HHS Public Access

Author manuscript

Curr Opin Chem Biol. Author manuscript; available in PMC 2024 July 02.

Published in final edited form as:

Curr Opin Chem Biol. 2023 October ; 76: 102373. doi:10.1016/j.cbpa.2023.102373.

Peptide-based molecules for the disruption of bacterial Hsp70 chaperones

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Abstract

DnaK is a chaperone that aids in nascent protein folding and the maintenance of proteome stability across bacteria. Due to the importance of DnaK in cellular proteostasis, there have been efforts to generate molecules that modulate its function. In nature, both protein substrates and antimicrobial peptides interact with DnaK. However, many of these ligands interact with other cellular machinery as well. Recent work has sought to modify these peptide scaffolds to create DnaK-selective and species-specific probes. Others have reported protein domain mimics of interaction partners to disrupt cellular DnaK function and high-throughput screening approaches to discover clinically-relevant peptidomimetics that inhibit DnaK. The described work provides a foundation for the design of new assays and molecules to regulate DnaK activity.

Introduction

The heat shock protein 70 (Hsp70) family aids in the maintenance of protein homeostasis, or proteostasis, across species. In bacteria, the major Hsp70, DnaK, is known as a central hub of the molecular chaperone network [1], as it collaborates with chaperonins (GroEL) [1,2], disaggregases (ClpB) [3], and other chaperones (HtpG) [4-6], as well as proteolytic machinery (Figure 1) [7]. DnaK plays an integral role in nascent protein folding [1], especially for multidomain proteins [8] and those with complex tertiary structures [1], and responds to stress by aiding in the resolution of misfolded and aggregated proteins [8,9]. Due to its varied roles, loss of *dnaK* can have pleiotropic effects. In *Escherichia coli*, Δ*dnaK* cells exhibit increased heat sensitivity [10], a hallmark of abrogated chaperone function due to proteome instability at higher temperatures. In the pathogen *Mycobacterium tuberculosis, dnaK* is essential for growth, as it is required for folding of proteins departing the ribosome [11]. In fact, mutation of *dnaK* affects the virulence of several pathogenic species [12,13], and impacts the evolution of antibiotic resistance mechanisms and antibiotic sensitivity [14-17]. Similar to the search for inhibitors of eukaryotic Hsp70s that are implicated in cancers, there has been a recent focus on the discovery and design of molecules that can disrupt bacterial DnaK activity due to its importance in survival and pathogenesis [18].

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Inhibitors of bacterial DnaK hold promise not only as antibiotics, but also as adjuvants that promote the bioactivity of existing antibiotics or counteract resistance mechanisms [19]. Finally, probes for DnaK would help to distinguish its seemingly redundant functions with other chaperones.

Targeting DnaK requires an understanding of its catalytic cycle, which is best studied in *E. coli*. DnaK is an ATP-dependent chaperone that cooperates with two co-chaperone or cofactor proteins, DnaJ, a J-domain protein, and GrpE, a nucleotide exchange factor [1,20-22]. DnaK is composed of a nucleotide binding domain (NBD) and substrate binding domain (SBD) connected by a flexible linker domain (Figure 1a). DnaJ binds non-native protein substrates and delivers them to DnaK in its ATP-bound "open" state, stimulating its ATPase activity and leading to formation of the ADP-bound "closed" state, which has up to 20-fold higher affinity for substrate than the open state (Figure 1b) [23-26]. It should be noted that DnaJ alone can interact with DnaK to stimulate its ATP hydrolysis activity [27,28]. GrpE then exchanges ADP for ATP, leading to substrate release. The cycle repeats to facilitate preferred folding trajectories and promote unfolding of misfolded states [8].

The SBDs of eukaryotic and bacterial Hsp70s show a preference for 5–7 amino acid sequence motifs with hydrophobic residues flanked by basic residues [29]. The heptameric DnaK/Hsp70 ligand NRLLLTG [30] was first discovered in peptide binding assays, and has since been studied in complex with various Hsp70 family members along with structurally similar peptides (Figure 1c). Structural work indicates that the peptide binding cleft in the SBD of Hsp70s is similar in bacteria and eukaryotes. SBDs make key contacts with five consecutive amino acid residues in protein substrates, as well as other peptide-based ligands [30-32].

While several recent reviews have highlighted current strategies for small molecule inhibition of bacterial and eukaryotic Hsp70s [18,33,34], here we focus on reports of peptide-based molecules that target different regions of DnaK. Many of these peptides have been inspired by primary sequences that are known to bind DnaK in nature, which we will discuss first. Based on growing structural information on DnaK, we highlight opportunities for Chemical Biologists to design new ligands that may represent antibiotic adjuvants or enable chemical genetic experiments to understand chaperone function across microbes.

Naturally-derived antimicrobial peptides non-selectively bind bacterial Hsp70s

Starting in the late 1980s, Proline-rich Antimicrobial Peptides (PrAMPs) were discovered from natural sources that were eventually shown to inhibit bacterial chaperones. PrAMPs are a subclass of antimicrobial peptides (AMPs) produced by insects and mammals, as detailed elsewhere [53-57]. These short (15–50 residue) host-defense peptides are primarily active against Gram-negative bacteria [54,58,59]. Their main structural characteristic is a high content of Pro residues (>25%) typically accompanied by an enrichment of basic residues in repeat sequence motifs [53]. While many sequences have a net positive charge, PrAMPs do not exhibit the non-specific membranolytic mechanism of action typically observed with other cationic AMPs [56]. In contrast, studied PrAMPs are stereospecific inhibitors that are

internalized by bacterial cells via transporter or permease activities [53,56]. Hence, PrAMPs have been proposed as an alternative strategy to existing antibiotics, as they do not lyse mammalian cells and do not show cytotoxicity at antibacterial concentrations used in host infection experiments [56]. Several well-studied PrAMPs are derived from insects, most notably pyrrhocoricin [60], drosocin [61], oncocin [62], and apidaecin, [63,64]; all of which have been shown to interact directly with DnaK (Table 1, Figure 2a) [42,46,47,63,65]. As a representative example, inspection of pyrrhocoricin-bound DnaK highlights that in addition to hydrogen-bonds with the peptide backbone, there is an abundance of van der Waals contacts between defined DnaK binding clefts and hydrophobic side chains of the peptide (Figure 2b) [42]. Across co-complex structures, the peptide binding cleft contains a buried surface area of approximately 600 $Å^2$ [42]. Structural analysis of *E. coli* DnaK bound to insect PrAMPs indicates that peptide ligands typically possess a Leu or Ile residue that fills a central deep hydrophobic pocket within the peptide binding cleft (Figures 2a-c), similar to key interactions between DnaK and NRLLLTG (Figure 1c). Interestingly, incubation of insect PrAMPs with DnaK can lead to activation of its ATPase activity, similar to the substrate analog NRLLLTG (Table 1). Some PrAMPs, such as bovine-derived Bac7, have been shown to inhibit *E. coli* DnaK-cofactor mediated protein folding *in vitro* at micromolar concentrations [36]. Hence, DnaK-PrAMP co-complexes provide an excellent resource for understanding peptide recognition by the SBD.

Cellular studies with PrAMPs have indicated that DnaK is not the primary target. Analysis of *E. coli* wild-type versus Δ*dnaK* strains showed that deletion strains remained susceptible to Bac7 [36], as well as apidaecin [45]. Photocrosslinking, binding, and translation inhibition experiments with apidaecin and oncocin analogs have demonstrated that the primary target is the 70S ribosome [45]. Other studies have indicated that PrAMPs can also disrupt the expression of chaperonins and outer membrane glycolipids in Gram-negative bacteria, as reviewed elsewhere [54]. Hence, there is opportunity for the development of engineered PrAMPs that possess greater selectivity for desired chaperone targets.

Modification of natural PrAMP scaffolds led to improved ligands for bacterial DnaK

Since PrAMPs have more than one molecular target *in vivo*, they are unsuitable for mechanistic studies on the role of DnaK in a cellular environment. Hence, several groups have sought to take inspiration from PrAMPs to produce peptides that selectively bind DnaK. Among the most promising is a PrAMP-like molecule called ARV-1502 that has reached the early stages of pre-clinical development. ARV-1502 was designed in 2005 through optimization of pyrrhocoricin-apidaecin chimeras using the sequences of thirteen insect-derived PrAMPs (Table 1, Figure 2d) [50]. An N-terminal amino-cyclohexyl carboxylic (Chex) moiety was added to the N-terminus to prevent aminopeptidase cleavage and weaken interactions with mammalian Hsp70s [65]. To promote cell permeability and proteolytic stability, ARV-1502 (also called Chex1-Arg20) was dimerized through a C-terminal 2,4-diaminobutyric acid linker, creating A3-APO (Figure 2d). ARV-1502 is believed to bind DnaK via interactions between the YLPRP motif and the peptide binding cleft of DnaK [42], along with possible contacts to the α-helical lid [69]. A3-APO is

viewed as a potential therapeutic, since it shows synergy with some antibiotics against Gram-negatives [70], and improves host survival in infection models, which has been reviewed by others [71].

Recently ARV-1502 has been further derivatized to improve its affinity for DnaK. Hoffmann and coworkers synthesized 182 derivatives to identify sequences that bound *E. coli* and *Staphylococcus aureus* DnaK by replacing at least one of the seven core residues with various side chains (Asp, Lys, Ser, Leu or Phe) (Figure 2d) [49]. One of the most hydrophobic sequences (peptide **102**) bound 10- and 20-fold better to *E. coli* and *S. aureus* DnaK, respectively, than ARV-1502. It should be noted that *E. coli* and *S. aureus* DnaK have approximately 58% sequence identity, yet can still be distinguished by a peptide-based ligand [72]. Peptide **102** showed weak binding to *E. coli* and *S. aureus* cofactors DnaJ and GrpE, and inhibited *E. coli* chaperone-cofactor folding activity. However, peptide **102** only slightly modulated the basal ATPase activity of *E. coli* and *S. aureus* DnaK. In cell-based assays, **102** showed two-fold increased minimum inhibitory concentration (MIC) values against *E. coli* compared to ARV-1502, but there was only a two-fold further increase in MIC when *dnaK* was deleted compared to wild-type. This data reinforces that DnaK is not the primary cellular target of PrAMPs. While this work provides information on a wealth of PrAMP-like sequences, it also demonstrates that different biochemical assays (*e.g.*, ATPase versus binding) might lead to conflicting predictions for optimal inhibitor sequences.

The same collection of ARV-1502 derivatives were then tested for targeting of bacterial ribosomes [73]. Fluorescently-labeled ARV-1502 bound to the 70S ribosome with almost equal affinity as observed for *E. coli* DnaK. The D_3KxxYLPRP$_{11}$ motif is thought to mediate interactions with the 50S subunit of the ribosome (Figure 2d). Only 3 out of the 182 peptides were found to bind the 70S ribosome, including a sequence with Leu_9 replaced with Lys (peptide **15**). Addition of positive charge generally promoted binding to the ribosome, perhaps through enhanced electrostatic interactions [74]. Accordingly, peptide **2**, which contains a Lys_3 residue, was one of the most potent inhibitors of the ribosome, and demonstrated slightly better growth inhibition of *E. coli* than ARV-1502 (Figure 2d). Interestingly, the hydrophobic peptide **102** showed modest inhibition (~ 25%) of translation *in vitro* at 50 μM, while an equal concentration of ARV-1502 had no effect, reinforcing that binding data does not always reflect effects in activity assays. Analysis of derivatives against *E. coli* strains with deletions in the *sbmA* and *mdtM* transporter genes thought to mediate cell uptake showed little change in growth compared to wild-type strains for most derivatives tested, suggesting alternative modes of uptake that have not yet been explored.

As an alternate approach to using PrAMP-like sequences, Dalphin et al. designed a 14-residue DnaK inhibitor, KLR-70 (Figure 2e), which resembles consensus client sequence motifs, to selectively target DnaK over ribosomal machinery [39]. A predictive algorithm for peptide substrate recognition based on rules developed by Rudiger et al. was used to computationally screen a library of 13–14 residue ligands against DnaK [29]. Then sequences were biased to bind DnaK over the cofactor DnaJ by replacing aromatic residues with aliphatic residues, and flanking positively charged residues were added [67,68]. Finally, Gly residues were introduced to reduce the charge density of the resulting peptide to avoid membrane lysis and/or non-specific electrostatic interactions. KLR-70

displays a stereo-selective, high nanomolar binding affinity for DnaK, as indicated by fluorescence polarization assays (Table 1). Since KLR-70, which lacks Pro residues, resembles NRLLLTG, it achieves enhanced binding to the peptide binding cleft, compared to the PrAMPs oncocin and pyrrhocoricin that may also bind elsewhere on DnaK [47] (Figure 2c). However, the exact binding site of KLR-70 was never confirmed. Micromolar concentrations of KLR-70 disrupted DnaK-cofactor-mediated refolding of a model substrate, but not translation in cell free extracts, and only showed slight perturbation of GroEL/ES-mediated folding. While KLR-70 binds DnaK at sub-micromolar concentrations, the authors rationalized that even small amounts (nM) of unbound DnaK can catalyze protein folding, necessitating the need for higher concentrations of peptide to inhibit chaperone activity. Further, KLR-70 must compete with DnaJ-delivered client protein in folding assays, which does not occur in DnaK binding experiments. Additionally, perhaps because *dnaK* is not essential in *E. coli*, KLR-70 is a less potent inhibitor of *E. coli* growth than the PrAMP oncocin. Interestingly, the all D-amino acid isomer of KLR-70 inhibits translation *in vitro* and shows greater toxicity against *E. coli*. While further testing is needed to confirm the targets of these peptides in living cells, along with analysis against other bacterial strains, the L- and D-KLR-70 sequences represent useful scaffolds to probe protein synthesis and folding in bacterial lysates.

KLR-70 is unique among other peptide ligands, as it shows high affinity for DnaK in the absence of any unnatural chemical modifications that often appear in designer PrAMPs [48], such as A3-APO. While it is unclear if derivatization of ARV-1502 can lead to improved selectivity for bacterial DnaK over other cellular targets, the non-proline containing KLR-70 sequence shows preference for binding to DnaK over the ribosome, indicating that DnaK-specific targeting in cells is possible.

Design of DnaJ proteomimetics enable multi-domain targeting of DnaK

While PrAMPs primarily interact with the SBD of DnaK and many small molecules that target Hsp70s bind to the NBD [33], larger molecules have the potential to target multiple domains of DnaK. Structural work has indicated that the N-terminal J-domain, which is conserved in DnaJ proteins, makes direct contacts with DnaK even in the absence of client [27,28]. In 2022, Nelson et al. synthesized mimics of mycobacterial DnaJ domains to disrupt DnaK-cofactor interactions that are essential in the pathogen *M. tuberculosis* (Mtb) [40] and other mycobacteria. Mtb contains two DnaJ proteins, DnaJ1 and DnaJ2, which each possess a conserved helix-turn-helix subdomain in the N-terminal J-domain that contacts DnaK (Figure 3a). Mutation of the His-Pro-Asp motif in the loop region is known to abrogate function [75-77]. Protein mimics of the helix-turn-helix of DnaJ1 and DnaJ2 were synthesized that maintained the turn region of each protein, and contained an optimized helix III to improve helical packing, and a consensus sequence of helix II of each DnaJ (Figure 3b). A rigid dibenzyl ether linker [78] could be installed via Cys residues to stabilize the proteomimetics. The resulting 30-mer peptide sequences, termed J1 and J2, were evaluated in both the constrained (C) and unconstrained (U) forms. The U-series lack the linker that is present in the C-series, and as a result, showed less α-helical content and stability in the presence of protease than their respective C-series sequences. While most J peptides showed low-micromolar inhibition of DnaK's cofactor-stimulated

ATPase activity (Table 1), only the constrained peptides (J1C and J2C) demonstrated high inhibition of chaperone activity at 50 μM (Figure 3b). Crosslinking experiments indicated that the structured peptides, J1C and J2C, formed contacts with both domains of DnaK [28,79], similar to native J-domains; however, the U-series mimicked unstructured substrate and only bound the SBD. Cell-based experiments using the non-pathogenic model organism *Mycobacterium smegmatis* showed that J1C was the most effective inhibitor of cell recovery following heat stress (>4-$\log_{10}$ loss of viability). J2C and the U-series only caused a 2-$\log_{10}$ and 1-$\log_{10}$ decrease in recovery, respectively. Deletion of either *dnaJ*did not impact the sensitivity of cells to J1C.

While J1C shows similar growth inhibition potency in mycobacteria as PrAMPs demonstrate against Gram-negatives (Table 1), it requires heat shock for cellular activity and additional cellular targets have not yet been evaluated. Further, the sequence of J1C is not optimized, as it differs from J2C by only three residues in the turn region. The ability to target multiple domains of DnaK using structures that are constrained and protease-resistant may prove to be advantageous compared to traditional peptides for downstream cellular experiments. Others have recently taken a cofactor proteomimetic approach to inhibit Hsp70 in cancer cells using the sequence of the cofactor HOP [80], similar to a previous study based on the interaction partner AIF [81], which provide additional examples of protein mimicry to inspire future work.

High-throughput screens for the discovery of peptidomimetic DnaK inhibitors

Due to the dearth of available chaperone ligands, many groups have sought to discover DnaK and Hsp70 inhibitors via high-throughput methods. The Gestwicki laboratory initially used ATPase activity as a readout in small molecule *E. coli* DnaK inhibitor and activator screens, as well as mechanistic studies [82-85]. Others have used computational-aided rational design [86] and NMR-based analysis [87] to discover small molecules that bind to eukaryotic Hsp70 or DnaK, respectively.

Recently, Hosfelt and Richards et al. conducted an ATPase-based high-throughput screen against Mtb DnaK and its cofactors DnaJ2 and GrpE to discover anti-infective leads against tuberculosis (TB) [35,88]. Out of >25K compounds, the authors showed that telaprevir (TP), a peptidomimetic first designed to target the Hepatitis C Virus NS3/4A protease, disrupted ATPase activity at low micromolar concentrations (Table 1, Figure 3c). TP binds with high nanomolar affinity to the peptide binding cleft of Mtb DnaK, as indicated by binding and fluorescence polarization assays, as well as photocrosslinking experiments using the analog, probe **7** (Figure 3c). In cell-based assays, TP decreased cell recovery following heat stress in *M. smegmatis* Δ*dnaJ1* cells that only contained DnaJ2, but did not affect wild-type or Δ*dnaJ2* cells. Further, folding experiments showed that DnaJ2-mediated chaperone reactions are more sensitive to TP than reactions containing DnaJ1 (Figure 3d, top). Concurrent work indicated that *dnaJ2* is necessary for chaperone-mediated resistance to the frontline TB drug rifampin in mycobacteria [14]. Accordingly, TP decreased the frequency of resistance of *M. smegmatis* to rifampin by ~ 10-fold (Figure 3d, bottom),

presumably through inhibition of DnaK-DnaJ2 function. Further, TP potentiated the growth inhibitory effect of aminoglycosides by 2- to 4-fold against mycobacteria. While TP has poor activity against wild-type *M. smegmatis* (MIC >100 μM), these results suggest that TP could be a promising adjuvant scaffold. Notably, TP has similar inhibitory activity against other members of the Hsp70 family, such as *E. coli* DnaK and human Hsc70, most likely due to high sequence [89] and structure conservation among homologs (Figure 1c), which precludes its use in host cells. Analysis of structural analogs of TP against various Hsp70s and DnaKs, along with studies on cellular uptake, may lead to more selective bacterial probes in the future.

The use of screening approaches offers expanded options to discover peptide-like ligands for bacterial versus eukaryotic Hsp70s. Others have recently used labeled NRLLLTG for fluorescence polarization-based assays to discover amino acid-based inhibitors of eukaryotic Hsp70s [18,90,91]. Oligopeptide libraries have been generated to discover ligands for the human Hsp70, HspA1A [92], along with yeast 2-hybrid-derived peptide aptamers for binding to Hsp70 in cancer cells [93]. Similar approaches for peptide library development could be leveraged for the selection of new bacterial DnaK peptide ligands.

Conclusions and implications

Although peptides are obvious scaffolds for the design of DnaK inhibitors, Chemical Biologists have only begun to expand upon peptide-based chaperone ligands and inhibitors. Much previous effort has focused on the discovery of allosteric small molecule inhibitors of DnaK that primarily bind the NBD. However, these molecules are typically micromolar inhibitors of DnaK with low selectivity, and few have been tested as antibacterials [100], as summarized elsewhere [33,34]. Many of the described DnaK peptide ligands bind to the SBD, as it contains a defined peptide binding cleft. The SBD is less conserved than the NBD across bacterial DnaKs and human Hsp70s [18]. Others have highlighted that this difference is due mainly to variation in the α-helical lid of the SBD and have proposed potential “hot spot” residues that may facilitate discrimination between different bacterial DnaKs [89]. A key advantage to targeting the SBD is that ligands do not need to compete with millimolar concentrations of cellular ATP [94,95], which is necessary for competitive inhibitors of the NBD active site [33]. However, a downside of targeting the DnaK peptide binding cleft with inhibitors is competition with client proteins that bind to the same site with low to mid-micromolar affinities [33,96-99]. Further, these unfolded substrates often increase in concentration under stress conditions. Since many of the peptide ligands described here only show high nanomolar to low-micromolar affinities to DnaK due to the shallow nature of the peptide binding cleft, a remaining challenge in the design and discovery of SBD inhibitors is improving affinities to the low nanomolar range. While some PrAMPs show sub-micromolar activity against bacterial cells, this is likely due to off-target effects. The behavior of PrAMPs with different eukaryotic Hsp70 isoforms and the effect of PrAMP glycosylation on Hsp70 interaction has also not yet been thoroughly explored. Further analysis of PrAMP cellular mechanisms might provide insight into how these peptides achieve a lack of toxicity in mammalian cells and promote specificity in targeting bacterial DnaK [54,56]. The optimization of cofactor proteomimetics, as described here, might facilitate higher potency and selectivity through increased contacts with both

the NBD, linker and SBD of DnaK. The decreased cellular permeability of larger ligands, however, may necessitate the addition of permease-targeting sequences for bacterial uptake to promote antibacterial activity [55,73,101].

Bacterial studies with ARV-1502 and TP indicate that chaperone inhibitors may serve as adjuvants that can be used to potentiate existing antibiotics or combat resistance mechanisms in Gram-negative and -positive bacterial species. Similar to connections between drug resistance and chaperones in eukaryotes, bacterial chaperone genes have recently been implicated in antibiotic resistance [14,15,102,103], which motivates continued efforts to find more potent and selective DnaK inhibitors. DnaK-binding assays with counter-screens against translation inhibition in a cell-based high-throughput format might facilitate the discovery of stable, cell-permeable molecules with desired function. There are a breadth of non-natural amino acid building blocks that could facilitate the development of leads to validate chaperones as clinically-relevant adjuvant targets.

Acknowledgements

The authors would like to thank Sterling Sandler for comments on the manuscript. TL would like to acknowledge funding from the National Institutes of Health (NIH) National Institute of General Medical Sciences (NIGMS) (5R35GM142887-02) and TL/AR would like to acknowledge NYU FAS for funding. Protein visualization and alignment were performed in PyMOL (Version 2.0, Schrodinger, LLC).

Data availability

No data was used for the research described in the article.

Abbreviations

Hsp70	70-kDa heat shock protein
NBD	nucleotide binding domain
SBD	substrate binding domain
PrAMPs	proline-rich antimicrobial peptides
TP	telaprevir
Mtb	*Mycobacterium tuberculosis*

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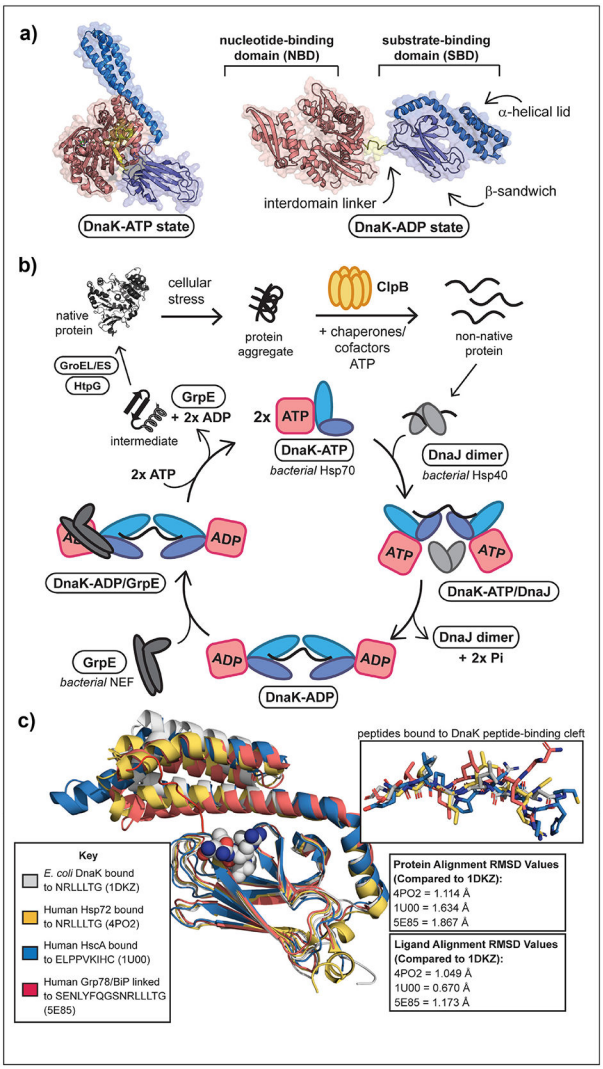

Figure 1. The DnaK cofactor network promotes protein folding.

(**a**) A cartoon depiction of *E. coli* DnaK bound to ATP (PDB: 5NRO) and ADP (2KHO). DnaK is composed of an N-terminal nucleotide binding domain (NBD, salmon) and a C-terminal substrate binding domain (SBD, blue) connected by a flexible linker (yellow). The SBD is further divided into a beta-sandwich subdomain (purple) and a C-terminal alpha-helical lid (blue). (**b**) Non-native and misfolded proteins are folded by DnaK and cofactor proteins DnaJ and GrpE. DnaJ dimers deliver non-native client proteins to DnaK in the ATP-bound state, which stimulates its ATPase activity and leads to a conformational change and increased affinity for client in the ADP-bound state. The nucleotide exchange factor (NEF) GrpE then helps exchange ADP for ATP to release client protein and restart the bind and release cycle until the protein is partially or fully folded. Binding of inhibitors to the NBD or SBD can interfere with the ATPase or chaperone activities of DnaK, leading to an increase in misfolded or unfolded proteins, which can be catastrophic for the cell. The representative protein substrate is shown in black (PBD: 3IEP). (**c**) Superposition of the crystal structures of the SBDs of *E. coli* DnaK, human Hsp72, human HscA, and human Grp78 bound to their representative peptide substrates. Root mean square deviation

(RMSD) values (all atoms) of the protein structures and peptide ligands were calculated separately based on comparison to *E. coli* DnaK SBD and ligand NRLLLTG, respectively. Alignment indicates structural similarity between *E. coli* DnaK and each Hsp70 homolog with complexed peptides. Parts (**a**) and (**b**) were modified from a previous report [35].

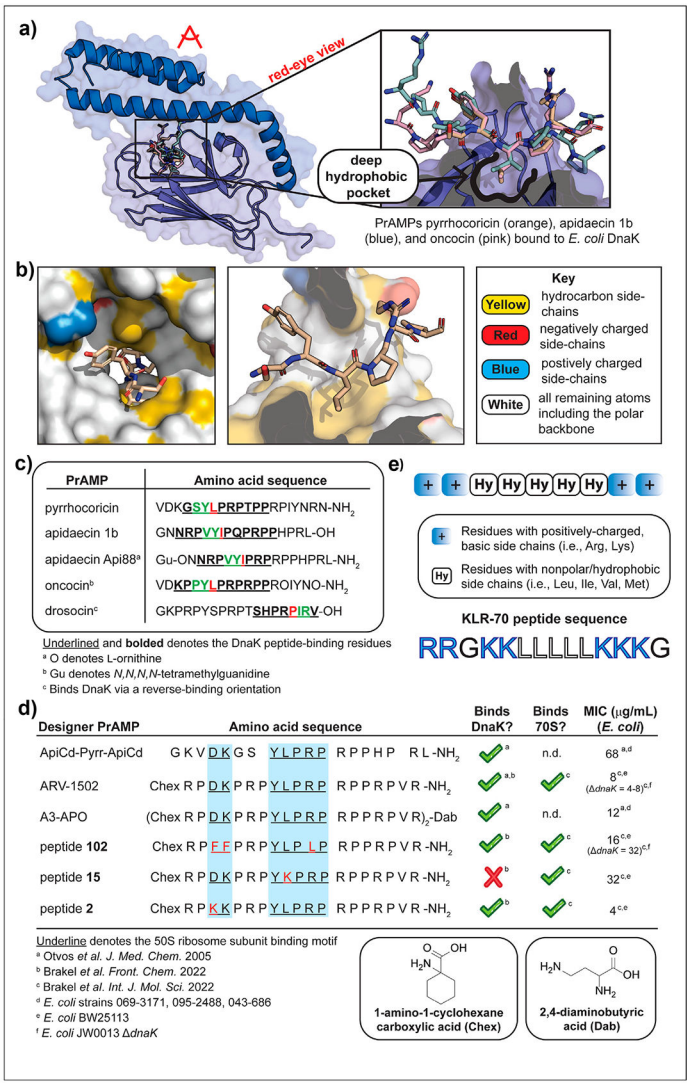

Figure 2. Proline-rich antimicrobial peptides (PrAMPs) interact with bacterial Hsp70s and other cellular targets.

(**a**) Superimposed crystal structures of the *E. coli* DnaK SBD bound to the PrAMPs pyrrhocoricin (orange, PBD: 4EZN), apidaecin 1b (blue, 4E81) and oncocin (pink, 3QNJ). (**b**) Hydrophobicity map of *E. coli* DnaK SBD bound to pyrrhocoricin (PBD: 4EZN) from (*left*) the entrance of the peptide-binding pocket and (*middle*) red-eye view of the pocket. The side-chains of the residues are colored as indicated (Hydrophobicity plugin used in PyMOL) [66]. (**c**) Table with the sequences of natural PrAMPs indicates that the deep hydrophobic pocket is typically occupied by nonpolar or hydrophobic residues (highlighted in red). Residues in green are hydrophobic residues that reside N-terminal to the deep hydrophobic pocket (except for drosocin, for which the residues are C-terminal to this pocket). Underlined and bolded residues indicate the DnaK-peptide binding region. (**d**) Table showing the sequences and behavior of designer PrAMPs that have been evaluated for interactions with DnaK and the 70S ribosome based on the chimeric PrAMP sequence in the first line. The predicted ribosome binding motif is underlined; residues that were varied in ARV-1502 derivatives are highlighted in blue. (**e**) (*top*) The predicted client peptide binding

motif that interacts with DnaK's SBD [29,39]. (*bottom*) The amino acid sequence of the KLR-70 peptide that targets bacterial DnaK but not the ribosome.

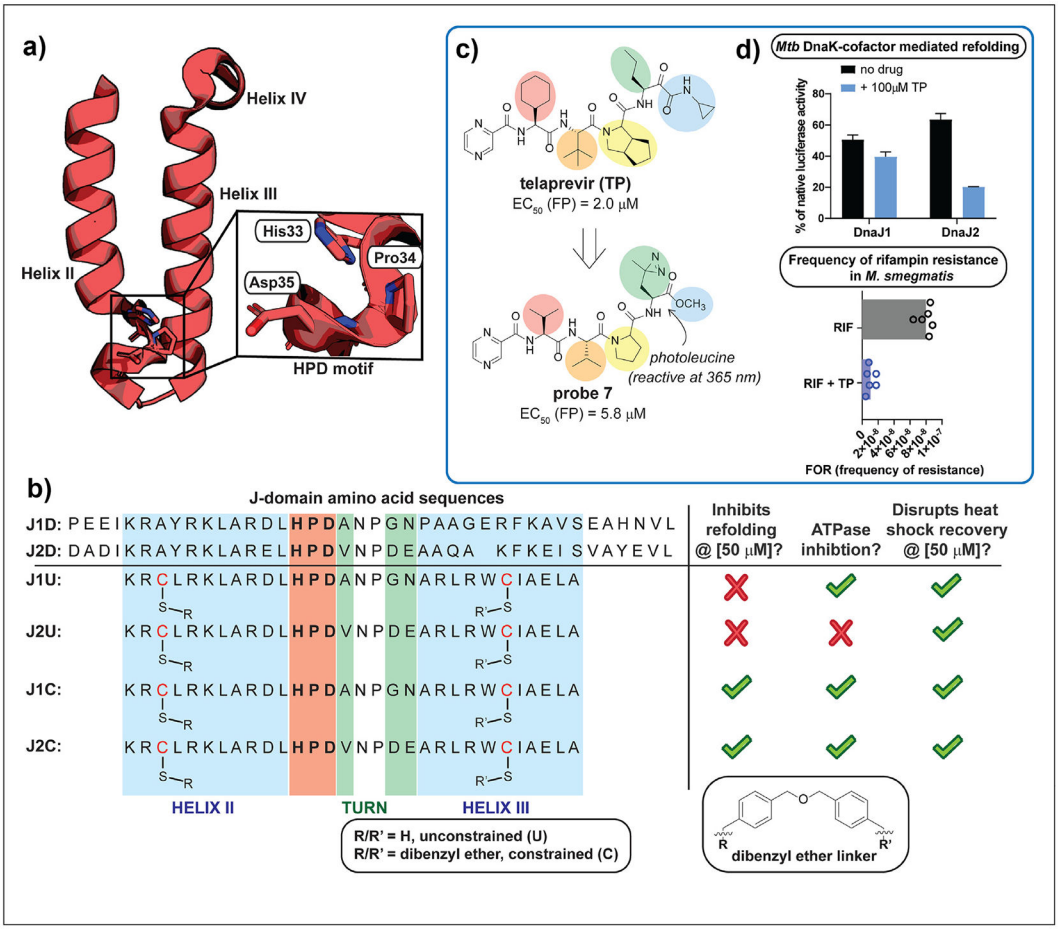

Figure 3. DnaK-cofactor interactions can be disrupted using mimics of cofactors or substrates.

(**a**) A cartoon representation of the *E. coli* N-terminal J-domain structure depicting helices II—IV, with the conserved HPD motif highlighted as sticks (PDB: 5NRO). DnaJ shown is from a co-complex with DnaK, which resembles the unbound DnaJ structure (PDB ID: 1BQ0, not shown). (**b**) Sequence alignment and behavior of the Mtb J-domain helix-turn-helix subdomains (J1D and J2D) with their respective unconstrained (J1U and J2U) and constrained (J1C and J2C) proteomimetics. Helices II and III are highlighted in blue while the HPD motif is highlighted in salmon. Residue differences between the J-proteomimetics are highlighted in green. See Table 1 for IC_{50} values for ATPase inhibition; inhibitory activity is indicated at 50 μM for *in vitro* and cellular assays based on a published report [40]. (**c**) Chemical structures of telaprevir (TP) and the photoreactive analog **7**, which interact with Mtb DnaK as indicated by fluorescence polarization (FP) and crosslinking assays. (**d**) (*top*) Denatured luciferase reactivation by Mtb chaperones DnaK and GrpE containing either DnaJ1 or DnaJ2 ± 100 μM TP demonstrates that DnaJ2 reactions are more sensitive to TP than DnaJ1 reactions. (*bottom*) TP reduces the frequency of resistance (FOR) of *M. smegmatis* exposed to high concentrations of rifampin (RIF). Data was originally reported by Hosfelt and Richards et al. [35].

Table 1

Peptide-based ligand scaffolds that bind and/or inhibit DnaK.

Ligand Name	IC_{50} (μM)[a]	K_D (μM)[b]	MIC[c]	Target Hsp70	Ref
Bactenecin-7 (Bac7) (1–35) [*]	10–50	3.3 (+ATP) 0.31 (+ADP)	0.5 μM (Δ*dnaK* = 0.5–1 μM)	*E. coli* DnaK	[36,37]
Drosocin (1–11) [*,j]	ND[d]	43.3 ± 8.7[i]	25 μM[e]	*E. coli* DnaK	[38,42]
KLR-70[j]	ND[d]	0.890 ± 0.390[h] (binding displacement) 0.158 ± 0.073[h] (gel-based assay)	ND[d]	*E. coli* DnaK	[39]
J1U[j]	10.9 ± 2.6[g]	ND[d]	ND[d]	Mtb DnaK	[40]
J2U[j]	128 ± 60[g]	ND[d]	ND[d]	Mtb DnaK	[40]
J1C[j]	14.7 ± 3.8[g]	185 ± 127[f,g]	4.17 ± 1.16 μM[g] (heat-shocked *M. smegmatis*	Mtb DnaK	[40]
J2C[j]	12.1 ± 3.9[g]	352 ± 224[f,g]	ND	Mtb DnaK	[40]
Telaprevir (TP)[j]	4.0[9]	0.590 ± 0.067[g]	>100 μM (*M. smegmatis*)	Mtb DnaK (also inhibits *E. coli*/ human Hsp70s)	[35]
Ligand Name	**AC_{50} (μM)[a]**	**K_D (μM)[b]**	**MIC[c]**	**Target Hsp70**	**Ref**
Apidaecin Api88 (1–13) [*,j]	273 ± 145[i]	5.8 ± 1.7[i]	0.44 μM (Δ*dnaK* = 0.27 μM)	*E. coli* DnaK	[41-45]
Oncocin (Onc72)[j]	31.0 ± 4.4[i]	4.0 ± 1.0[i]	6.7 μM (Δ*dnaK* = 6.7 μM)	*E. coli* DnaK	[42,45,46]
Pyrrhocoricin[j]	59.5 ± 5.9[i] 0.61 ± 0.01[i] −5.54 ± 0.19[i]	1.33 ± 0.08[i] −2.2 ± 0.4[i]	514 ± 14 μg/mL[i]	*E. coli* DnaK	[47,48]
NRLLLTG	73.0 ± 4.5[i]	1.0 ± 0.3[i]	ND[d]	*E. coli* DnaK (also binds bacterial/ eukaryotic Hsp70s)	[30,32]
ARV-1502[j]	27.9 ± 2.8[i]	0.41 ± 0.01[i]	8 μg/mL (Δ*dnaK* = 4–8 μg/mL)	Bacterial DnaK	[49-52]

[*] Refers to residues that were studied for indicated PrAMPs.

[a] IC_{50} refers to the half maximal inhibitory concentration, while AC_{50} refers to the half maximal activation concentrations, which are measured using ATPase or chaperone activity assays.

[b] Binding affinities measured by isothermal titration calorimetry or fluorescence displacement assays unless otherwise noted.

[c] MIC indicates minimum inhibitory concentration measured against *E. coli* unless otherwise noted.

[d] ND indicates “not determined”.

[e] Measurement was made for full-length Drosocin (full sequence provided in Figure 2c).

[f] Measurement signifies binding affinity of indicated J-domains (not proteomimetics).

[g] Error shown represents 95% confidence intervals.

[h] Error shown represents standard deviation (SD).

[i] Error not specified.

[j] Sequences of relevant peptides are shown in Figures 2 and 3.