

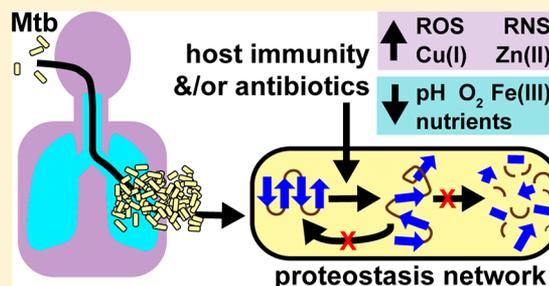
Targeting the Proteostasis Network for Mycobacterial Drug Discovery

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ABSTRACT: Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains one of the world's deadliest infectious diseases and urgently requires new antibiotics to treat drug-resistant strains and to decrease the duration of therapy. During infection, Mtb encounters numerous stresses associated with host immunity, including hypoxia, reactive oxygen and nitrogen species, mild acidity, nutrient starvation, and metal sequestration and intoxication. The Mtb proteostasis network, composed of chaperones, proteases, and a eukaryotic-like proteasome, provides protection from stresses and chemistries of host immunity by maintaining the integrity of the mycobacterial proteome. In this Review, we explore the proteostasis network as a noncanonical target for antibacterial drug discovery.

KEYWORDS: proteostasis, proteolysis, chaperones, proteasome, proteases, protein aggregation, protein folding, protein misfolding, proteostasis network, *Mycobacterium tuberculosis*, antibiotics, host immunity



Antibiotic-resistant bacterial pathogens, including *Mycobacterium tuberculosis* (Mtb), the etiological agent of tuberculosis (TB), present a serious health crisis. TB is now the leading cause of death from an infectious disease and at the forefront of diseases in dire need of new antibiotics. Treatment of drug-sensitive TB requires 6–9 months of therapy with up to four antibiotics, including the first-line drugs rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (EMB). Multidrug resistant TB (MDR-TB), defined by resistance to RIF and INH, and extremely drug resistant TB (XDR-TB), defined by resistance to RIF, INH, a fluoroquinolone, and one of three injectable second-line drugs (amikacin, kanamycin, capreomycin), may require up to an additional 1.5 years of treatment (www.cdc.gov/tb).¹ Other treatment options for TB often lack extensive data on efficacy and/or long-term safety, including bedaquiline (BDQ), delamanid (DLM), linezolid (LZD), clofazimine (CFZ), and β -lactams paired with β -lactamase inhibitors, such as meropenem–amoxicillin–clavulanate.^{1,2} Bleak reports from the front lines of drug discovery indicate that the antibiotic pipeline is dwindling.³ To discover new antibiotics for the treatment of drug-resistant strains and to shorten therapy, we must consider adopting new drug discovery strategies. In this Review, we explore the proteostasis network and evaluate its suitability as a noncanonical target for the treatment of tuberculosis.

IN PURSUIT OF NONCANONICAL TARGETS FOR ANTIBIOTIC DRUG DISCOVERY

Most antibiotics were discovered by their ability to interfere with the synthesis of macromolecules such as DNA, RNA, lipids, proteins, and peptidoglycan and the vitamin folate, when

bacteria are replicating in a synthetic growth medium in a laboratory.^{3–5} Antibiotics discovered using this strategy may fail to translate to a human host where conditions are often not conducive for Mtb replication.⁵ Mtb coevolved with the human host for tens to hundreds of thousands of years,⁶ and TB transpires from a complex interplay between Mtb, host immunity, host microenvironments, and host chemistries. Mtb can survive in a latent state for decades in a human.^{7,8} From the time Mtb is inhaled in an aerosolized droplet until the time the bacilli are eradicated by host immunity and/or with antibiotics, Mtb faces numerous cell types and host chemistries. Upon entering the respiratory tract, Mtb is ingested by alveolar macrophages and traffics to a phagosome. T-cells produce interferon- γ (IFN γ) that activates Mtb-infected macrophages, which is accompanied by a dramatic transcriptional change of both Mtb and the macrophage,^{9,10} and the Mtb-containing phagosome matures into a hostile environment that can slow or abrogate Mtb growth. Mtb can encounter suboptimal growth conditions in the caseum of a granuloma composed of necrotic macrophages, neutrophils, T cells, and B cells.¹¹

Mtb has adapted to endure a diverse set of microenvironments in the host, including spatial compartments (lung, spleen, liver, brain, and adipose tissue), extracellular compartments (blood, granulomas, and caseum), and cell types (macrophages, dendritic cells, and neutrophils). Each microenvironment's unique combination of carbon and nitrogen sources, micronutrients, pH, metal concentrations, gases, such

Special Issue: Drug Discovery for Global Health

Received: November 14, 2017

Published: February 21, 2018

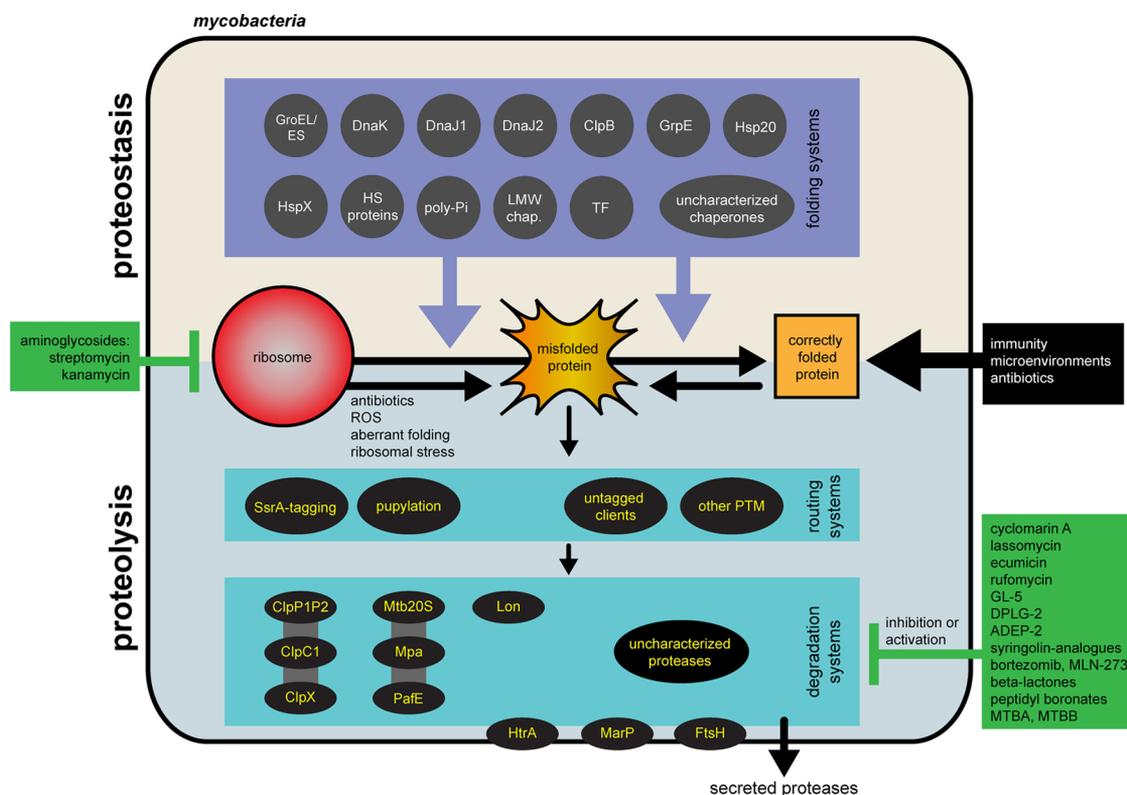


Figure 1. Schematic of the mycobacterial proteostasis network. Mycobacteria possess numerous systems for maintaining protein structure and function via proteostasis (tan) or proteolysis (light blue). While proteolysis systems have become a rich source of antibiotic candidates, there are no described inhibitors of any members of mycobacterial chaperones or their accessory proteins. ClpP1P2 is associated with ClpC1 and ClpX, and Mtb20S is associated with Mpa and Paf. Lon is not found in pathogenic Mtb. The list of inhibitors targeting the Mtb proteolysis system is not comprehensive. HS proteins = heat shock proteins; poly-Pi = polyphosphate; LMW chaperones = low molecular weight chaperones; HspX = Acr1/ α -crystallin 1; Hsp20 = Acr2/ α -crystallin 2; TF = Trigger Factor; PTM = post-translational modification; ROS = reactive oxygen species.

as oxygen, carbon monoxide, carbon dioxide, and nitric oxide, and host immune effectors, encourages Mtb to proliferate, cease replicating and remain metabolically active, remain metabolically active and nonculturable, die by starvation for essential nutrients, or die after insult by host immune effectors.⁵

In many patients with TB, the foregoing immune mechanisms fail to eradicate Mtb without the assistance of antibiotics. TB is recalcitrant to antibiotic treatment, in part, due to populations of phenotypically tolerant Mtb (nongenetic drug resistance)^{5,12} generated by host immune chemistries and host microenvironments. Drug-tolerant Mtb persisters exist in animal models of tuberculosis^{13–15} and in humans.¹⁶ Phenotypic drug tolerance may result from arrest of replication for individual bacteria within a larger population (Class I persistence) or synchronous arrest of replication of a population of bacteria in response to an extrinsic stress (Class II persistence). While often associated with nonreplication, Class I drug tolerance may also result from phenomena unrelated to nonreplication,^{17,18} including stochastic expression of INH's activating enzyme, catalase–peroxidase (KatG),¹³ or mistranslation of RIF's target, RNA polymerase subunit RpoB.^{19,20} The mechanisms leading to Class II persistence are different from those leading to Class I persistence.¹⁸ For example, nonreplicating Class II persisters may have different uptake, retention, and metabolism of drugs.^{5,18,21} The Class II definition of persistence is further demarcated by the ability of bacteria, upon removal of the persistence-inducing stress, to recover as colonies on solid agar (Class IIa) or recover exclusively in liquid medium upon serial

dilution (Class IIb).^{22,23} Bacteria exhibiting Class I phenotypic tolerance to one drug will often remain sensitive to other antibiotics,²⁴ while bacteria exhibiting Class II phenotypic tolerance are broadly resistant to numerous antibiotics.^{18,25–28} Defining Class I and Class II persisters lays a foundation for designing rational strategies to treat human TB with antibiotics.^{12,18} Thus, in addition to targeting actively replicating Mtb, decreasing the time of TB treatment will require additional antibiotics that eradicate Class I, IIa, and IIb drug-tolerant Mtb persisters.^{5,12}

TB drug discovery may benefit from embarking on a risky journey into target space enriched for noncanonical essential processes or pathways whose essentiality only becomes apparent during nonreplication and/or infection of a host.^{4,12} In fact, the first new TB drug in the last 40 years, BDQ, has a noncanonical target and kills both replicating and nonreplicating forms of Mtb by disrupting energy production via the C-subunit of ATP synthase.^{29,30} Other noncanonical antibiotic targets include redox homeostasis, cofactor/vitamin synthesis, metal homeostasis and uptake, signaling, and virulence factor production.⁴ Some examples of noncanonical target enzymes/pathways are thioredoxin reductase,^{31,32} dihydrolipoamide acyltransferase,³³ depolarization of the bacterial membrane,^{34–36} and the proteolytic machinery.^{37–40} Of these noncanonical targets, the proteostasis network, consisting of chaperones that help fold and maintain client protein structure (proteostasis) and proteases that degrade client proteins (proteolysis), is particularly intriguing due to its

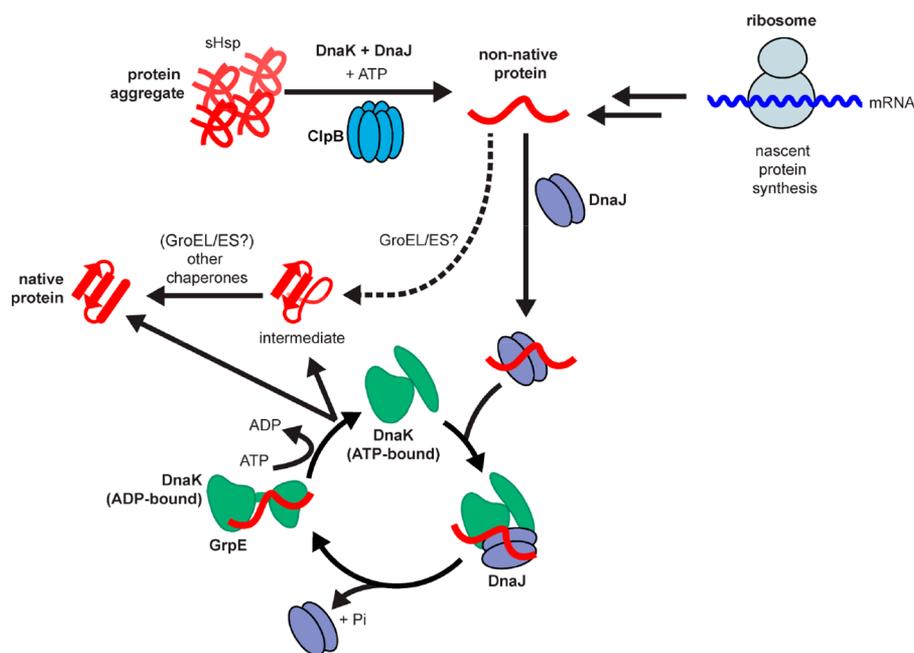


Figure 2. Function of mycobacterial protein chaperones includes both nascent protein folding and protein aggregate reactivation. Protein aggregates resulting from stress due to aging or antibiotic treatment are thought to be bound by small heat shock proteins (sHsps) prior to unfolding by the chaperone DnaK, its cofactor DnaJ, and the disaggregase ClpB in an ATP-dependent process that releases non-native proteins. These non-native proteins, which also result from ribosomal synthesis, are bound by DnaJ proteins, which then interact with DnaK in an ATP-bound state to stimulate ATP hydrolysis by DnaK. Once DnaK is converted to an ADP-bound state with attached protein substrate, the nucleotide-exchange factor GrpE binds to DnaK, causing release of ADP and binding of ATP to restart the catalytic cycle. The cofactor binding cycle repeats until the substrate is either released as a partially folded intermediate that is handled by other chaperones (such as Mtb GroEL/ES or HtpG) or released in a fully folded state. Mtb GroEL/ES may also act at earlier stages in the folding process, perhaps interacting with non-native proteins from the ribosome. Adapted with permission from Kim, Y. E., Hipp, M. S., Bracher, A., Hayer-Hartl, M., and Hartl, F. U. (2013) *Annu. Rev. Biochem.* 82, 323–355.⁴⁶ Copyright 2013 Annual Reviews.

potential to disrupt homeostatic levels of hundreds of client proteins.⁴¹

■ THE MYCOBACTERIAL PROTEOSTASIS NETWORK

The proteostasis network plays a critical role in Mtb survival against host immune stresses. In the absence of a functional proteostasis network to protect proteins, immune insults may irreversibly damage Mtb proteins and ultimately cause its death. For this critical role in protecting protein homeostasis, Mtb encodes over 100 proteins predicted or experimentally demonstrated to have a role in protein degradation (proteases, amidohydrolases, and the proteasome) or protein folding (chaperones and accessory proteins). The mycobacterial proteostasis network is summarized in Figure 1.

Protein Maintenance by Chaperones and Their Cofactors. The molecular players in mycobacterial proteostasis are important in maintaining protein structure and function during normal cell growth and become essential in preventing damage and repairing proteins during periods of stress. DnaK (encoded by *rv0350*), the prokaryotic homologue of heat shock 70 kD proteins (Hsp70s), is an ATP-powered protein chaperone that is conserved from bacteria to humans. Several Hsp70-family members exist in eukaryotes, spanning different cellular compartments, with functions that include folding of nascent proteins, prevention and reactivation of protein aggregates, and intracellular trafficking.^{42–45} Along the same lines, *Escherichia coli* DnaK has been shown to be an important hub in the bacterial proteostasis network, as it is involved in protein folding, stabilizing non-native proteins for eventual folding by other chaperones, and mediating transfer of unfolded

proteins to proteases for degradation.^{46,47} It has only recently been revealed that the specific roles of DnaK relative to other protein chaperones differ in mycobacteria compared to *E. coli*. In *Mycobacterium smegmatis* (Msm), a nonpathogenic, fast-growing mycobacterial saprophyte used as a model organism, DnaK is essential for cell growth and folding of nascent proteins following synthesis by the ribosome.⁴⁸ Whole genome transposon-insertion mutant library analysis predicts that *dnaK* is also essential for viability of Mtb.⁴⁹ However, DnaK is only conditionally essential in bacteria such as *E. coli*, in which deletion mutants can grow at low temperature (30 and 37 °C) but not at high temperature (42 °C).⁵⁰ Additionally, the essential function of *E. coli* DnaK overlaps with that of the chaperone Trigger Factor (TF, encoded by *rv246c/tig*), as suggested by the observation that *dnaK* and *tig* form a synthetic lethal pair.^{51–53} Hence, the cellular roles of DnaK in mycobacteria cannot be extrapolated from studies in other organisms.

In mycobacteria, along with some Gram-negative and other high-GC-content Gram-positive bacteria, the repressor HspR (encoded by *rv0353*) regulates the expression of DnaK and a number of other heat shock-associated proteins.^{54,55} HspR is encoded on the same operon as *dnaK* and controls *dnaK*'s expression by binding an upstream HAIR (HspR-associated inverted repeat) sequence. DnaK modulates HspR binding to DNA through a proposed interaction with the unfolded C-terminal tail of HspR following denaturation by high temperatures.^{56,57} HspR also negatively regulates the expression of *clpB*, which encodes the protein disaggregase ClpB (encoded by *rv0384c*), and *acr2* (encoded by *rv0251c*), which encodes a

small heat shock protein called α -crystallin 2, or Hsp20. While *hspR* is under standard replicating conditions, infection of a mouse model with Mtb deficient in *hspR* revealed decreased viability compared to wild-type during the chronic phase of infection.⁵⁸ One explanation for this observation is that the Δ *hspR* mutant's lack of repression led to the overproduction of chaperones and increased protein secretion, which in turn stimulated the host's immune response. Indeed, purified recombinant mycobacterial DnaK bound to peptide can act as an adjuvant *in vitro*.^{59–61}

The *dnaK* operon also contains the protein cofactor-encoding genes *dnaJ1* (encoded by *rv0352*) and *grpE* (encoded by *rv0351*). In bacteria, DnaK's protein folding activity is modulated by these cofactors, DnaJ (the homologue of eukaryotic Hsp40) and GrpE, a nucleotide exchange factor (NEF). DnaJ–proteins bind aggregated or non-native proteins prior to their delivery to DnaK in its ATP-bound state. Upon binding, the DnaJ–substrate complex activates ATP hydrolysis by DnaK, which is converted to an ADP-bound state. GrpE then mediates release of ADP by DnaK, which then binds ATP, allowing the reaction cycle to repeat until the substrate protein is folded to its native form or handed off to other chaperones in a semifolded state⁴⁶ (Figure 2). The structural architecture of Hsp70s from all domains of life is highly conserved, with an N-terminal nucleotide binding domain (NBD) and a C-terminal substrate-binding domain (SBD) connected by a flexible linker.⁴⁴ Cofactors predominantly interact with the N-terminal domain of Hsp70s.⁶² While eukaryotes possess many DnaJ–proteins, this is not the case in prokaryotes, where only GC-rich Gram-positive actinobacteria⁶³ such as mycobacteria have more than one annotated *dnaJ* gene. Mtb has two copies of DnaJ, encoded by *dnaJ1* and *dnaJ2* (encoded by *rv2373c*). Studies on the overexpression of Mtb DnaJ1 and DnaJ2 suggest that each has a different cellular role⁶⁴ and their encoding genes evolved separately. Genetic studies have recently shown that Msm *dnaJ1* and *dnaJ2* are individually dispensable but collectively required for cell growth.⁶⁵ *grpE* is essential in Msm⁴⁸ and anticipated to be essential in Mtb, which has only a single annotated NEF in the genome.

The other conserved protein chaperone genes regulated by HspR, *clpB* and *acr2*, encode molecules that prevent and resolve protein aggregates in cooperation with the DnaK/cofactor system. Mtb *acr2* regulation by HspR is modulated by interaction of the repressor with the response regulator PhoP.⁶⁶ Bacterial ClpB, a homologue of eukaryotic Hsp104, is a hexameric disaggregase AAA+ ATPase (ATPase associated with various cellular activities) that hydrolyzes ATP while mediating protein unfolding through its central pore. DnaK and DnaJ–proteins deliver partially unraveled aggregated substrates to ClpB through direct interaction with the NBD of DnaK that competes for binding with GrpE.⁶⁷ Small heat shock proteins (sHps) like mycobacterial α -crystallin 2 (Hsp20) and α -crystallin 1 (HspX; encoded by *rv2031c*) are typically annotated as HSPB (heat shock protein family B) in humans and Ibp (inclusion body protein) proteins in *E. coli*. Mtb lacks a third *acr* gene found in other mycobacteria such as Msm, *M. marinum* and *M. leprae*.⁶⁸ sHps form oligomers that, in a nucleotide-independent manner, prevent aggregation by binding to proteins or assist other chaperones in protein disaggregation.⁶⁹ Comparison of transcriptomes of Mtb under various growth and stress conditions shows that *clpB* expression is highly correlated with *acr2* expression,⁷⁰ suggesting that the encoded proteins cooperate in their cellular functions. While *clpB* is not

essential under normal growth conditions, Mtb lacking *clpB* is defective for growth and virulence in a mouse model.⁷¹ Similarly, while neither *acr1* nor *acr2* is essential in Mtb under standard growth conditions, deletion of *acr2* reduces the pathogenesis of Mtb in infected mice.⁶⁸ Hence, these stress response genes are not required under standard laboratory conditions; however, under stresses that damage biomolecules, such as host infection, sublethal antibiotic treatment, or nonreplication, these genes encode proteins that constitute important pathways that repair aggregated or modified proteins.

DnaK, cofactor proteins, ClpB, and sHps play important roles in reactivating damaged proteins. Microscopy studies in live mycobacteria have shown that ClpB and DnaK are recruited to a fluorescently tagged aggregating protein sequence (ELK16) and that tagged chaperones form foci that colocalize with these aggregates.^{48,71} Using aggregated luciferase as a model protein substrate, biochemical reconstitution of this proteostasis network consisting of Mtb DnaK, cofactors, ClpB, and Hsp20 showed that GrpE and at least one DnaJ–protein is required for luciferase refolding and that DnaJ1 and DnaJ2 exhibit nonredundant roles in protein folding.⁶⁵ The *in vitro* requirements for these proteins in refolding reactions are consistent with *grpE*'s essentiality and *dnaJ1* and *dnaJ2*'s synthetic lethality in Msm. Differences in the activity of mycobacterial DnaJ proteins were also evident in ATPase assays that showed DnaJ1 and DnaJ2 activate ATP hydrolysis by DnaK in the presence of GrpE to different degrees. Point mutations of a conserved three amino acid (histidine–proline–aspartate) motif^{72–74} in the N-terminal J-domain of DnaJ1 or DnaJ2 abrogate the ability of J-proteins to activate DnaK's ATPase activity, inhibit aggregate reactivation when added to the reconstituted ClpB/DnaK/cofactor proteostasis system, and result in loss-of-function of DnaJs in mycobacterial cells. Since the DnaJ2 point mutant in this motif binds DnaK with affinity similar to the wild-type protein, these observations suggest that the native DnaK–DnaJ interaction can be disrupted. Targeting this interaction with selective small molecules could be lethal in mycobacteria,⁷⁵ similar to what has been proposed for homologues in eukaryotes,⁷⁶ in which the function of human Hsp70 proteins is essential in certain cell types, including some cancer cells.⁷⁷

In bacteria, the GroE chaperone system often functions in parallel and downstream of the DnaK system. GroE consists of the chaperonin GroEL (Hsp60) and cochaperonin GroES (Hsp10) and is the only temperature-independent essential chaperone system in *E. coli*.^{78,79} (Table 1). The GroE system binds hydrophobic patches on polypeptides that are exposed during translation and helps stabilize folding intermediates by encasing them in a central channel.^{46,80} The GroE system also helps fold newly synthesized proteins that TF and DnaK cannot

Table 1. Select Chaperone Network Proteins and Their Essentiality for Optimal Growth *in Vitro*

protein	<i>E. coli</i>	<i>M. smegmatis</i>	<i>M. tuberculosis</i>
Trigger Factor	nonessential ⁵³	nonessential ⁴⁸	nonessential ^a
DnaK	nonessential ⁵⁰	essential ⁴⁸	essential ^a
GrpE	nonessential ²³²	essential ⁴⁸	essential ^a
DnaJ	nonessential ⁷⁴	DnaJ1 and DnaJ2 collectively essential ⁶⁵	DnaJ1 and DnaJ2 essential ^a
ClpB	nonessential ²³³	nonessential ⁷¹	nonessential ⁷¹

^a<https://mycobrowser.epfl.ch/>.^{49,101}

fold. GroE consists of two stacked heptameric rings, forming a barrel-like structure that is capped by two heptameric GroES subunits on either end.⁸¹ The canonical GroE system typically has strong ATPase activity and forms higher oligomeric structures.⁸² GroE from *Mtb*, however, is a dimer with weak ATPase activity that can partially refold proteins *in vitro*.⁸² Mycobacteria and other actinomycetes are unique in that they encode two copies of the chaperonin GroEL: GroEL1 (encoded by *rv3417c*) and GroEL2 (encoded by *rv0440*). *groEL1* is in an operon with *groES* (encoded by *rv3418c*), and *groEL2* is encoded elsewhere in the genome. Only GroEL2 is essential for *in vitro* survival of *Mtb*,⁸³ suggesting its clients are essential proteins. Interestingly, the GroEL chaperonins are potent immunomodulators. GroEL2 is an immunodominant mycobacterial antigen,⁸⁴ and both GroEL1 and GroEL2 play a role in cytokine induction and stimulating the host's immune response to *Mtb* infection.^{83,85,86}

The ClpB/DnaK and GroE system in *Mtb* is important for the proper folding of proteins and polypeptides under standard growth conditions as well as under conditions of stress. The expression of *dnaK*, *clpB*, *dnaJ1*, *grpE*, *acr2*, *groEL1*, and *groEL2* is upregulated at elevated temperature.⁵⁴ Upon phagocytosis in macrophages, *groEL1*, *groEL2*, and *acr* are upregulated in mycobacteria.^{8,5}

Other annotated chaperones present in the genome in *Mtb* have not been well-studied. For example, HtpG,⁸⁸ the homologue of the eukaryotic protein chaperone Hsp90, is encoded by *rv2299c* in *Mtb* and is absent in *Msm*. While Hsp90 interactions in a larger protein network have been well-described,⁸⁹ little is known of the interaction between HtpG and other proteostasis proteins in *Mtb*; hence, much remains to be determined in the network of proteins responsible for forming and maintaining protein structures in mycobacteria.

Protein Degradation by Proteases and the Proteasome. In addition to proteostasis systems that maintain proteins in their native conformation, the proteostasis network encompasses proteases that degrade damaged, aggregated, and overexpressed protein or proteins no longer needed by the cell.

Actinomycetes, including mycobacteria, encode four major cytosolic compartmentalized ATP-dependent proteases: ClpP, HslUV, Lon, and the proteasome. Neither HslUV nor Lon are present in *Mtb*. We will focus on the two cytosolic compartmentalized, two-component ATP-dependent proteases present in the pathogen *Mtb*: ClpP and the proteasome.

Under standard growth conditions, many *Mtb* proteins are degraded by the Clp (caseinolytic) protease.⁹⁰ The Clp protease is comprised of a barrel shaped protease (ClpP) and an AAA+ ATPase (ClpC and ClpX in *Mtb*; see below).⁹¹ Clp proteases are present in eubacteria as well as in chloroplasts and mitochondria.⁹² In addition to misfolded or abnormal protein substrates, the Clp protease recognizes substrates that have been cotranslationally modified at the C-terminus with the 11 amino acid SsrA tag.^{91,93} Under standard growth conditions, in *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus*, the homotetradecamer Clp protease is nonessential; however, ClpP has a critical role for virulence (*S. aureus*) or under stresses.^{91,94} In *Mtb*, the Clp protease consists of a heterotetradecamer comprised of two subunits, ClpP1 and ClpP2, that possess chymotrypsin and caspase-like activities.⁹⁵ The ClpP1P2 protease of *Mtb* is essential both for *in vitro* replication⁹⁰ and in a mouse model of *Mtb* infection, where genetic knockdown of ClpP1P2 leads to decline in CFU in mouse lungs during the acute phase of infection and leads to

the rapid elimination in mice by day 30.^{90,96,97} Under nonstress conditions, over 100 candidate substrates were identified in a regulated knockdown of ClpP1P2 in *Mtb*.⁹⁸ This indicates that targeting ClpP1P2 with small molecules could cause dysregulation of numerous proteins under standard growth conditions and additionally lead to secondary effects by dysregulation of transcription factors, such as WhiB3, and their target genes.⁹⁸

The Clp protease complex also has AAA+ ATPases, similar to ClpB, that interact with protein targets and use the energy from ATP hydrolysis to unfold protein substrates and feed them into the protease's central pore. In *Mtb*, ClpC1 (encoded by *rv3596c*) interacts with ClpP1P2 to degrade a model substrate and has ATPase and chaperone activities (prevention and reactivation of luciferase aggregates).^{99,100} ClpX (encoded by *rv2457c*) is another AAA+ ATPase that interacts with and serves as a chaperone for ClpP1P2 in *Mtb*.⁹⁹ While both ClpC1 and ClpX are predicted to be essential for *Mtb* survival *in vitro*, to date, no reports have confirmed this experimentally.^{101,102}

The proteasome (PrcBA, herein Mtb20S), encoded by genes *rv2110c* and *rv2109c* (β and α subunits, respectively), was first identified in bacteria based on its sequence similarity to the eukaryotic proteasome.¹⁰³ The eukaryotic proteasome degrades the majority of damaged, unfolded, or unwanted proteins in the cell and plays an essential role in cell survival.¹⁰⁴ The eukaryotic core particle (CP) consists of heteroheptameric 14 α and 14 β subunits with peptidase activity afforded by the N-terminal threonine nucleophile of the β -subunit. In addition to the CP, the eukaryotic proteasome consists of a regulatory particle that flanks one or both ends of the CP and recognizes poly ubiquitin (Ub)-tagged substrates, removes Ub from tagged substrates, unfolds the substrates, and then delivers the substrates to the CP for degradation.¹⁰⁵ The prokaryotic proteasome is present in archaea but limited to the bacterial orders Actinomycetales and Nitrosporaes. The bacterial CP consists of homoheptameric 14 α and 14 β subunits with peptidase activity similar to that of eukaryotes.¹⁰⁶ Unlike the eukaryotic proteasome, where the majority of the accessory factors have been characterized, the bacterial proteasome accessory factors are still being identified. Mpa (mycobacterial proteasome ATPase, encoded by *rv2115c*), an AAA+ ATPase, recognizes pupylated substrates, unfolds them, and delivers at least some for degradation by the CP.^{107,108} Pup (prokaryotic ubiquitin-like protein, encoded by *rv2111c*) is a protein tag with function analogous to that of poly-Ub but without sequence or structural homology. Pup is intrinsically disordered^{109,110} and PafA (proteasome accessory factor A, encoded by *rv2097c*) attaches Pup to the ϵ -amino group of lysine residues on proteins^{111,112} after Pup activation by Dop (deamidase/depupylase of Pup, encoded by *rv2112c*).^{113,114} Dop also serves as a depupylase and removes Pup from protein substrates prior to entry into the proteasome. PafE (proteasome accessory factor E, encoded by *rv3780*), an ATP- and Pup-independent unfolding machine, associates with and activates proteolysis through the proteasome in *Mtb*.^{115,116} Similar to Mpa,¹¹⁷ PafE contains a C-terminal hydrophobic-tyrosine-X-motif (HbYX motif) that docks into the proteasome CP. *In vitro*, PafE enhances the CP-dependent degradation of unstructured proteins such as casein and is thought to deliver partially unfolded proteins (likely formed under conditions of stress) into the CP for degradation in the cell.^{115,116} Interestingly, PafE-deficient *Mtb* is unable to degrade HspR and is heat-sensitive, suggesting it is a regulator of the heat shock response in *Mtb*.¹¹⁵ Therefore, PafE may be both

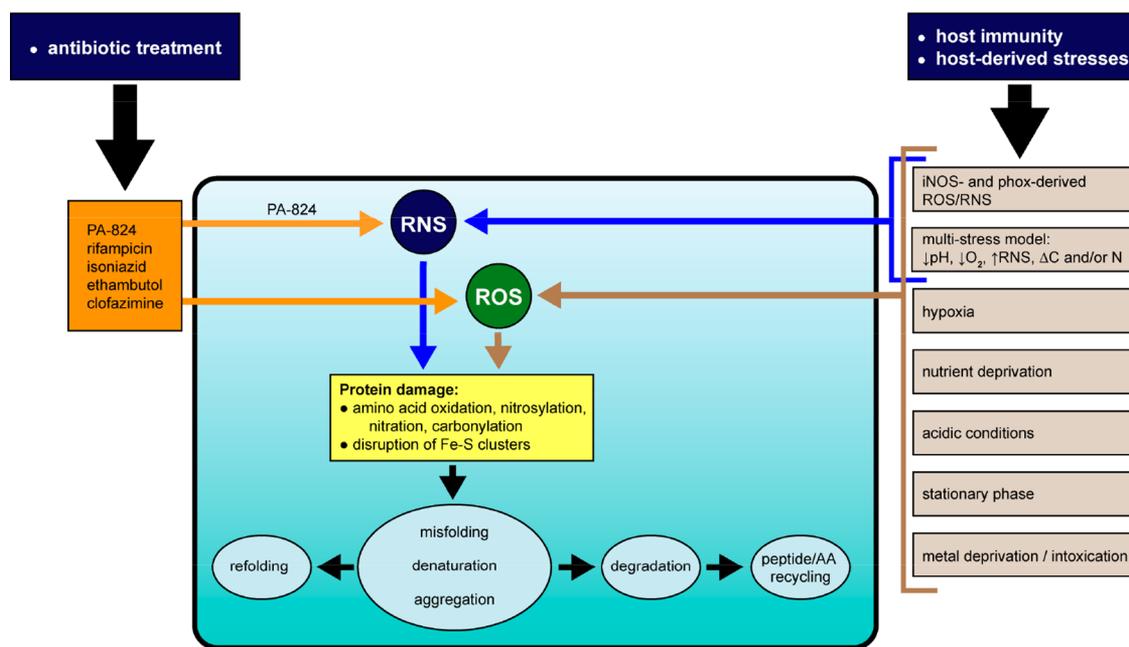


Figure 3. Host microenvironments and immune chemistries, and antibiotic treatment, converge on the mycobacterial proteostasis network. In this example, antibiotics used to treat TB and/or host microenvironments and immune chemistries directly or indirectly generate ROS or RNS, which may damage proteins. Proteins damaged by ROS and/or RNS are often enzymatically inactivate and must be repaired or degraded. Killing of Mtb by the antibiotic PA-824 (pretomanid) is mediated in part via generation of RNS.

responsible for the degradation of misfolded proteins formed during heat stress and responsible for activating the chaperone response to heat stress. Under standard culture conditions, the Mtb proteasome degrades only a handful of proteins and is not essential.¹¹² However, the proteasome and its accessory factors play a critical role in surviving host-relevant stress conditions such as nutrient starvation, prolonged stationary phase, reactive nitrogen species and heat.^{115,118–121} In the acute phase of a mouse model of infection, Mtb lacking the proteasome CP achieves bacterial burden equivalent to Mtb with a wild-type proteasome, but during the chronic phase of infection, the proteasome-deficient mutant was unable to sustain infection.^{119,120} It was recently proposed that the mycobacterial proteasome is important for amino acid recycling under nutrient starvation, in particular, nitrogen starvation in Msm.^{122,123}

■ ROLE OF THE PROTEOSTASIS NETWORK IN PROTECTING Mtb FROM HOST IMMUNITY

In addition to general protein quality control, the Mtb proteostasis network is responsible for degrading damaged or prematurely terminated proteins, as well as supplying amino acids under conditions of stress. Mtb is reliant on the proteostasis network to survive host immunity and host chemistries.

To control Mtb growth, macrophages produce ROS and RNS via the phagocyte oxidase (phox)¹²⁴ and the inducible isoform of nitric oxide synthase (iNOS),¹²⁵ respectively. As demonstrated in Figure 3, numerous host stresses associated with phenotypic tolerance and treatment with antibiotics converge on the formation of ROS and RNS.^{71,126–129} RNS have been implicated in the antimycobacterial activity of the nitroimidazole drug, PA-824.¹³⁰ RNS (including •NO, ONOO⁻, ONOOH, •NO₂, N₂O₃, N₂O₅) and ROS (including •O₂⁻, H₂O₂, ROOH, •OH, ¹O₂, O₃, HOCl, HOBr, HOI) may

damage proteins by oxidation of cysteine and methionine, disruption of iron in heme and Fe–S clusters, oxidation of arginine, lysine, proline, and threonine, nitration of tyrosine, and S-nitrosylation of cysteine residues.^{5,131,132} Some protein damage inflicted by RNS/ROS, such as oxidation of methionine or cysteine, and S-nitrosylation of cysteine, is enzymatically reversible.^{133,134} Other damage to proteins, such as carbonylation or tyrosine nitration, is irreversible and may provoke protein misfolding, aggregation, degradation, or irreversible oxidation of proteins to protease resistant or protease inhibiting forms.^{45,135–137} Whole genome expression analysis of Mtb following addition of nitric oxide (NO) or hydrogen peroxide (H₂O₂) shows different expression patterns of proteostasis genes. In response to ROS, the *dnaK* operon, *clpB*, and *acr2* are significantly upregulated; while in response to RNS, only *acr2* and *clpB* show increased expression among the proteostasis and proteolysis genes described here.¹³⁸ In particular, *acr* expression is induced in activated versus naïve macrophages following infection with Mtb over the course of 2 days, highlighting its role in responding to host stress environments.⁹

After stimulation by with IFN γ , the pH of the macrophage's phagosomal compartment in which Mtb resides decreases to ~4.5.¹³⁹ *rv2224c*, which encodes a protease involved in GroES processing, is important for survival in acidic conditions.^{140–143} The MarP protease plays a role in helping Mtb survive the acidic environment and maintain its intrabacterial pH.^{141,144} A member of a family of proteases involved in protecting proteins from acid stress, HtrA, was identified as the target of a benzoxazinone screening hit that arose from an HTS of over 300 000 small molecules for MarP protease inhibitors.¹⁴⁵

Mtb faces varying degrees of hypoxia in a host.¹⁴⁶ The nitroimidazole drug metronidazole kills Mtb under severe hypoxia (<0.1 ppm of O₂) *in vitro*^{147,148} and trends toward displaying higher activity in the animal models of TB with hypoxic lesions.⁵ Exposure to hypoxic conditions for 4 to 7 days

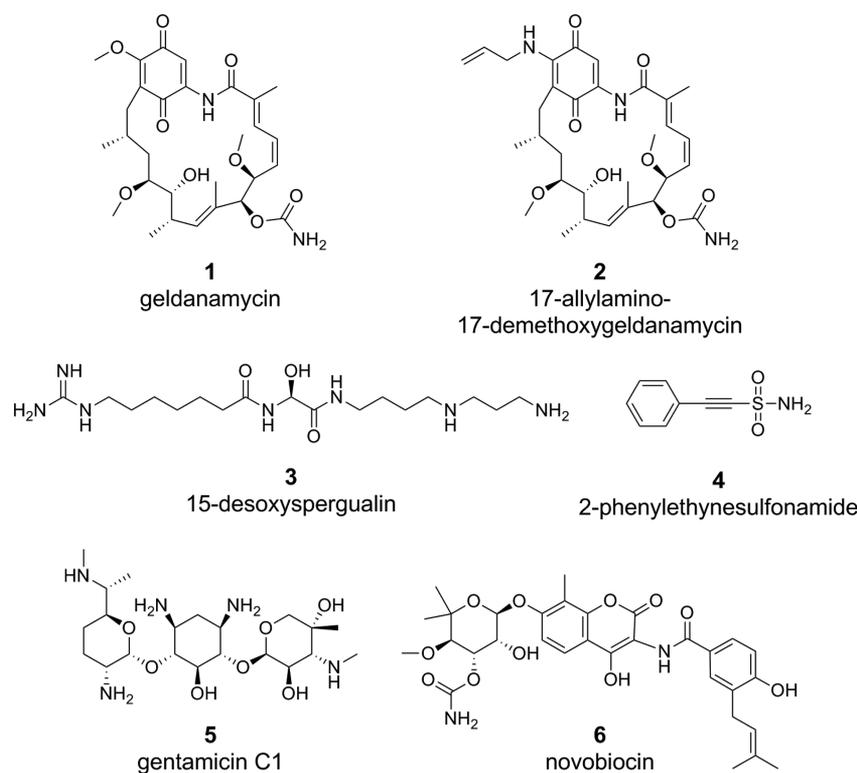


Figure 4. Examples of compounds targeting human Hsp70 or Hsp90.

increases expression of *dnaK*, *clpB*, and *acr2* compared to nonstressed, log-phase cells.¹⁴⁹

Mycobacterial metal homeostasis, in particular surviving copper or zinc intoxication and iron starvation, is critical during host infection.^{150–155} Iron starvation-induced growth arrest in Mtb leads to nonreplication and phenotypic tolerance to antibiotics. The chaperone ClpB plays a critical role in surviving stresses associated with nonreplication imposed by iron starvation.¹⁵⁶

Mtb adapts to various degrees of nutrient starvation or changes to carbon and nitrogen availability or sources in the host. In one example of prolonged stationary phase (>100 days), DnaK protein was induced in Msm.¹⁵⁷ In Mtb, the proteins GrpE, DnaK, and HspX (α -crystallin) were induced by 6-week starvation in phosphate-buffered saline (PBS).¹⁵⁸ Nonreplication imposed by nutrient starvation or depletion of carbon and nitrogen sources can result in oxidative carbonylation of proteins.⁷¹ Thus, these data suggest that antibiotics targeting Mtb's proteostasis network will sensitize it to proteotoxic stress from host chemistries and synergize with host immunity.

■ TARGETING THE PROTEOSTASIS NETWORK: CHAPERONES AND THEIR COFACTORS

Chaperones have emerged as exciting targets for cancer chemotherapy, and numerous inhibitors of eukaryotic protein chaperones have entered clinical trials.^{159–161} Within their respective microenvironments, there are parallels to the stresses encountered by cancer cells in solid human tumors and Mtb in activated macrophages and/or granulomas. For example, solid tumors are nutrient deprived, hypoxic, and mildly acidic, and the degree of each stress is dependent on a cancerous cell's location within the tumor and relative to the tumor's vasculature.^{162–164} Cancer cells produce more ROS than

wild-type cells.^{165,166} The microenvironmental conditions found in solid tumors lead to variable growth rates, and some or the majority of cancer cells may become tolerant to anticancer agents. Many of the predominant stresses, nutrient deprivation, hypoxia, acidity, and ROS, are associated with both drug resistance of cancer cells and phenotypic drug tolerance in Mtb.

Cells in solid tumors depend on the proteostasis network for survival. Chaperones may be highly induced on the transcriptional and protein level in cancer cells, and chaperones such as Hsp90 may exist in activation states and complexes that make them 100-fold more sensitive than wild-type cells to small-molecule inhibition.^{164,167} This parallels the upregulation of components of the proteostasis network in Mtb upon infection of macrophages or exposure to individual components of host immunity. Eukaryotic chaperones help malignant cells survive host stresses in solid tumors and help maintain pro-oncogenic proteins in a functional state that serves to keep cancer cells alive. Some examples of compounds with efficacy against Hsp70 and Hsp90 include geldanamycin (1), 17-allylamino-17-demethoxygeldanamycin (2), 15-desoxyspergualin (3), 2-phenylethynesulfonamide (4), gentamicin (5), and novobiocin (6) (Figure 4).

The strategy of treating cancer with chaperone inhibitors might translate to new strategies for bacterial drug discovery¹⁶⁸ and, in particular, for TB. For example, the mycobacterial chaperone ClpC1 has become an exciting drug target and is discussed in further detail below. In other bacteria, the proteostasis network, including the GroE system, HtpG, and DnaK, has been disrupted by small molecules and peptides, many of which exert bactericidal activity. In light of a lack of understanding of which pharmacophores engage chaperones, we assembled structures of compounds targeting bacterial chaperones and their cofactors as a reference to serve as a

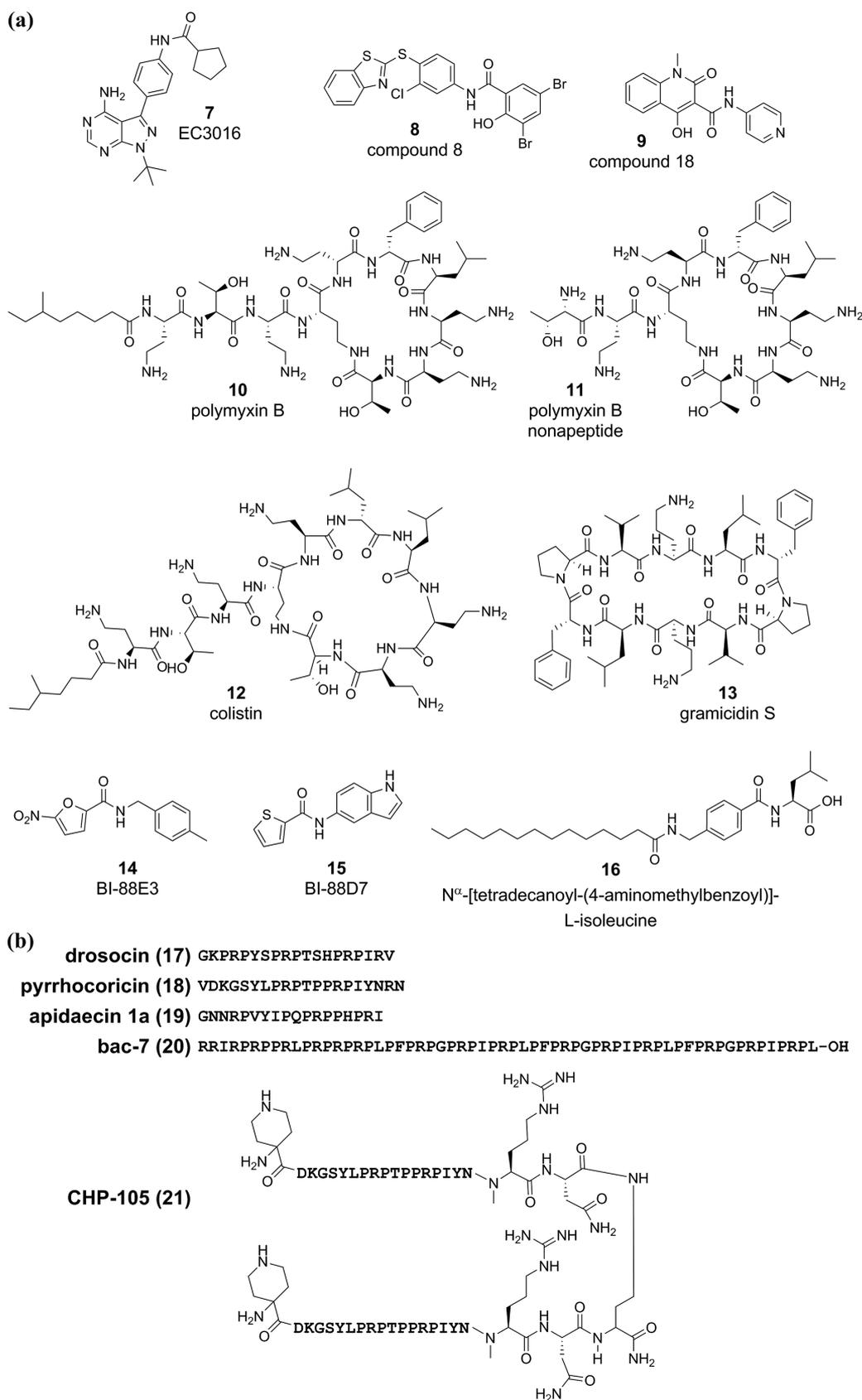


Figure 5. Examples of compounds targeting bacterial chaperones. Representative structures are shown of (a) small molecules or (b) amino acid sequences of proline-rich peptides that inhibit bacterial GroEL/ES, HtpG, and DnaK. Some proline-rich peptides like Bac-7 and pyrrhocoricin may mediate antibacterial activity by inhibiting translation. To date, there are no examples of compounds or peptides targeting mycobacterial protein folding machinery.

Table 2. Summary of Select Inhibitors or Activators of ClpC1, ClpP1P2, or Mtb20S^a

compound class	compound	synthetic (S) or natural product (NP)	target	method of identification	activity on R Mtb	activity on NR Mtb	activity in mouse model of TB	FOR	references
peptide, cyclic	cyclomarin A	NP	ClpC1	WCS against <i>M. bovis</i> BCG	yes	yes	NT	<10 ⁻⁹	39, 234
peptide, cyclic	ecumicin	NP	ClpC1	WCS vs actinomycetes extracts against Mtb	yes	yes	yes	1 × 10 ⁻⁸	37
peptide, cyclic	rufomycin	NP	ClpC1	WCS vs actinomycetes extracts against Mtb	yes	NT	NT	NT	196
peptide, lasso	lassomycin	NP	ClpC1	WCS of NP extracts against Mtb	yes	yes	NT	NT	38
acyldeipeptide	ADEP-2	NP	ClpP1P2	N/A; previously shown to be active against <i>S. aureus</i> ⁴¹	yes	NT	NT	1 × 10 ⁻⁶ (for <i>S. aureus</i>)	41, 97, 195
peptidyl boronate	examples: N-(2-(3,5-difluorophenyl)acetyl)-Trp-boroMet; N-(Picolinoyl)-Ala-Lys-boroMet	S	ClpP1P2	blocked tripeptide-AMC library to determine substrate preferences followed by rational design of inhibitors	yes	NT	NT	NT	206
peptidyl boronate	bortezomib (Velcade), MLN-273	S	Mtb20S and ClpP1P2 (bortezomib); Mtb20S (MLN-273)	bortezomib: target-based whole-cell screen against <i>M. smegmatis</i> strain carrying SsrA-tagged GFP; MLN273: N/A	yes	yes	NT	NT	106, 118, 202
β -lactones	β -lactone 7	S	ClpP1P2	whole-cell screen of racemic mixture (14 scaffolds) of β -lactones against <i>M. smegmatis</i>	yes	NT	NT	NT	200
N, C-capped dipeptide	DPLG-2	S	Mtb20S	purified recombinant Mtb20S-OG; hydrolysis of AMC-linked substrate	no	yes	NT	NT	210
oxathiazol-2-one	GL-5	S	Mtb20S	purified recombinant Mtb20S-OG; hydrolysis of AMC-linked substrate	no	yes	NT	NT	40
stryl-oxathiazol-2-one	compound 17	S	Mtb20S	purified recombinant Mtb20S-OG; hydrolysis of AMC-linked substrate	no	yes	NT	NT	209
macrolactam	syringolin analogues-compound 13	S based on NP	Mtb20S	purified recombinant Mtb20S-OG; hydrolysis of AMC-linked substrate	NT	yes	NT	NT	212
epoxyketone Peptide	epoxomicin	NP	Mtb20S	NA	NT	yes	NT	NT	118
lipopeptide aldehyde	fellutamide B	NP	Mtb20S	purified recombinant Mtb20S-OG; hydrolysis of AMC-linked substrate	NT	NT	NT	NT	208, 235
NA	MTBA, MTBB	S	Pup/Mpa interaction	in-cell STINT-NMR	NT	yes	NT	NT	211

^aAbbreviations: NA = not applicable; NT = not tested or not found in the literature; S = synthetic; NP = natural product; FOR = frequency of resistance; Mtb20S = 20S proteasome (PrcBA); OG = open gate; AMC = aminomethyl coumarin; GFP = green fluorescent protein; R = replicating; NR = nonreplicating.

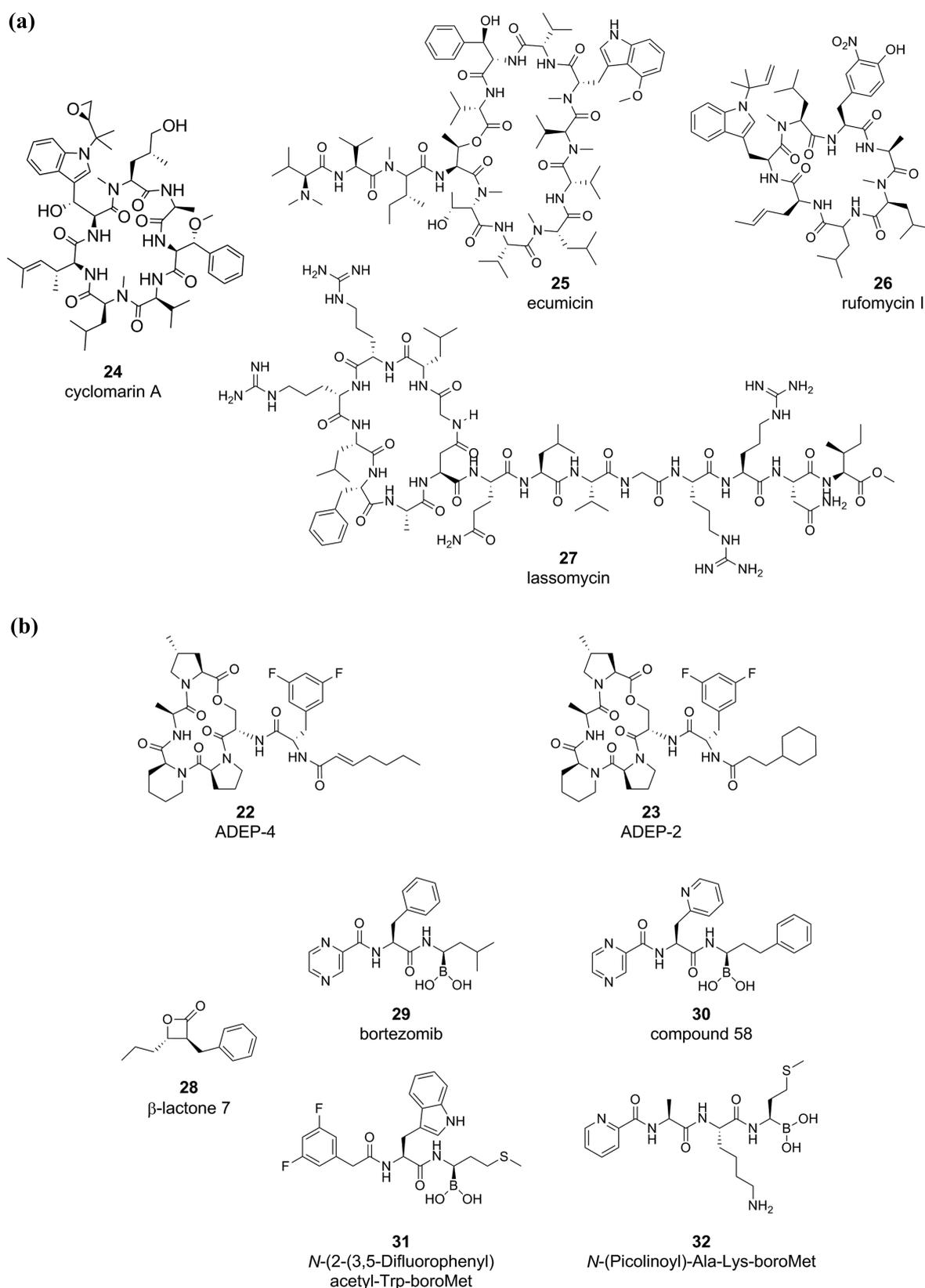


Figure 6. Examples of compounds targeting mycobacterial protein degradation. Molecules targeting protein degradation in Mtb inhibit or activate (a) ClpC1 or (b) ClpP1P2.

starting point for mycobacterial chaperone inhibitors (structures and amino acid sequences are shown in Figure 5a,b). Compound EC3016 (7) is an inhibitor of both ATPase and chaperone activity of a modified *E. coli* GroEL/ES variant.¹⁶⁹

Nontoxic inhibitors of GroEL/ES (compounds 8 (8) and 18 (9)) have bactericidal activity against *E. coli*.¹⁷⁰ Large cyclic, lipopeptide antibiotics such as polymyxin B (10), polymyxin B nonapeptide (11), colistin (12), and gramicidin S (13) inhibit

chaperone activity of HtpG in the model organism *Synechococcus elongatus* PCC7942 by binding its N-terminus and inducing oligomerization and/or aggregation.¹⁷¹ Compounds BI-88B3 (14) and BI-88D7 (15) bind the substrate-binding β -domain of the *E. coli* DnaK, and BI-88B3 (14) had weak antimicrobial activity against *E. coli* and *Yersinia pseudotuberculosis*.¹⁷² N^α-[Tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine (16) inhibits the secondary peptide cis-trans isomerase activity of the *E. coli* DnaK¹⁷³ but has poor antimicrobial activity.^{173,174} The proline-rich antibacterial peptides of insect origin (Figure 5b), including drosocin (17), pyrrocoricin (18), apidaecin 1a (19), bac-7 (20), and CP-105 (21), block the ATPase and chaperone activities of the *E. coli* DnaK.^{168,175–179} The proline-rich peptide CHP-105 (21) synergizes with levofloxacin to kill levofloxacin-sensitive and -resistant *Klebsiella pneumoniae* and *E. coli*.¹⁸⁰ Pyrrocoricin (18) and a stabilized pyrrocoricin (Chex-pyrrocoricin-Dap(Ac)) protected mice from *E. coli* septicemia.¹⁸¹ While the proline-rich antimicrobial peptides have weak to no activity against Gram positive pathogens,¹⁸² modifying peptide length and amino acid composition may improve anti-Mtb activity.¹⁸³ Some proline-rich peptides, including pyrrocoricin (18) and Bac-7 (20), were found to kill bacteria by blocking peptide exit from the 70S ribosome,^{184,185} indicating that at least some proline-rich peptides exert antimicrobial activity by inhibiting translation.

While targeting the chaperone network is an active area of research for cancer therapy and has been explored for bacterial drug discovery, targeting chaperones for TB drug discovery is in its infancy. In fact, to our knowledge, there only has been one report of drug discovery efforts targeting protein-folding chaperones in Mtb.¹⁸⁶ We propose further exploration of mycobacterial chaperones and their cofactors for TB drug discovery.

■ TARGETING THE PROTEOSTASIS NETWORK: ClpC1, ClpP1P2, AND Mtb20S

The majority of drug discovery work in the proteostasis field has focused on targeting barrel-shaped proteases and their Hsp100-family chaperone partners. Excellent reviews have extensively explored drug discovery for the bacterial proteolysis machinery.^{187–191} We have summarized information regarding inhibitors and activators of the Mtb proteolysis systems in Table 2. Targeting bacterial proteolysis provides a unique situation in which either inhibiting or activating the target pathway results in bacterial death.^{189,190} Inhibition or activation of proteases or their activators may alter the levels of client proteins (damaged, nascent, or “normal”), resulting in dysregulation, cell stress, and death.

The discovery that ADEP-4 (22) (Figure 6a) activates proteolysis and kills both replicating and phenotypically drug tolerant *S. aureus* and pairs with RIF to eradicate a deep-seated thigh infection of *S. aureus* in mice indicates that proteolysis may be an exciting target pathway for latent diseases such as Mtb.^{41,192} In bacteria such as *B. subtilis* and *E. coli*, ADEPs bind ClpP and open the ClpP tetradecamer pore to permit unregulated degradation of nascent and flexible proteins.¹⁹³ ADEP-bound ClpP subunits also fail to associate with their Hsp100 chaperones and subsequently lose tight regulation of ClpP substrates.¹⁹³ Dysregulated degradation of the essential cell-division protein FtsZ plays a major role in ADEP-dependent bactericidal activity against *B. subtilis* and *S. aureus*.¹⁹⁴ ADEPs have relatively weak activity against mycobacteria, with

ADEP2 (23) having an MIC₉₀ of 16–25 μ g/mL against Mtb and *M. bovis* BCG.^{97,195} In the absence of ClpC1, ADEPs open the Mtb ClpP1P2 pore and increase protease activity, and in the presence of ClpC1, ADEPs interfere with the ClpC1–ClpP1P2 interaction.¹⁹⁵ Dysregulated FtsZ proteolysis is not the cause of ADEP-dependent death in Mtb¹⁹⁵ and the degradation of specific client proteins has not been elucidated. The activity of ADEPs against mycobacteria can be improved with efflux pump inhibitors or ClpP1P2 depletion,^{97,195} suggesting cell entry may be a limiting factor for activity. Thus, ADEPs appear to have a poor prognosis for further development for TB.

There has been progress in the discovery of molecules that target ClpC1 and phenocopy treatment of other bacterial pathogens with ADEPs. In fact, experimental data generated with a handful of cyclic peptides support ClpC1's candidacy as a high-priority target in Mtb (Figure 6a). The antimycobacterial peptides cyclomarin A (24),³⁹ ecumicin (25),³⁷ rufomycin (26),¹⁹⁶ and lassomycin (27)³⁸ target Mtb ClpC1 via different mechanisms. Cyclomarin A and rufomycin activate ClpC1-mediated, ATP-dependent proteolysis by ClpP1P2.³⁹ Ecumicin and lassomycin uncouple ClpC1's ATP hydrolysis,^{37,38} which ultimately prevents ClpC1's ability to feed damaged and client proteins into ClpP1P2. Unregulated ATP hydrolysis can also deplete critical cellular ATP. In essence, ecumicin and lassomycin arrest ClpP1P2 protein degradation and function as ClpP1P2 inhibitors. The ClpC1-targeting peptides boast nanomolar to micromolar potency against replicating and nonreplicating Mtb, with limited toxicity to eukaryotic cells. Ecumicin and lassomycin were active against MDR and XDR Mtb,^{37,38} and ecumicin killed Mtb in both macrophages and mice.³⁷ When tested, the microbial spectrum of activity was highly specific to Mtb. For example, lassomycin was inactive against Gram-positive bacteria such as *S. aureus*, *Bacillus anthracis*, and *Klebsiella pneumoniae*.³⁸ The ClpC1-targeting peptides are natural products isolated from actinomycetes, including cyclomarin A (*Streptomyces* spp. CNB-982), ecumicin (*Nonomuraea* spp. MJM5123), lassomycin (*Lentzea kentuckyensis* spp. IO0009804), and rufomycin (*Streptomyces atratus* ATCC 14046). The biosynthesis of these peptides is complex; for example, the synthesis of cyclomarin A is mediated by over 23 genes (over 47 kb of DNA).³⁹ The high prevalence of unnatural amino acids,^{191,197} including D-amino acids, methoxylated amino acids, N-methylleucine, N-methylhydroxyleucine, β -methoxyphenylalanine, 2-amino-3,5-dimethylhex-4-enoic acid, N-(1,1-dimethyl-2,3-epoxypropyl)- β -hydroxytryptophan, N-dimethylallyltryptophan, trans-2-crotylglycine, and 3-nitrotyrosine, indicated that it is unlikely that similar compounds or peptides exist in synthetic compound collections. A current and future challenge is in isolating and/or modifying complex natural products. Structure activity relationship studies may help identify critical pharmacophores that retain ClpC1 binding¹⁹⁸ and permit identification of similar structures in screening decks. Recent developments in elucidating pathways encoding nonribosomally synthesized peptides¹⁹⁹ may offer new avenues to discover and synthesize natural products targeting the ClpC1/ClpP1P2 in mycobacteria. To our knowledge, there are no described inhibitors or activators for the other ClpP1P2 accessory proteins, ClpC2 and ClpX.⁹⁹

Compounds that directly inhibit or activate the ClpP1P2 tetradecamer include ADEPs, β -lactones, bortezomib, and peptidyl boronates (Figure 6b). Some β -lactones (28) inhibit ClpP1P2 and have antimycobacterial activity.²⁰⁰ Caution must

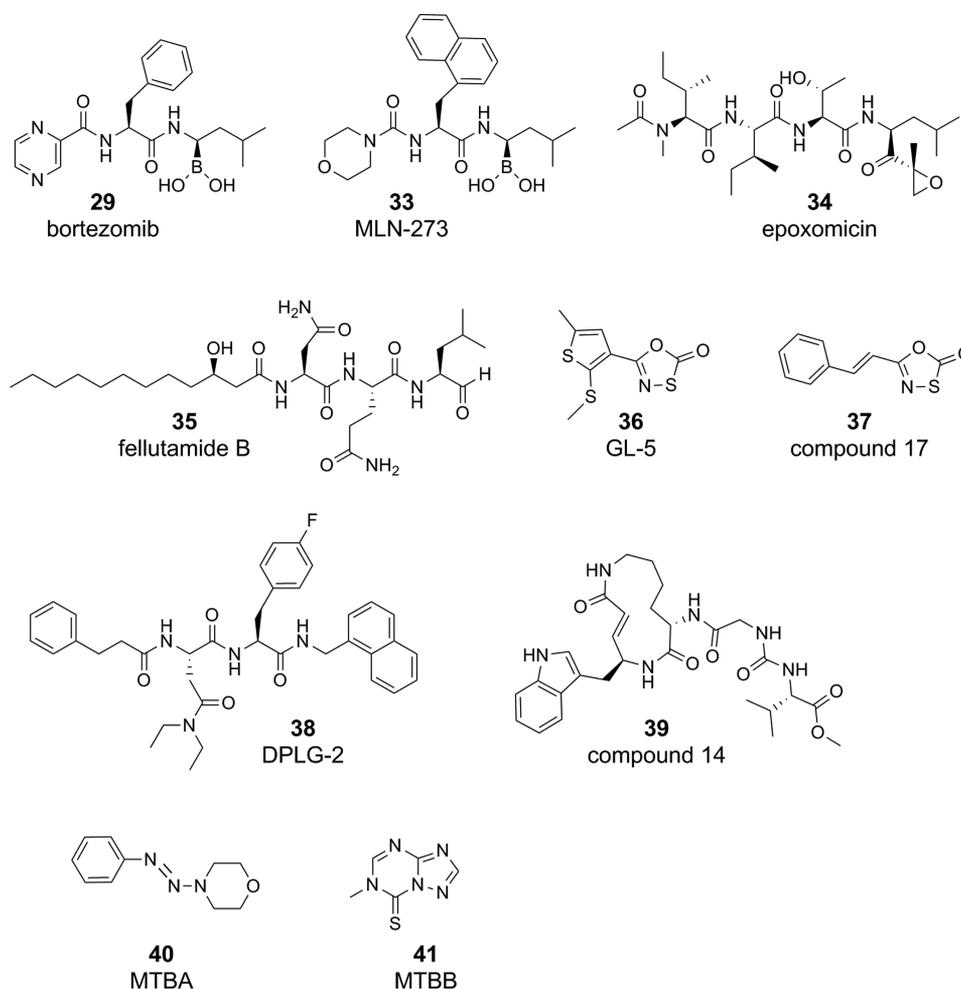


Figure 7. Examples of compounds targeting Mtb's eukaryotic-like proteasome, Mtb20S, and proteins serving the proteasome pathway, such as Pup and Mpa.

be taken with β -lactones as ClpP1P2 inhibitors; as recently, a different member of the β -lactone class exerted antimycobacterial activity by inhibiting mycolic acid biosynthesis.²⁰¹ While these β -lactones also associated with ClpP1P2 in pull-down experiments, further characterization demonstrated that they had poor inhibitory activity against recombinant ClpP1P2.²⁰¹ A general concern of ClpP1P2-targeting β -lactones is that they lack structural features to generate target selectivity and risk indiscriminate reactivity with cellular nucleophiles. Another ClpP1P2 inhibitor, bortezomib (**29**), is a peptidyl boronate antineoplastic drug originally found to target the human 20S proteasome and was identified as an inhibitor of both ClpP1P2²⁰² and Mtb20S.²⁰³ SAR campaigns have identified bortezomib analogues, including compound **58** (**30**), with improved selectivity toward ClpP1P2 and Mtb20S over the human proteasome.²⁰⁴ Other bortezomib analogues, in which the boronate was replaced with a chloromethyl ketone warhead, had negligible activity against the human proteasome.²⁰⁵ Numerous di- (example, *N*-2-(3,5-difluorophenyl)acetyl-Trp-boro-Met, **31**) and tri- (example, *n*-(picolinoyl)-Ala-Lys-boro-Met, **32**) peptidyl boronates with potent nM affinities for ClpP1P2 were inhibitory to Mtb at low μ M concentrations.²⁰⁶ The stark difference in IC_{50} against the recombinant proteins and the MIC_{50} against Mtb indicates poor uptake into the bacillus or metabolism of the compound to inactive species. These peptidyl boronates inhibited human mitochondrial ClpP

to a lesser extent than the Mtb ClpP1P2, and a select set were found to be nontoxic to human MM.1S myeloma cells.²⁰⁶ In summary, the majority of compounds directly binding ClpP1P2 can kill replicating Mtb *in vitro*, and to our knowledge, there is no evidence that they possess activity in animal models of TB. As stated above, one of the most promising cyclic peptides targeting ClpC1, ecumicin, phenocopies ClpP1P2-inhibiting molecules and kills Mtb in murine models of TB.

The discovery of a eukaryotic-like proteasome in mycobacteria with an important role in defending Mtb against stresses of host immunity, such as RNI,¹¹⁸ spurred intense efforts to find species-selective Mtb20S inhibitors (Figure 7). A major concern pursuing the Mtb20S was the existence of the essential human proteasome. In fact, the first few characterized inhibitors of the Mtb proteasome were originally developed to target the human 26S proteasome, including bortezomib (**29**; described above), MLN-273 (**33**), and epoxomicin (**34**).¹¹⁸ While the cocrystal of MLN-273 and Mtb20S was solved, MLN-273 was not pursued due to lack of specificity for the mycobacterial proteasome.²⁰⁷ Similarly, fellutamide (**35**), the most potent Mtb proteasome inhibitor to date with a K_i under ~ 7 nM, was not pursued as a lead candidate due to its potent inhibition of the human proteasome.²⁰⁸ The discovery of the oxathiazol-2-ones (**36**)⁴⁰ importantly demonstrated the possibility of developing highly selective, irreversible Mtb proteasome inhibitors. Analogues of oxathiazol-2-ones, including styryl-

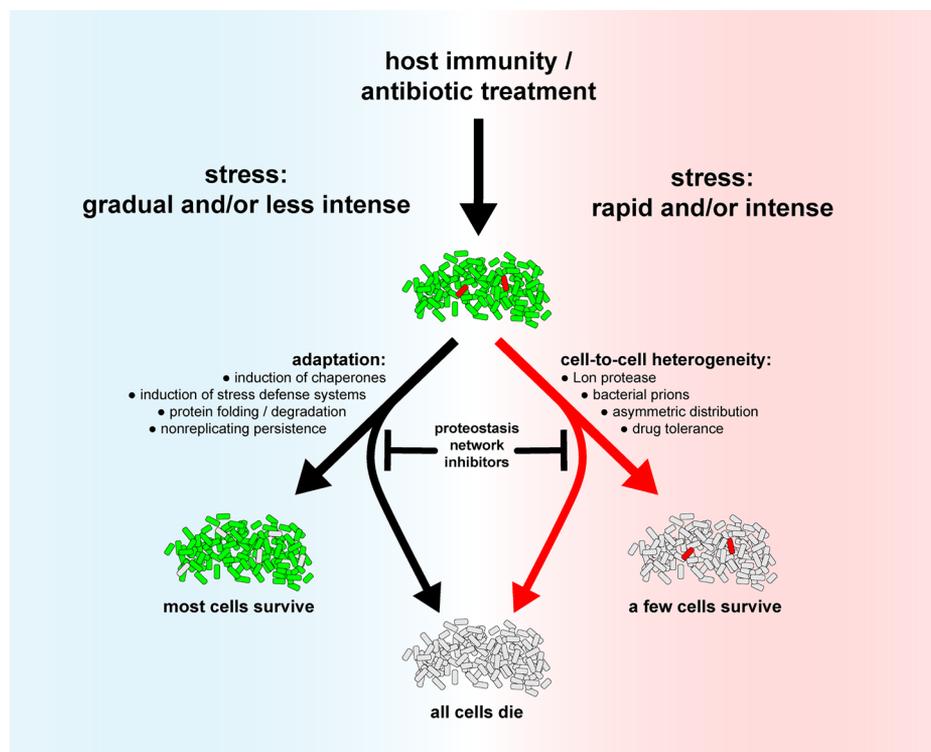


Figure 8. Targeting the proteostasis network may sensitize Mtb to host immunity and antibiotics. In situations when host stresses or antibiotics reach Mtb slowly or at low intensity (left side), Mtb has ample time to adapt by inducing defense mechanisms. However, in situations in which stresses or antibiotics attain Mtb rapidly and/or with high intensity (right side), Mtb may not have time to adapt. The only surviving bacilli may be rare cells that are different from the general cell population that display phenotypic tolerance to stresses or antibiotics. The proteostasis network, via chaperones and the protein degradation machinery, contributes to adaptation (left side) or cell-to-cell heterogeneity (right side). Cell color key: alive, green; dead, light gray; rare outlier cells that are different from an average cell, red.

oxathiazol-2-ones (37), were developed with improved antimycobacterial activity.²⁰⁹ The N,C-capped dipeptides, including DPLG-2 (38), are reversible inhibitors that gained selectivity for the Mtb proteasome by exploiting key differences revealed in the human and mycobacterial proteasome active sites.²¹⁰ Recently, progress to identify novel pharmacophores for Mtb-selective proteasome inhibitors was inspired by syringolin, a natural product made by the plant pathogen *Pseudomonas syringae*.²¹² Compound 14 (39) was over 70-fold selective for the Mtb proteasome versus the human proteasome.²¹² Finally, the compounds MTBA (40) and MTBB (41) target the Pup/Mpa interface and prevent degradation of FabD, a physiological Mtb20S substrate. MTBA and MTBB kill *M. bovis* BCG under nitrosative stress conditions and were noncytotoxic.²¹¹ Unlike compounds targeting ClpC1 and/or ClpP1P2, *in vitro* the Mtb20S inhibitors require coadministration of additional stresses, usually RNS generated under mildly acidified conditions, to exert bactericidal activity.^{118,210} This corroborates genetic evidence that PrcBA (Mtb20S) is nonessential under standard replicating conditions and conditionally essential under nitrosative stress.^{118–120} To date, there is no evidence that compounds targeting Mtb20S have efficacy in TB animal models.

In addition to potent activity against replicating bacilli, many of the compounds targeting ClpC1, ClpP1P2, and Mtb20S also have activity against Mtb rendered nonreplicating by stresses that mimic components of host immunity described above. For example, Mtb rendered nonreplicating by acid/RNS stress is killed by proteasome inhibitors MLN-273, epoxomicin, GL-5,

DPLG-2, and the syringolin-like compound 14 (39).^{40,118,210,212} Under nutrient starvation or stationary phase, Mtb is susceptible to ClpP1P2 inhibitors such as lassomycin and proteasome inhibitors such as compound 17 (37),^{38,209} presumably due to an increased demand on recycling proteins for amino acids. Under nonreplicating hypoxic conditions, Mtb is killed by ClpC1-targeting cyclic peptides, including cyclomarin A, ecumicin, and lassomycin.^{37–39}

Although the frequencies of resistance (FOR) to ADEPs in nonmycobacterial organisms are borderline unacceptable for use as an antibiotic ($\sim 1 \times 10^{-6}$ in *S. aureus*⁴¹), molecules targeting the Mtb ClpC1 have attractive FORs on par with clinically used antibiotics such as RIF (cyclomarin A, $< 1 \times 10^{-9}$;³⁹ lassomycin, 3×10^{-7} ³⁸). At least one compound, bortezomib, targets both ClpP1P2 and the proteasome, an interesting feature that may ultimately lead to a lower frequency of resistance than targeting either the protease or proteasome individually.^{202,204} Standard methodologies do not permit determination of the FORs for compounds that only kill when Mtb has been rendered nonreplicating with host-mimicking stresses such as RNI, nutrient starvation, or hypoxia.

■ PROSPECTIVE: COULD DISRUPTING THE PROTEOSTASIS NETWORK SERVE AS ADJUNCTIVE THERAPY?

Targeting the proteostasis network may serve as an adjunctive therapy to help our current arsenal of TB drugs to kill Mtb that has survived stresses imposed by host immunity and/or to kill Mtb drug tolerant persisters. During the course of its infectious

cycle, *Mtb* faces stresses that vary in nature and magnitude, and at all stages of infection, the proteostasis network contributes directly or indirectly to *Mtb*'s survival.

The proteostasis network plays an important role in allowing the majority of a population to adapt to stress conditions in a manner that permits their survival. However, how do cells handle host stresses or antibiotic stresses that arrive rapidly, with high intensity, and preclude bacterial adaptation? In these cases, pre-existing cell-to-cell heterogeneity permits survival of a minority of cells whose physiology differs from those of average cells in a normally distributed population (Figure 8). The proteostasis network bolsters diversity of phenotypic traits of individuals within a population. For example, it is possible that stochastic variations of antitoxin degradation of toxin–antitoxin systems by the Lon protease permit a minority of cells in a population, with higher toxin levels, to survive exposure to a stress such as antibiotics. In *E. coli*, mutants deficient in the Lon protease produce less drug tolerant persisters than a wild-type strain.²¹³ *Mtb* does not have a Lon protease. Since some antitoxins are pupylated in *Mtb*,¹¹² some of *Mtb*'s 80 toxin–antitoxins (TAs) are likely regulated by proteasomal degradation.^{213,214} Therefore, inhibition of the proteasome may kill some persister cells. Likewise, *Mtb* persists surviving INH exposure had increased expression of *dnaK*, *grpE*, *clpB*, *clpX*, and *groE*.²¹⁵ Cells expressing higher levels of *dnaK* represented the majority of the persister population and were more resistant to INH.

Prions are another example of the proteostasis network controlling cell-to-cell heterogeneity. Prions are self-propagating protein aggregates that confer protein-based phenotypic traits. The transcriptional terminator of *Clostridium botulinum*, Rho, is a native prion that can switch to a loss-of-function amyloidogenic form that causes genome-wide transcription.²¹⁶ Propagation of the yeast Sup35 prion in *E. coli* required ClpB disaggregase activity.²¹⁷ As prion transmission from parental to progeny cells requires the chaperone ClpB,²¹⁷ inhibiting ClpB in prion-containing bacteria might decrease cell-to-cell heterogeneity, which has been associated with enhanced killing by antibiotics.²¹⁸

TB patients requiring antibiotics represent a situation in which host immunity failed to kill and eradicate *Mtb*. In this case, *Mtb* must recover and adapt from damage by chaperone-mediated refolding of damaged proteins or degrading them with Clp proteases and the proteasome. If damaged proteins can neither be repaired nor degraded, *Mtb* deploys a last bastion of defense against proteotoxic stress, the ClpB-associated buffering, sequestration, and distribution between progeny cells of irreversibly oxidized proteins (IOP).⁷¹ Similar to the previous reasoning for prions, inhibiting ClpB may cripple the pathway by which *Mtb* eliminates damaged macromolecules. Interfering with metabolic processes might impact the ability of bacteria to sequester and asymmetrically distribute aggregates of damaged proteins or propagate prions. This could occur by impacting large particle (protein aggregates) diffusion in the cytosol or protein organization and compartmentalization by interfering with cytoplasmic fluid-to-glass or proteinaceous liquid–liquid phase transition.^{219,220} This raises the possibility that small molecules targeting metabolism may indirectly perturb proteostasis pathways.

During chronic infection, *Mtb* must adapt to its new environmental niche. A comparison of global transcriptomic data from four models of *Mtb* nonreplicating phenotypic tolerance, including persisters surviving D-cycloserine treat-

ment, two models of hypoxia (the enduring hypoxic response and the Wayne model), and nutrient starvation, found *acr2* was upregulated in all dormancy models, and *clpB* was upregulated in three of the four models.²²¹ Mutations in genes encoding GrpE and TF were correlated with a high-persister phenotype in *Mtb*.¹⁶ *Mtb* deficient in α -crystallin (encoded by *hspX*) were cleared from mouse lungs more rapidly than the wild-type by a three-drug combination in the Cornell model of persistence (INH, RIF, PZA), and the *hspX* mutants had a decreased relapse rate upon treatment of mice with steroids.²²² Targeting these pathways might affect *Mtb*'s ability to adapt to its host compartments.

Finally, in some instances, targeting the proteostasis network might have a detrimental impact by rendering *Mtb* more resistant to host stress or antibiotics. For example, knockdown of the *Mtb* proteasome¹²⁰ or mutations in genes encoding accessory proteins Mpa and PafA¹¹⁸ led to hydrogen peroxide resistance. In another example, the proteostasis network may dictate the stability of target enzymes of antibiotics.²²³ PZA is a first-line drug that kills nonreplicating *Mtb* by inhibiting translation and the synthesis of lipids, pantothenate, and coenzyme A,^{224–228} and two independent studies identified PZA resistance-conferring mutations in ClpC1.^{229,230} It is possible that the mutant ClpC1 has decreased affinity for client protein(s) that are the targets of PZA. This might contribute to PZA resistance by increasing the amount of PZA target enzyme in the cell. In another example, amikacin- and kanamycin-resistant *Mtb* had increased protein levels of chaperones TF (encoded by *rv2462c*) and α -crystallin (encoded by *rv2031c*) and proteolysis protein proteasome subunit alpha (encoded by *rv2109*).²³¹

■ CONCLUSIONS

The proteostasis network repairs or degrades *Mtb* proteins damaged during infection. Some individual components of the proteostasis network, such as the proteasome, are vulnerable to selective inhibition over their eukaryotic counterparts,⁴⁰ and we anticipate that species-selective inhibitors could be selected for most of the components of the pathway. Disrupting the *Mtb* proteostasis network as a noncanonical target pathway of small molecules is anticipated to have multiple effects on *Mtb* during human TB. First, targeting the proteostasis network will prevent *Mtb* from repairing damage to mycobacterial proteins that had already been inflicted by the host. Second, inhibiting or activating the proteostasis network may sensitize *Mtb* to stresses of host immunity, including ROS, RNS, hypoxia, mild acidity, nutrient starvation, and metal sequestration or intoxication, in part by preventing *Mtb* stress adaptation and/or generation of cell-to-cell heterogeneity. Finally, compounds inhibiting the proteostasis network may prevent the formation of drug-tolerant persister cells and serve as adjunctive therapy to boost the efficacy of conventional antibiotics. The ClpP1P2 accessory chaperone ClpC1 has emerged as an extremely promising noncanonical drug target.^{37–39,192} Of the compounds targeting mycobacterial proteolysis, only ecumicin has demonstrated efficacy in a murine model of TB.³⁷ The next major hurdle will be to develop compounds with pharmacologic properties suitable for testing in animal models of TB, which will enable further testing of the hypothesis that targeting the proteostasis network renders *Mtb* hypersusceptible to host stresses during infection. To date, the majority of studies have focused on developing antibiotics targeting components of the *Mtb* proteolysis machinery, in particular ClpP1P2/ClpC1 and

the eukaryotic-like proteasome, Mtb20S. However, the development of inhibitors of bacterial proteostasis in other bacteria, including GroEL/ES, GrpE, and DnaK, suggest that these proteins could be targeted in mycobacteria as well. Future studies will determine if molecules targeting the proteostasis network will impact Mtb pathogenesis in animal models or a human host.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Drs. Carl Nathan and Gang Lin (Weill Cornell Medicine) for critical discussions, chemistry guidance, and expert review of the manuscript. We thank Drs. Michael Glickman and Allison Fay (Memorial Sloan Kettering) and Drs. Nader Fotouhi, Khisi Mduli, Anna Upton, Christopher Cooper, and Takushi Kaneko (Global Alliance for TB Drug Development) for excellent ideas and discussions. We apologize to those authors whose references were omitted due to space constraints. This work was supported by the TB Drug Accelerator Program of the Bill and Melinda Gates Foundation, the Abby and Howard P. Milstein Program in Chemical Biology and Translational Medicine, and an NIH TB Research Unit (U19 AI111143). The Department of Microbiology and Immunology is supported by the William Randolph Hearst Foundation. T.J.L. was supported by a Helen Hay Whitney and Simons Foundation Postdoctoral Fellowship.

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