Mol. Life Sci.* 63 (2006) 2560–2570.
- [20] P. Walsh, D. Bursac, Y.C. Law, D. Cyr, T. Lithgow, The J-protein family: modulating protein assembly, disassembly and translocation, *EMBO Rep.* 5 (2004) 567–571.
- [21] F. Hennessy, M.E. Cheatham, H.W. Dirr, G.L. Blatch, Analysis of the levels of conservation of the J domain among the various types of DnaJ-like proteins, *Cell Stress Chaperones* 5 (2000) 347–358.
- [22] C. Sahi, E.A. Craig, Network of general and specialty J protein chaperones of the yeast cytosol, *Proc. Natl. Acad. Sci. USA* 104 (2007) 7163–7168.
- [23] D.F. Smith, L. Whitesell, E. Katsanis, Molecular chaperones: biology and prospects for pharmacological intervention, *Pharmacol. Rev.* 50 (1998) 493–514.
- [24] S. Chaudhury, T.R. Welch, B.S. Blagg, Hsp90 as a target for drug development, *ChemMedChem* 1 (2006) 1331–1340.
- [25] Y.L. Janin, Heat shock protein 90 inhibitors. A text book example of medicinal chemistry? *J. Med. Chem.* 48 (2005) 7503–7512.
- [26] S. Ansar, J.A. Burlison, M.K. Hadden, X.M. Yu, K.E. Desino, J. Bean, L. Neckers, K.L. Audus, M.L. Michaelis, B.S. Blagg, A non-toxic Hsp90 inhibitor protects neurons from Abeta-induced toxicity, *Bioorg. Med. Chem. Lett.* 17 (2007) 1984–1990.
- [27] W. Xu, L. Neckers, Targeting the molecular chaperone heat shock protein 90 provides a multifaceted effect on diverse cell signaling pathways of cancer cells, *Clin. Cancer Res.* 13 (2007) 1625–1629.
- [28] C.-K.S. Arthur, G. Schultz, Chemistry of Naturally Occurring Polyamines. 1. Total Synthesis of Celastrol, Celastrol, and Maytenine, *J. Org. Chem.* 45 (1980) 2041–2042.
- [29] S. Komesli, C. Dumas, P. Dutartre, Analysis of in vivo immunosuppressive and in vitro interaction with constitutive heat shock protein 70 activity of LF08-0299 (Tresperimus) and analogues, *Int. J. Immunopharmacol.* 21 (1999) 349–358.
- [30] S.G. Nadler, D.D. Dischino, A.R. Malacko, J.S. Cleaveland, S.M. Fujihara, H. Marquardt, Identification of a binding site on Hsc70 for the immunosuppressant 15-deoxyspergualin, *Biochem. Biophys. Res. Commun.* 253 (1998) 176–180.
- [31] S.G. Nadler, A.C. Eversole, M.A. Tepper, J.S. Cleaveland, Elucidating the mechanism of action of the immunosuppressant 15-deoxyspergualin, *Ther. Drug Monit.* 17 (1995) 700–703.
- [32] S.W. Fewell, B.W. Day, J.L. Brodsky, Identification of an inhibitor of hsc70-mediated protein translocation and ATP hydrolysis, *J. Biol. Chem.* 276 (2001) 910–914.
- [33] S.W. Fewell, C.M. Smith, M.A. Lyon, T.P. Dumitrescu, P. Wipf, B.W. Day, J.L. Brodsky, Small molecule modulators of endogenous and co-chaperone-stimulated Hsp70 ATPase activity, *J. Biol. Chem.* 279 (2004) 51131–51140.
- [34] M. Liebscher, G. Jahreis, C. Lucke, S. Grabley, S. Raina, C. Schiene-Fischer, Fatty acyl benzamido antibacterials based on inhibition of DnaK-catalyzed protein folding, *J. Biol. Chem.* 282 (2007) 4437–4446.
- [35] C. Avila, M.K. Hadden, Z. Ma, B.A. Kornilayev, Q.Z. Ye, B.S. Blagg, High-throughput screening for Hsp90 ATPase inhibitors, *Bioorg. Med. Chem. Lett.* 16 (2006) 3005–3008.
- [36] C. Avila, B.A. Kornilayev, B.S. Blagg, Development and optimization of a useful assay for determining Hsp90's inherent ATPase activity, *Bioorg. Med. Chem.* 14 (2006) 1134–1142.
- [37] R. Howes, X. Barril, B.W. Dymock, K. Grant, C.J. Northfield, A.G. Robertson, A. Surgenor, J. Wayne, L. Wright, K. James, T. Matthews, K.M. Cheung, E. McDonald, P. Workman, M.J. Drysdale, A fluorescence polarization assay for inhibitors of Hsp90, *Anal. Biochem.* 350 (2006) 202–213.
- [38] M.G. Rowlands, Y.M. Newbatt, C. Prodromou, L.H. Pearl, P. Workman, W. Aherne, High-throughput screening assay for inhibitors of heat-shock protein 90 ATPase activity, *Anal. Biochem.* 327 (2004) 176–183.
- [39] L. Galam, M.K. Hadden, Z. Ma, Q.Z. Ye, B.G. Yun, B.S. Blagg, R.L. Matts, High-throughput assay for the identification of Hsp90 inhibitors based on Hsp90-dependent refolding of firefly luciferase, *Bioorg. Med. Chem.* 15 (2007) 1939–1946.
- [40] T.P. Geladopoulos, T.G. Sotiroidis, A.E. Evangelopoulos, A malachite green colorimetric assay for protein phosphatase activity, *Anal. Biochem.* 192 (1991) 112–116.
- [41] D.K. Fisher, T.J. Higgins, A sensitive, high-volume, colorimetric assay for protein phosphatases, *Pharm. Res.* 11 (1994) 759–763.
- [42] E.B. Cogan, G.B. Birrell, O.H. Griffith, A robotics-based automated assay for inorganic and organic phosphates, *Anal. Biochem.* 271 (1999) 29–35.
- [43] M. Zyllicz, J.H. LeBowitz, R. McMacken, C. Georgopoulos, The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an in vitro DNA replication system, *Proc. Natl. Acad. Sci. USA* 80 (1983) 6431–6435.
- [44] K. Mensa-Wilmot, K. Carroll, R. McMacken, Transcriptional activation of bacteriophage lambda DNA replication in vitro: regulatory role of histone-like protein HU of *Escherichia coli*, *EMBO J.* 8 (1989) 2393–2402.
- [45] M. Zyllicz, T. Yamamoto, N. McKittrick, S. Sell, C. Georgopoulos, Purification and properties of the dnaJ replication protein of *Escherichia coli*, *J. Biol. Chem.* 260 (1985) 7591–7598.
- [46] K. Linke, T. Wolfram, J. Bussemer, U. Jakob, The roles of the two zinc binding sites in DnaJ, *J. Biol. Chem.* 278 (2003) 44457–44466.
- [47] C.O. Kappe, High-speed combinatorial synthesis utilizing microwave irradiation, *Curr. Opin. Chem. Biol.* 6 (2002) 314–320.
- [48] S.Y. Stevens, S. Cai, M. Pellicchia, E.R. Zuiderweg, The solution structure of the bacterial HSP70 chaperone protein domain DnaK(393-507) in complex with the peptide NRLLLTG, *Protein Sci.* 12 (2003) 2588–2596.
- [49] J.S. McCarty, A. Buchberger, J. Reinstein, B. Bukau, The role of ATP in the functional cycle of the DnaK chaperone system, *J. Mol. Biol.* 249 (1995) 126–137.
- [50] T.K. Barthel, J. Zhang, G.C. Walker, ATPase-defective derivatives of *Escherichia coli* DnaK that behave differently with respect to ATP-induced conformational change and peptide release, *J. Bacteriol.* 183 (2001) 5482–5490.
- [51] A.J. Ryan, N.M. Gray, P.N. Lowe, C.W. Chung, Effect of detergent on “promiscuous” inhibitors, *J. Med. Chem.* 46 (2003) 3448–3451.
- [52] L.S. Chesnokova, S.V. Slepnev, S.N. Witt, The insect antimicrobial peptide, L-pyrrolicin, binds to and stimulates the ATPase activity of both wild-type and lidless DnaK, *FEBS Lett.* 565 (2004) 65–69.
- [53] J.L. Brodsky, G. Chiosis, Hsp70 molecular chaperones: emerging roles in human disease and identification of small molecule modulators, *Curr. Top. Med. Chem.* 6 (2006) 1215–1225.
- [54] C.T. Zhang, T.D.Y. Chung, K.R. Oldenburg, A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays, *J. Biomol. Screen* 4 (1999) 67–73.
- [55] P. Zuck, G.T. O'Donnell, J. Cassaday, P. Chase, P. Hodder, B. Strulovici, M. Ferrer, Miniaturization of absorbance assays using the fluorescent properties of white microplates, *Anal. Biochem.* 342 (2005) 254–259.
- [56] Y. Harada, C. Sato, K. Kitajima, Complex formation of 70-kDa heat shock protein with acidic glycolipids and phospholipids, *Biochem. Biophys. Res. Commun.* 353 (2007) 655–660.