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REVIEW ARTICLE

Bacterial proteases, untapped antimicrobial drug targets

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Bacterial proteases are an extensive collection of enzymes that have vital roles in cell viability, stress response and pathogenicity. Although their perturbation clearly offers the potential for antimicrobial drug development, both as traditional antibiotics and anti-virulence drugs, they are not yet the target of any clinically used therapeutics. Here we describe the potential for and recent progress in the development of compounds targeting bacterial proteases with a focus on AAA+ family proteolytic complexes and signal peptidases (SPs). Caseinolytic protease (ClpP) belongs to the AAA+ family of proteases, a group of multimeric barrel-shaped complexes whose activity is tightly regulated by associated AAA+ ATPases. The opportunity for chemical perturbation of these complexes is demonstrated by compounds targeting ClpP for inhibition, activation or perturbation of its associated ATPase. Meanwhile, SPs are also a proven antibiotic target. Responsible for the cleavage of targeting peptides during protein secretion, both type I and type II SPs have been successfully targeted by chemical inhibitors. As the threat of pan-antibiotic resistance continues to grow, these and other bacterial proteases offer an arsenal of novel antibiotic targets ripe for development.

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INTRODUCTION

The antibiotic crisis shows no sign of being resolved in the short term. Resistance to our arsenal of clinically approved antibiotics continues unabated, increasing in global reach and affecting all drugs. In addition, the lack of new antimicrobial drugs coming to market and the paucity of companies investing in this therapeutic area conspire to threaten our ability to treat and prevent infectious diseases. One of the reasons for the lack of new antibiotics is a feeling that the traditional molecular target for antibiotics, cell wall biosynthesis, protein and DNA/RNA synthesis have perhaps been over-mined.[1] Over two decades of access to complete bacterial genomes and associated genome-scale tools promised a more rational target-based strategy to antibiotic discovery and the exploitation of new molecular targets; however, this approach has yet to fulfill its touted promise. Nevertheless, the current antibiotic crisis demands solutions including a renewed consideration of potential antibiotic targets and alternate therapeutic strategies. Bacterial proteases offer one such set of underexploited targets for new antimicrobial agents.

Proteases have a number of key roles in bacterial physiology and biochemistry, as well as in pathogenicity. The *Escherichia coli* essential gene set includes several proteases including *ftsH*, an ATP-dependent metallo-protease that regulates the outer membrane biosynthesis enzyme LpxC,[2] *rseP*, an integral membrane enzyme involved in σ^E activation during stress response,[3] and the signal peptidases (SPs) I (*lepB*) and II (*lspA*), which are required for targeting and exit of proteins from the cell.[4] In addition to such essential genes, many proteases such as caseinolytic protease (ClpP) and Lon are tightly regulated to avoid uncontrolled proteolysis in the bacterial cell that, unlike eukaryotes, cannot isolate enzyme activities by compartmentalization in organelles. Interfering with these enzyme and regulatory activities offers a route to untapped antibiotic targets.

Proteases are also essential to the ability of many bacteria to infect the host and cause disease. Among the most celebrated is Lethal Factor, a Zn^{2+}-dependent protease required for infection by *Bacillus anthracis*.[5] Blocking such virulence factors to prevent infection has been on the radar of thought leaders in infectious disease for decades; however, as yet there are no approved drugs with this mode of action.[6,7] As reliable diagnostics to identify pathogens are increasingly being called for, targeting proteases essential to virulence is becoming increasingly viable and worthy of investigation as drug candidates.

PROTEASES AS DRUG TARGETS

Protein-degrading enzymes are mechanistically, structurally and functionally highly diverse. They operate by one of five general chemical mechanisms. Ser proteinases activate the primary hydroxyl of an active site Ser side chain, commonly through Ser-His-Asp triad or Ser-Lys dyad relays, to increase its nucleophilicity and position it for attack on a peptide amide bond (Figure 1a).[8] The resulting covalent enzyme complex is then cleaved by hydrolysis. Cys and Thr proteinases

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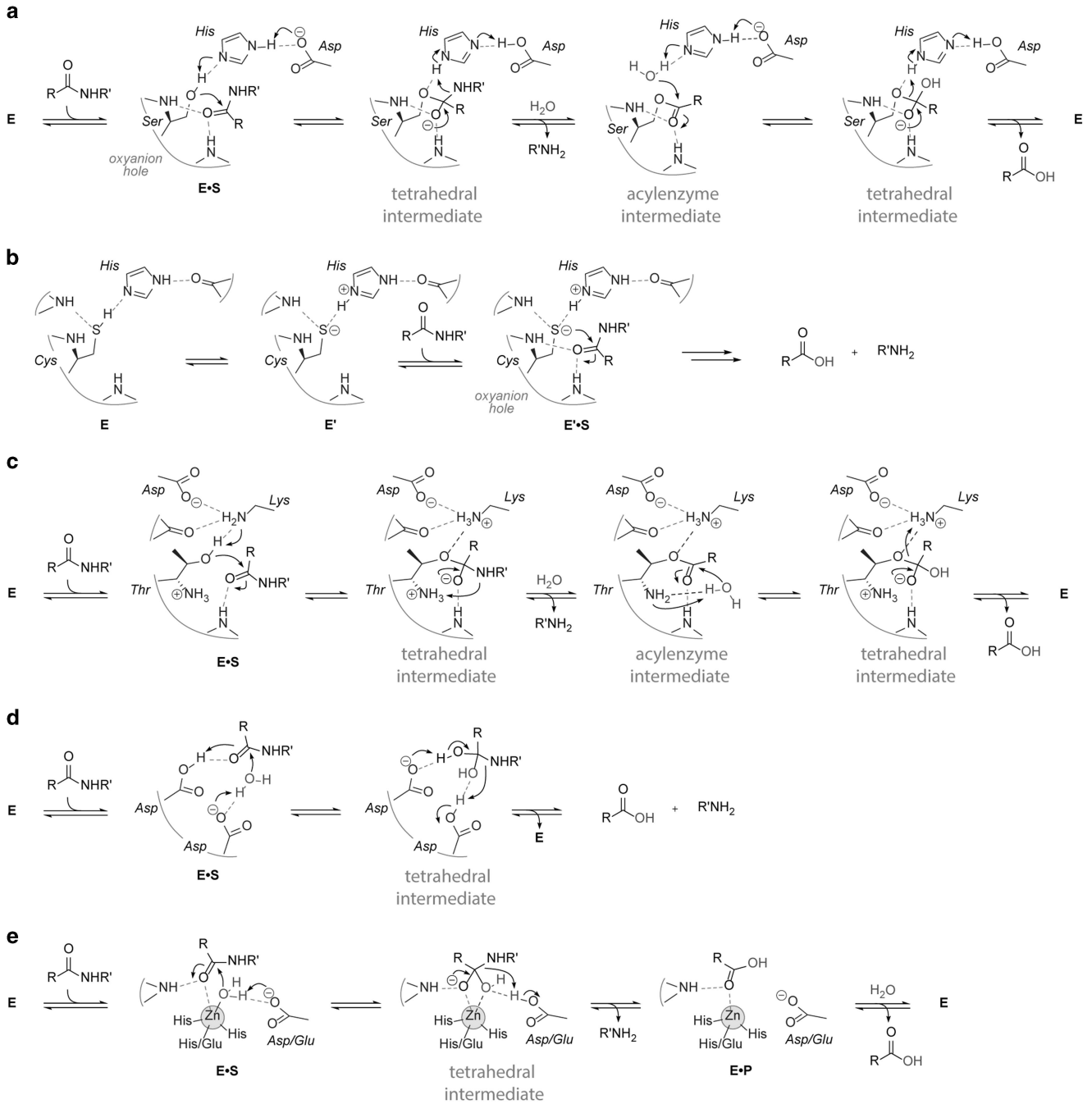


Figure 1 Protease catalytic mechanisms. All proteases cleave peptide bonds by proceeding through tetrahedral intermediates. Serine (**a**), cysteine (**b**) and N-terminal threonine (**c**) proteases form stable covalent acyl-enzyme complexes that are subsequently hydrolyzed by water. Aspartate (**d**) and zinc metalloproteases (**e**) use a non-covalent acid-base mechanism. Ser proteases can activate the nucleophilic Ser-hydroxyl through a His-Asp dyad as shown in (**a**) or through the primary amine of Lys. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

(Figures 1b and c) operated by an analogous mechanism where the more reactive Cys thiol[9] or N-terminal Thr hydroxyl[10] is the active site nucleophile. Asp and metallo-proteinases, on the other hand, do not operate through a covalent mechanism, but rather activate a water molecule by electrophilic catalysis, positioning the equivalent of hydroxide ion for amide bond cleavage (Figures 1d and e).[11,12]

Protease functional diversity is accompanied by a wide range of structural diversity that can be broadly grouped based on whether they are found extracellularly or intracellularly. Extracellular proteases are generally monomeric with high substrate specificity for a single protein or family of proteins.[13] They are often synthesized as inactive zymogens, protecting the cell from unregulated activity before secretion. In contrast, intracellular proteases are multimeric complexes with little substrate specificity but whose activity and substrate selection are tightly regulated.[13] To achieve this level of regulation, catalytic sites are hidden in barrel-like structures and gaining access requires substrate selection and unfolding. In this way, the protease is able to specifically target a wide range of substrates, including not only

damaged or misfolded proteins, but also transcription factors and signaling proteins required to coordinate complex cell responses.

In mammals, proteases have diverse, clinically relevant roles and are the target for an estimated 5–10% of all drugs being developed.[14] Extracellular secreted proteases are involved in roles ranging from blood pressure regulation (ACE and renin), blood coagulation (thrombin and factor Xa) and blood glucose regulation (dipeptidyl peptidase 4).[14] These proteases are the target of therapeutics such as the blood pressure drugs captopril (Capoten; Brystol-Myers Squibb) and aliskiren (Tekturna/Rasilez; Novartis/Speedel), and anticoagulants destirudin (Revasc/Iprivask; Novartis) and rivaroxaban (Xarelto; Bayer).[14] Intracellular proteases are also popular therapeutic targets in mammalian systems, owing to their involvement in viral infection, cancer and neurodegeneration. For example, tipranavir (Aptivus; Pfizer/Boehringer Ingelheim) targets the HIV protease, boceprevir (Victrelis; Merck) targets the hepatitis C virus NS3-4A protease and bortezomid (Velcade; Milennium) is a proteasome inhibitor used for the treatment of multiple myeloma and mantle cell lymphoma.[14]

As in mammalian systems, bacterial proteases have key roles in cell physiology, replication and survival. Of clinical relevance, extracellular proteases are involved in virulence, where they are responsible for the destruction of host tissue and the degradation of host defense proteins such as IgA1 immunoglobin.[15] Meanwhile, some intracellular proteases are essential genes required for cell viability and many are required for virulence. Their role in virulence includes regulating virulence factor production and secretion, and coordinating stress responses important for survival inside the host. These critical roles make proteases a prime target for antibacterial drug development, either as a traditional antibiotic promoting cell death or an antivirulence drug. Finally, the success of drugs targeting mammalian proteases validates their druggability. Indeed, proteases have complex structures with potential drug binding pockets in active sites, protein–protein interaction sites, cofactor-binding sites or other allosteric sites.

Despite these advantages, there are currently no approved antimicrobial agents targeting bacterial proteases. Common drug discovery strategies employed may be behind this contrast to the success of drugs targeting mammalian and viral proteases. Almost all mammalian and viral protease inhibitors bind in the active site by mimicking peptide substrates. Most of these were discovered by studying the natural substrate preferences of a protease and developing a similar synthetic molecule that married substrate recognition elements with structures that inhibit enzyme activity. For example, this strategy proved effective in the development of the renin inhibitor aliskiren, the ACE inhibitor capropril and the HIV protease inhibitor ritonavir. More recently, combinatorial libraries and fragment-based design strategies are being adopted. However, all of these methods continue to rely on small synthetic molecules. The history of antibiotic development suggests that such methods are unlikely to be fruitful against bacteria, which have mechanisms to sense and avoid small molecules including efflux pumps and complex membrane structures such as those of Gram-negative bacteria.[16] Furthermore, they are unlikely to have the structural complexity required to specifically bind a bacterial protease and not its mammalian homolog. In contrast to synthetic drug-like molecules, natural products are highly complex chiral molecules that have been sculpted by millions of years of evolution to enter bacterial cells.[16] These may be the critical leads required to propel bacterial proteases into the realm of *bona fide* antimicrobial drug targets.

INTRACELLULAR PROTEOLYTIC COMPLEXES

There are four families of intracellular proteolytic complexes ubiquitous in eubacteria: Lon, HslUV (ClpQY), ClpXP and FtsH. In addition to these five, HtrA (DegP) is a periplasmic/secreted proteolytic complex, whereas the prokaryotic proteasome is found only in actinomycetes. The structure, function and role in pathogenesis of each protease has been reviewed previously.[13,17] Several of these complexes have been investigated as potential antibacterial targets, including Lon,[18] ClpXP,[19] HtrA[20] and the proteasome.[21] Of these, ClpXP has been most extensively investigated and is the only complex for which natural product inhibitors have been found thus far.

The clp proteolytic complex

ClpPs are well conserved in most bacterial species and have an important role in protein turnover. In addition to protein homeostasis and degradation of misfolded proteins, ClpP is also involved in numerous regulatory processes by targeting transcriptional regulators and remodeling of the proteome.[22–25] Indeed, ClpP has been found to have a key role in regulating processes such as cell division, stress tolerance, virulence, morphological differentiation and antibiotic resistance.[22–24] ClpP's role in these processes relies on its strict selection of protein substrates, which it achieves by restricting access to its catalytic sites. Each ClpP complex is formed by stacking two heptameric rings to create a tetradecamer (Figure 2).[26] The channel that is formed houses 14 serine protease catalytic sites that cannot be accessed without passage through the axial openings. Furthermore, apo-ClpP adopts an inactive, compressed conformation in which its catalytic triad is misaligned.[27] Controlling conformational activation and access to axial openings are Hsp100 proteins of the AAA+ superfamily of ATPases: ClpA or ClpX in Gram-negative bacteria and ClpC or ClpX in Gram positives. These accessory ATPases form hexameric rings that bind the tetradecamer's axial faces using tripeptide (L/I)GF motifs that fit into hydrophobic pockets.[28] Binding in this hydrophobic pocket regulates ClpP activity in two ways: first, by stabilizing the complex in an active, extended conformation with the catalytic triad aligned and, second, through protein substrate unfolding. AAA+ ATPases interact with protein targets, either directly or through a cooperating adaptor protein, and use the energy provided by ATP to unfold the substrate and feed it into the central pore where it is hydrolyzed in an energy-independent manner. As folded proteins are otherwise too large to enter the channel, these AAA+ partners tightly regulate which protein substrates are targeted for degredation.[28]

Most bacterial species, including *E. coli*, *Bacillus subtilis* and *Staphylococcus aureus*, have one *clpP* gene that, along with their associated AAA+ ATPases, are nonessential for cell viability.[25,29–32] Nonetheless, it has been observed that *clpP* deletion in these species increases their susceptibility to antibiotics such as linezolid and rifampicin, and decreases virulence in pathogens such as *Listeria monocytogenes* and *S. aureus*.[23,33] Loss of virulence in ClpXP-deficient strains has been linked to major perturbations in global virulence transcriptional factor levels such as the *sar/agr* regulatory network in *S. aureus*.[23] Interestingly, the effect of ClpP inactivation on several of these virulence regulators appears to be strain dependent and, moreover, some *S. aureus* strains deficient in ClpXP function appear to have decreased susceptibility to vancomycin, daptomycin and β-lactam antibiotics.[34–36]

In contrast to most bacteria, two or more copies of *clpP* are found in actinobacteria and cyanobacteria and at least one functional copy is essential for viability.[37,38] In *Mycobacterium tuberculosis*, *clpP1* and *clpP2* form an operon and both genes are essential. These isoforms

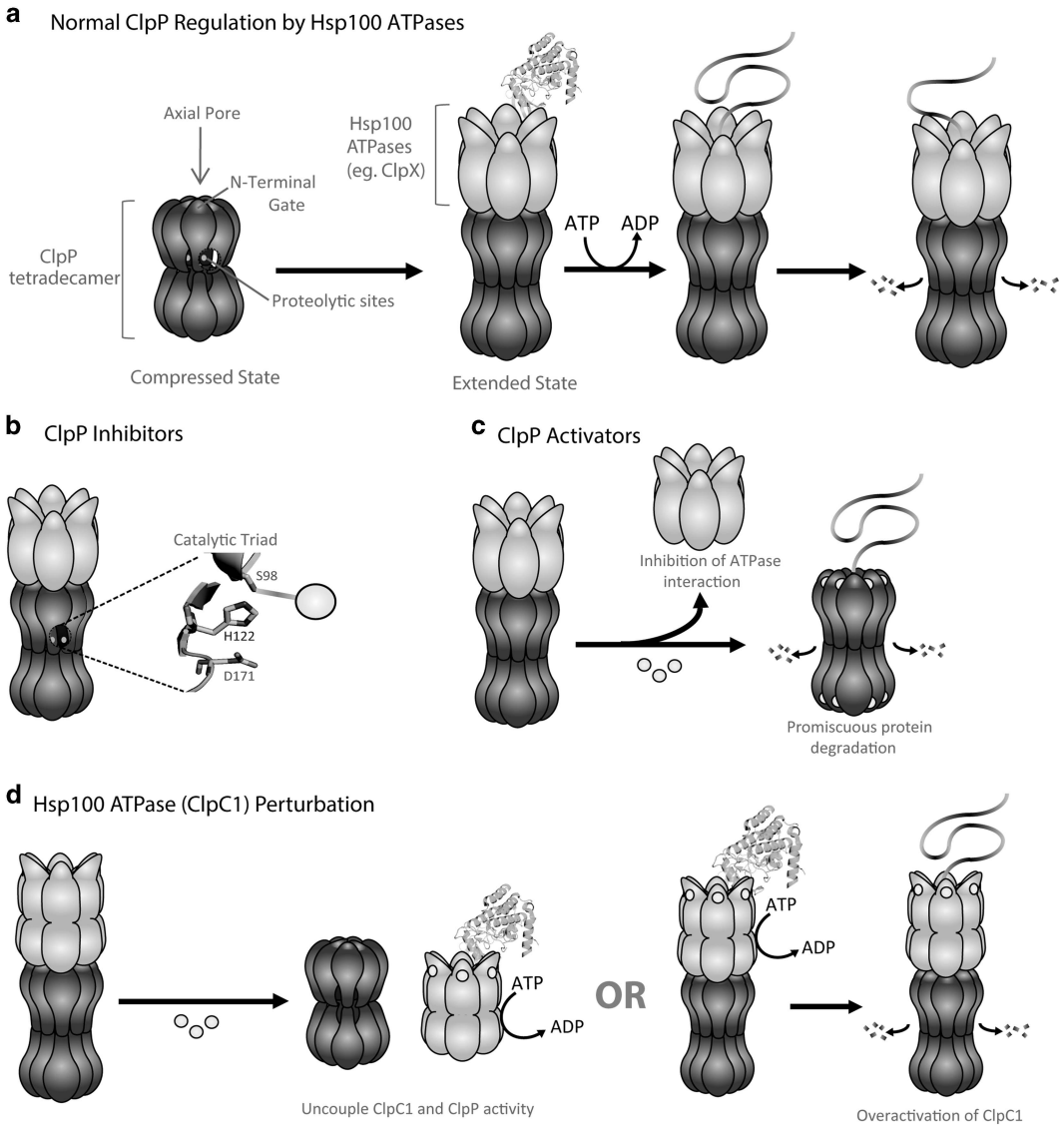


Figure 2 Mechanisms of perturbing the ClpP proteolytic system. (**a**) ClpP tetradecamers (shown in red) housing serine proteolytic sites (shown in green) are tightly regulated by Hsp100 ATPase hexamers (shown in blue). (**b**–**d**) Drugs (shown in yellow) can cause perturbation in one of three ways, leading to cell death or reduced virulence. Drugs activating the ATPase activity of ClpC1 in *M. tuberculosis* (**d**) are thought to either uncouple it from ClpP, inhibiting proteolysis or lead to an increase in protein degradation. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

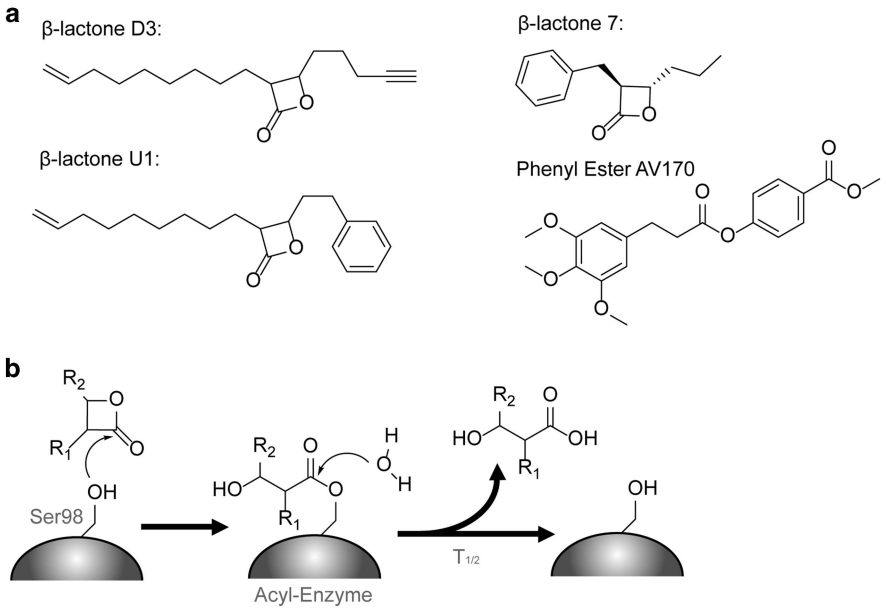
work together to create a functional protease by stacking ClpP1 and ClpP2 homoheptamers into a heterotetradecamer.[37,39] Of the four AAA+ ATPases in *M. tuberculosis*, ClpX and ClpC1 are essential for viability.[40,41]

Given its role in cell viability and virulence, the ClpP system is a promising target for novel antibacterials with three possible mechanisms for deregulation (Figure 2): inhibition of ClpP proteolysis, activation of ClpP proteolysis or perturbation of partner AAA+ ATPases.

ClpP inhibitors

Perhaps the most obvious approach to targeting the ClpP system is to develop an active site inhibitor of ClpP. Proof of principle of this mode of action has been demonstrated in *S. aureus*, *Streptococcus pneumoniae* and *L. monocytogenes*, where *clpP* knockouts are unable to cause skin infection abscesses, lung infections or *in vivo* macrophage parasitism, respectively.[22,42,43] This loss of virulence has been associated with decreased activity of extracellular proteases, lipases, DNases and α-hemolysin in *S. aureus*, and α-listerolysin and listerial phospholipase in *L. monocytogenes*.

The pioneering efforts of Böttcher and Sieber[44] to target ClpP led to the development of a series of β-lactone inhibitors. Inspired by the reactivity of β-lactones found in nature, they synthesized a library of alkyne-tagged derivatives (for example, Lactone D3; Figure 3a), which were screened against several bacterial proteomes. Click chemistry on the alkyne tag was used to attach a fluorophore and allow for the identification of reactive enzymes. In this way, ClpP was identified as a highly specific target that forms a covalent adduct between its active site Ser98 and the β-lactone, thereby irreversibly inhibiting proteolytic activity (Figure 3b).[44] Subsequent characterization demonstrated the ability of β-lactones to reduce virulence factor activity, including α-hemolysin and listerolysin, in *S. aureus* and *L. Monocytogenes*, respectively.[45,46] This reduction in virulence factors correlated with the ability of optimized β-lactone scaffolds (U1; Figure 3a)



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Compound	Acyl-Enzyme Half Life (Hr)	ClpP Inhibition - IC50 (μM)	Notable Bioactivity
B-lactone D3 [44]	5 ± 0.4 [49]	ND	Hemolysis reduction (EC50 = 34 μM) [46]
β-lactone U1 [46]	>40 [27]	7 [44]	Hemolysis reduction (EC50 = 30 μM) [46]
Phenyl ester AV170 [49]	8.2 ± 0.8 [44]	0.3 [44]	Partial hemolysis reduction [44]
β-lactone 7 [48]	ND	ND	Anti-*M. tuberculosis* (MIC = 28 μg/ml)[48]

Figure 3 ClpP inhibitors. (**a**) Chemical structures of highly active β-lactones and phenyl esters. β-Lactone D3 was originally used as a probe for ClpP inhibition by click chemistry and subsequent optimization yielded β-lactone U1. β-Lactone 7 was optimized for *M. tuberculosis* growth inhibition. (**b**) Mechanism of proteolytic active site inhibition of β-lactones by covalent modification of Ser98. Hydrolysis of the acyl-enzyme complex is described by its half-life. (**c**) Comparison of β-lactone and phenyl ester stability, potency and activity. Hemolysis reduction refers to that quantified by clearing on blood agar plates caused by *S. aureus* cultures. (ND, not determined). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

to significantly reduce *S. aureus* infection after subcutaneous administration in a skin abscess model and *L. monocytogenes* growth in macrophages.[46,47] Furthermore, β-lactone derivatives (compound 7; Figure 3a) were found to be among the privileged group of compounds able to enter *M. tuberculosis* in order to effectively inhibit growth with an MIC of 28 μg ml^{-1}.[48] Ultimately, however, low plasma stability due to rapid hydrolysis of the cyclic ester precluding further clinical development.

More recently, Sieber and colleagues[49] has discovered a new class of potent ClpP inhibitors, the phenyl esters (AV170; Figure 3a). Discovered using an unbiased screen of 137 000 synthetic compounds in a fluorogenic assay for ClpP activity, phenyl esters act by the same covalent modification of Ser98 as β-lactones. Interestingly, some enantiomers are also able to trigger deoligomerization of the ClpP tetradecamer into heptamers, a favorable mode of action given that conformational control of the serine catalytic triad yields it inactive in ClpP's heptameric form. Despite the phenyl esters' improved potency of protease inhibition over β-lactones, their anti-virulence activity is reduced, as they are only able to diminish and not abolish α-hemolysin production (Figure 3c). Efforts to increase potency revealed a trade-off between stability and reactivity.[49]

Although ClpP inhibition shows promise as a mechanism of action, further development and discovery of novel scaffolds is clearly required. Moreover, given recent evidence of drug resistance in certain *clpP* knockout strains,[34,36] some caution down this path may be warranted.

ClpP activators

Activation of the ClpP protease system is a particularly intriguing therapeutic option. A hallmark of intracellular proteases is strict regulation of activity to avoid off target protein degradation. In eukaryotes, this is often accomplished by compartmentalization in organelles. In contrast, bacteria have developed tight regulatory protein complexes to control protease activity. By activating proteolytic activity to indiscriminately degrade proteins, a drug would be able to cause cell death not only in those species where ClpP is essential, but also those where ClpP is dispensable. Mutations abolishing ClpP's activity, while theoretically conferring resistance, would be fatal in cells where ClpP is essential and impair virulence in cells where ClpP is dispensable. Finally, the unprecedented mode of action by activation rather than inhibition of its target may allow such a drug to be effective against dormant persister cells.

Serendipitous proof of principle of this unique mechanism of action was reported with the discovery of acyldepsipeptides (ADEPs; Figure 4a). ADEPs were first described in a patent in 1985 as the 'A54556 complex,' a group of eight closely related compounds produced by *Streptomyces hawaiiensis* NRRL15010.[50] ClpP was identified as ADEP's molecular target in 2005 when Brötz-Oesterhelt

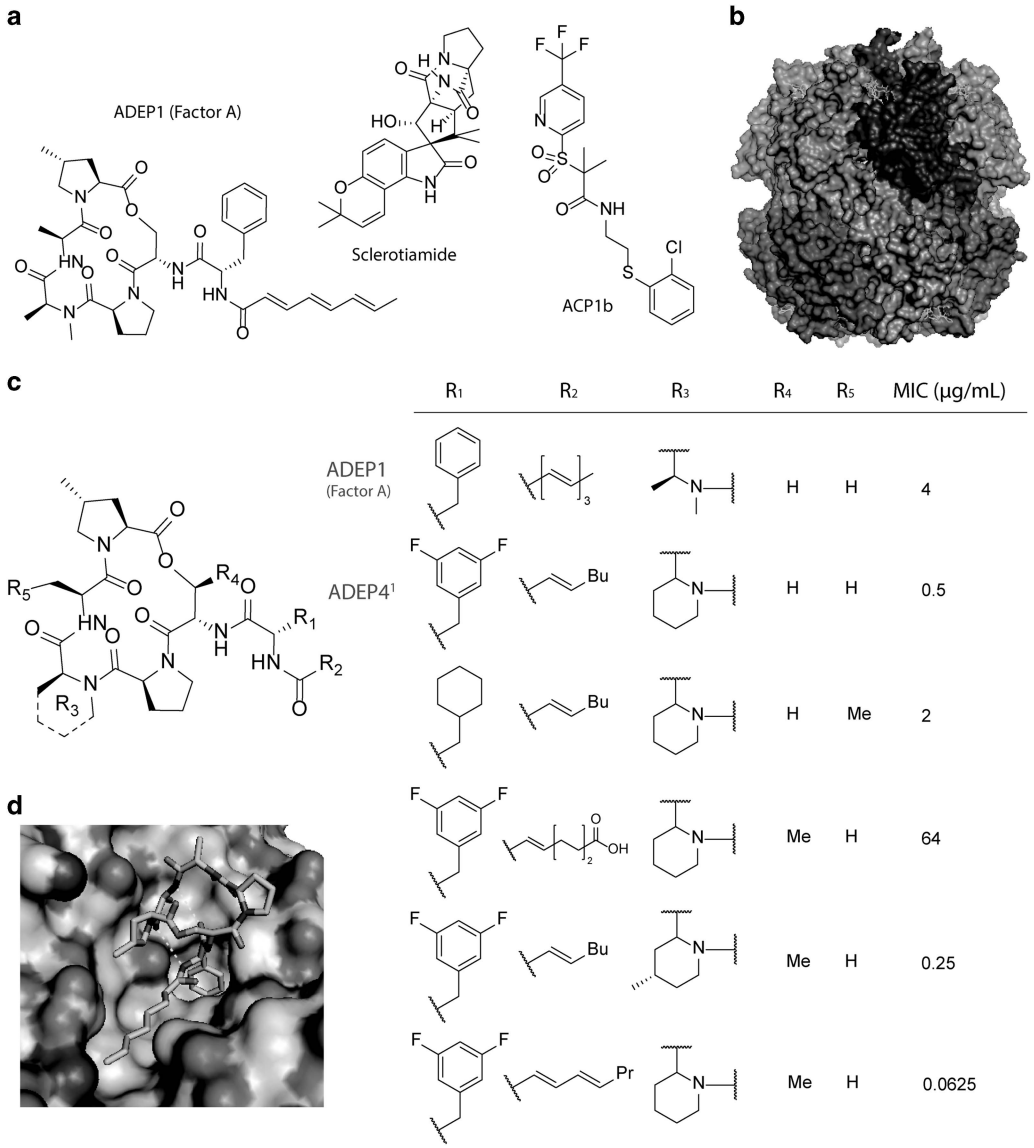

Figure 4 ClpP Activators. (**a**) Chemical structures of natural product ClpP activators ADEP1 and sclerotiamide, and the optimized synthetic ClpP activator ACP1b. (**b**) Crystal structure of *E. coli* ClpP tetradecamer in complex with ADEP (PDB 3MT6)[55] Stacking heptamers are shown in light/dark colours, ClpP monomers are shown in alternating red and blue, and ADEP molecules are shown in green. (**c**) Representative structures of ADEP's SAR culminating from several medicinal chemistry efforts. MICs against *S. aureus* are listed.[54] (**d**) ADEP docking site at the interface of two ClpP monomers, shown in light pink/blue. Nitrogens are marked in dark blue while oxygens are marked in red. Two reported transannular H bonds are shown by yellow dashed lines. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

et al.[51] applied reverse genomics to an ADEP-resistant *E. coli* strain to identify the resistance determinant as a mutation in ClpP that renders it inactive. This finding suggested that ADEPs activate ClpP, causing inappropriate protein degradation. Indeed, *in vitro* studies measuring cleavage of fluorogenic peptides and *in vivo* proteomic analysis confirmed that ADEP-activated *B. subtilis* ClpP was able to degrade proteins independent of AAA+ regulation or ATP hydrolysis.[51]

Insight into this loss of regulation has been provided by crystal structures of ADEP-activated ClpP from *E. coli*, *B. subtilis*, *M. tuberculosis* and *Neisseria meningitidis* (Figure 4b).[52–55] An ADEP molecule binds to each monomer in the ClpP tetradecamer in the same hydrophobic pocket that is used by the AAA+ ATPases, thus inhibiting their interaction (Figure 4d).[52,55,56] Binding mimics the ATPase's conformational control of ClpP, inducing alignment of the serine catalytic triad and a rigid body rotation of the ClpP monomers, widening the axial pore from 10–12 Å to 20 Å in *E. coli*.[27,52,55] A gating mechanism is also provided by the N-terminal domains, which move from a down, or closed conformation to an up, or open conformation on ADEP binding.[52,55] ADEP activation does not allow stably folded proteins to be degraded, as they are still too large to enter ClpP's axial lumen, but does allow unstable proteins and nascent chains emerging from the ribosome to be degraded, especially if they fold slowly.[57] Cell death is thought to be a result of this indiscriminate degradation, as well as the inhibition of normal ClpP function.

ADEPs are active against a range of Gram-positive bacteria including clinically relevant methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci and penicillin-resistant *S. pneumoniae*, as well as the Gram-negative pathogens *N. meningitidis* and *Neisseria gonorrheae*.[51,54] Semisynthetic ADEP derivatives are

effective against *Enterococcus faecalis*, *S. aureus* and *S. pneumoniae* in mouse and rat models with activity superior to linezolid,[51] and activity against MRSA in a murine periotinitis model is superior to vancomycin.[58] Notably, resistance to ADEPs develop at a frequency as high as 10^{-6} in these bacterial species by mutations diminishing the function of ClpP, which is non-essential. Nevertheless, using *clpP* mutants' decreased fitness as an advantage, combination therapies of ADEP and rifampicin have been shown to completely eradicate a vancomycin resistant MRSA population *in vitro*.[59]

Even more promising is the ability of ADEPs to eliminate persister cells.[59] Conlon *et al.*[59] first described this property when an ADEP effectively killed both stationary phase *S. aureus* and persister *S. aureus* left after ciprofloxacin treatment. This finding has implications for the use of ADEPs against latent tuberculosis infections caused by drug-tolerant *M. tuberculosis* persisters. To date, activity against *M. tuberculosis* persisters has not been described, but ADEPs have been shown to slow the growth of *M. tuberculosis* when combined with two efflux pump inhibitors, reserpine and verapamil.[39]

Although ADEPs are promising leads for drug candidates, they have unfavorable pharmacological properties including poor water solubility, rapid systemic clearance and chemical instability.[51,60] Following the discovery of ADEPs' mode of action, a medicinal chemistry SAR program was launched by Bayer AG, to develop a more stable and potent derivative of ADEP. This effort resulted in the development of ADEP4, a highly potent ADEP1 derivative with three important modifications: Phe is replaced by 3,5-difluorophenylalanine, which is thought to form H bonds with ClpP, the acyl polyene is replaced by an α,β-unsaturated hexenoyl tail to improve stability and N-MeAla is replaced by pipecolate, which increases ADEP's rigidity.[60] Activity of ADEP4 was further improved by Carney *et al.*[61] by replacing Ser with Allo-Thr and Pip with 4-MePip to further rigidify ADEP. By quantifying hydrogen-deuterium exchange rates using ^{1}H NMR, rigidification of the ADEP molecule was shown to strengthen the transannular H bonds, thereby reducing the entropic costs of binding to ClpP. Carney's ADEP derivative has 600–1200-fold greater potency than ADEP1 against Gram-positive pathogens.[61]

Despite the increased potency of these ADEP derivatives, they still only have limited activity against Gram-negative bacteria and are efficiently removed from the cell by active efflux, especially in *M. tuberculosis*.[39,62] Numerous other synthetic chemistry efforts have explored motifs to overcome these limitations, but most have resulted in diminished activity (Figure 4c).[54,63–67] Given the intimate binding of ADEP in its hydrophobic pocket, this result is perhaps predictable and suggests that modification on the ADEP scaffold may have reached an impasse.

Two high-throughput screens have been mounted to identify novel ClpP activators.[65,68] Both identified small-molecule activators of *E. coli* ClpP by using an *in vitro* assay measuring increases in fluorescence caused by cleavage of fluorescein isothiocyanate–casein, a model substrate for ClpP. In the first, Leung *et al.*[65] screened 60 000 drug-like synthetic chemicals, identifying five compounds termed ACP1–5 (Figure 4a). The most active of these compounds were 10–20-fold less potent than ADEP1 at activating ClpP and only displayed modest antibacterial activity even in the presence of permeabilizing agents. Lavey *et al.*[68] instead screened $>20\,450$ fungal and bacterial extracts or metabolites and identified a single ClpP activator, sclerotiamide (Figure 4a). This paraherquamide-related indolinone was 73-fold less potent than ADEP1 at activating EcClpP and failed to inhibit growth of efflux deficient *E. coli* or *Pseudomonas aeruginosa.*

Despite the limited efficacies of the ACPs and sclerotiamide, these studies provide novel scaffolds for derivatization and open the door to future studies to identify ClpP activators. One could imagine, for example, identifying alternate activation binding sites on ClpP. ClpP's regulation involves not only control of substrate entry by pore widening upon AAA+ ATPase association, but also formation of the serine catalytic site through oligomerization into tetradecamers.[69] Perhaps a small molecule that induced active site formation while maintaining ClpP as a heptamer could allow for easy access to this active site by indiscriminate substrates.

AAA+ ATPase uncouplers as therapeutics

As ClpP relies on AAA+ ATPases to select and unfold protein substrates, perturbation of these partners can also deregulate the ClpP proteolytic system. In particular, this is true in *M. tuberculosis* where the ClpC1 and ClpX ATPases are essential for cell viability.[40,41] Indeed, each of the three compounds targeting ClpC1 characterized to date were discovered by screening natural product extracts for anti-*M. tuberculosis* activity. The first of these to be discovered is cyclomarin A (cymA), a cyclic heptapeptide produced by the marine bacterium *Streptomyces* sp. CNB-982 (Figure 5a).[70] Although it was described in 1999 as a potent anti-inflammatory agent with cytotoxicity against cancer cells, it was not until 2011 that activity against *M. tuberculosis* was discovered during a natural product whole-cell screen.[71] In a first attempt to identify cymA's molecular target, a reverse genomics approach was taken. However, after no spontaneous resistant *M. tuberculosis* mutants could be recovered, affinity chromatography was instead used to show that cymA targets ClpC1 with high specificity. Subsequent co-crystalization of cymA with ClpC1's N-terminal domain identified residues important for binding and, despite the inability to generate spontaneous resistant mutants, allowed for the creation of ClpC1 mutants conferring resistance to cymA.[72]

In 2014, ecumicin, a macrocyclic tridecapeptide from *Nonomuraea* sp. MJM5123,[73] and lassomycin, a 16-membered lasso-peptide from *Lentzea kentuckyensis* sp.,[74] were isolated both by screening crude actinomycete extracts (Figure 5a). The N-terminal domain of ClpC1 was identified as the molecular target of these compounds by reverse genomics on spontaneous resistant mutants. Despite cymA, ecumicin and lassomycin sharing a common target, structural characterization and the position of mutations conferring resistance to each compound suggest that each bind at a slightly different position on the N-terminal domain of ClpC1 (Figure 5b). For example, in contrast to cymA and ecumicin, lassomycin is highly basic, containing several Arg residues, and docks in a highly acidic region of ClpC1.[74]

CymA, ecumicin and lassomycin are all bactericidal against replicating *M. tuberculosis*, a range of other mycobacterial species, and multidrug-resistant *M. tuberculosis*. Importantly, they are also active against nonreplicating *M. tuberculosis.* Consistent with the lack of essentiality of AAA+ ATPases, each lacks activity against other Gram-positive and Gram-negative species such as *S. aureus* and *P. aeruginosa.* This specificity does have benefits, as they also lack activity against commensal members of the human microbiota.

In order to cause cell death in *M. tuberculosis*, ecumicin and lassomycin appear to stimulate ATPase activity, but uncouple it from protein degradation.[73,74] In this way, degradation of natural substrates is inhibited and leads to their buildup and toxicity, similar to the actions of both ClpP inhibitors and activators in *M. tuberculosis*. In contrast to ecumicin and lassomycin, cymA has been suggested to increase protein degradation, as demonstrated by a decrease in LeuAspAsp tripeptide-tagged green fluorescent protein fluorescence

targeted to ClpC1 on incubation with cymA.[71] However, it is possible that this decrease in fluorescence is the result of green fluorescent protein unfolding by ClpC1 rather than degradation and cymA may therefore have the same uncoupling mechanism as ecumicin and lassomycin. Several questions remain unanswered about the mechanism of action of these drugs, including their effects on protein unfolding and how ATPase activity is stimulated and proteolysis inhibited, for example, by inhibiting interaction with ClpP1P2. Furthermore, characterization is still underway for yet another ClpC1 inhibitor recently discovered, the rufomycin analog RUF-I.[75]

Despite the relatively high potency of cymA, ecumicin and lassomycin against *M. tuberculosis*, optimization of pharmacological properties is required. For example, cymA exhibits hepatic clearance and a short half-life in mice, and ecumicin has limited solubility and poor intestinal absorption.[13,73] Recent total syntheses and fermentation optimization may aid in these developments.[76–78]

Although drugs targeting ClpC1 are an intriguing possibility for anti-*M. tuberculosis* therapeutics, they generally do not have bactericidal activity against species other than actinobacteria. In these species where ClpP and associated AAA+ ATPases are dispensable, it is possible that targeting these ATPases would have antivirulence effects similar to those observed with ClpP inhibitors. However, such a compound would likely need to be able to target multiple ATPase partners in order to have as widespread an effect as direct action on ClpP. These potential antivirulence effects have not been investigated for cymA, lassomycin or ecumicin.

Achieving specificity

Of the bacterial proteolytic complexes, all but HslUV has a human ortholog and many are in fact intensely studied as potential anticancer targets. For example, mitochondrial proteases LONP1, ClpXP and m-AAA (FtsH homolog) have important roles in quality control in the mitochondria, especially during respiratory stress.[79] Mutations in m-AAA are also implicated in spastic paraplegia, a hereditary neurodegenerative disease.[80] As such, it is vital that antimicrobial agents be able to target their bacterial homolog specifically.

In the case of protease inhibitors aiming to act as suicide substrates, achieving specificity can be challenging, owing to conserved catalytic mechanisms. Indeed, a lack of specificity has been encountered in efforts to develop inhibitors of both the prokaryotic proteasome and the bacterial Lon protease. The prokaryotic proteasome in *M. tuberculosis* makes for a promising target for inhibition, as it is dispensable for growth *in vitro* but is essential for survival of nitric oxide stress[81] and persistence in mice.[62,82] Many attempts have been made to develop a drug against the mycobacterial proteasome, usually

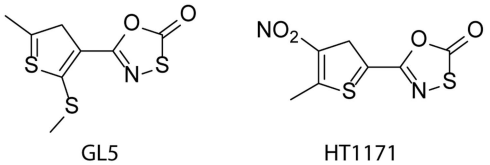

Figure 6 *M. tuberculosis* proteasome inhibitors of the oxathiazole-2-one family.

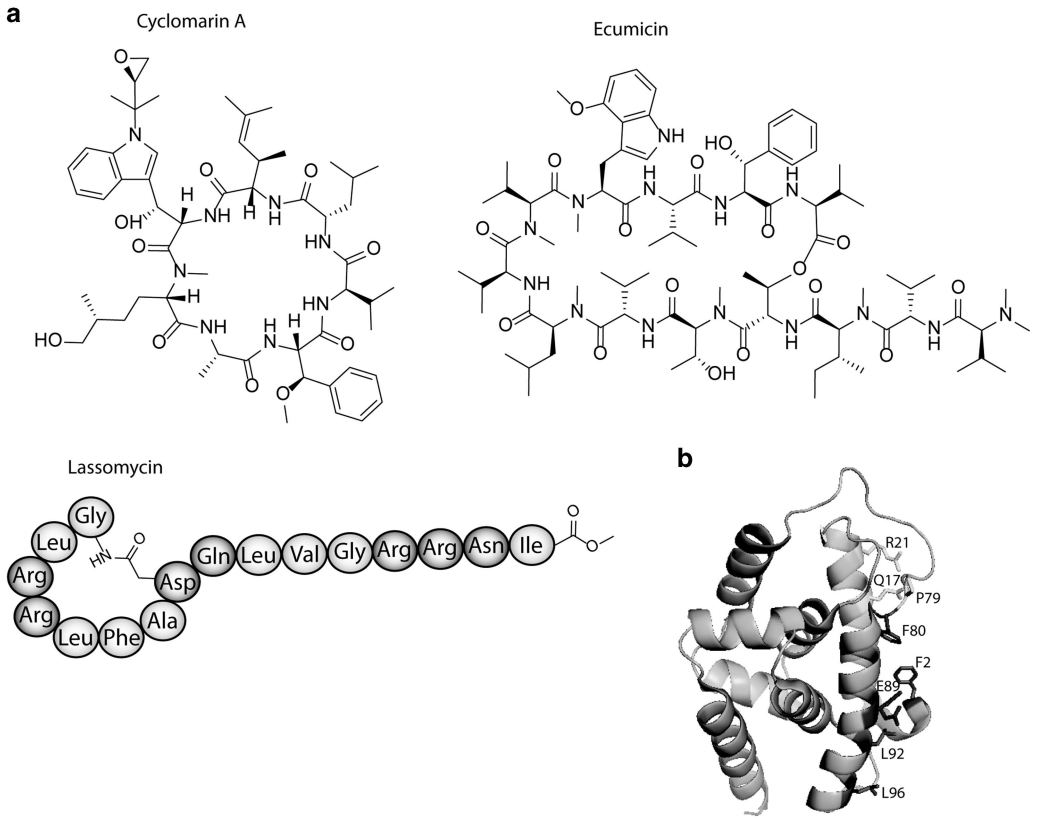

Figure 5 ClpC1 Activators. (**a**) Chemical structures of cymA, ecumicin and lassomycin. Basic amino acids are shown in red, and aliphatic/aromatic are shown in blue. (**b**) Crystal structure of *M. tuberculosis* N-terminal domain of ClpC1 (PDB 3WDC). The distinct binding sites of each activator are demonstrated by the location residues involved in activator binding, shown in yellow (lassomycin), blue (cyclomarin) and red (ecumicin). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

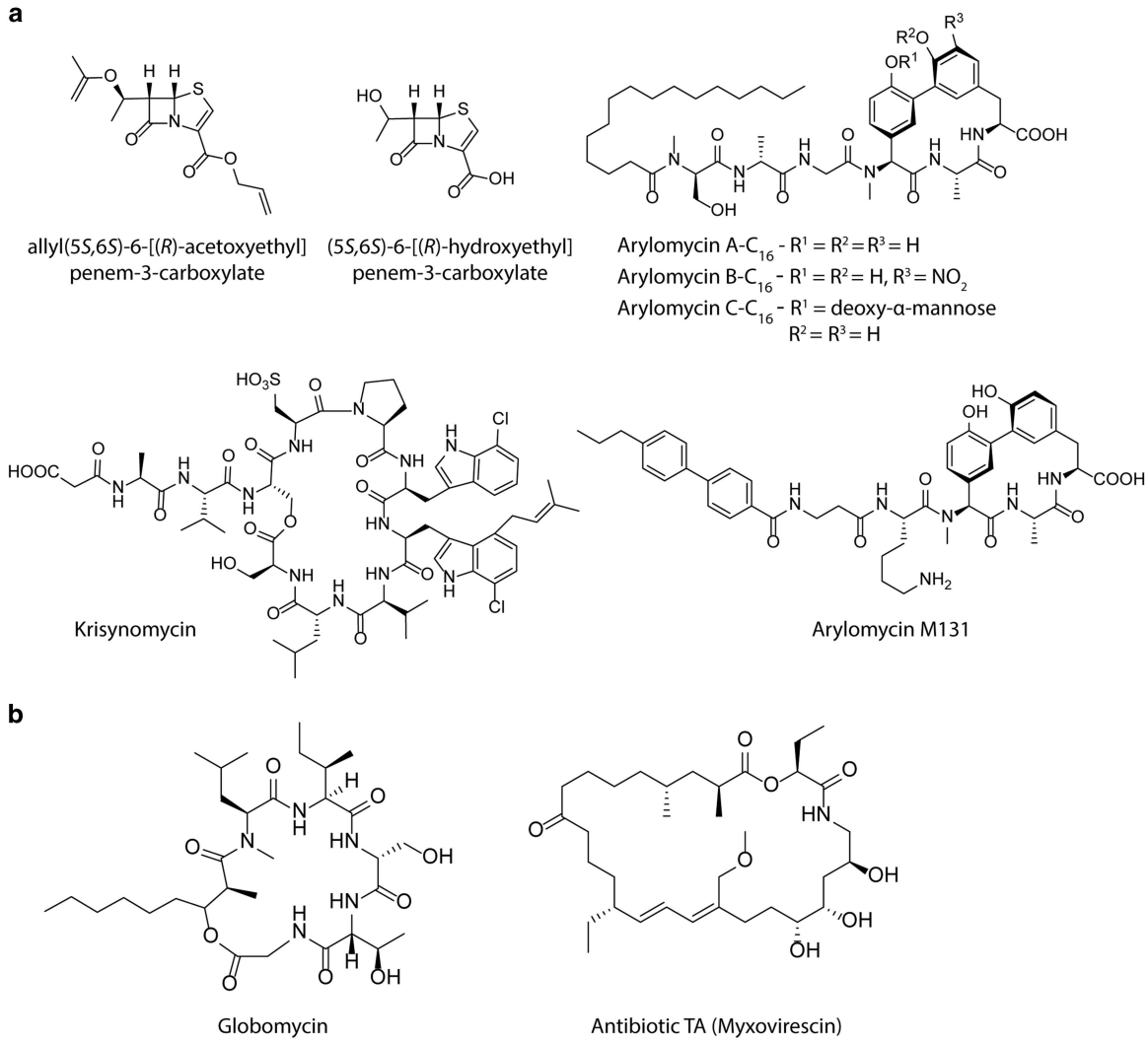

Figure 7 Signal peptidase inhibitors. Chemical structures of (**a**) SPI inhibitors and (**b**) SPII inhibitors.

as peptidyl epoxyketones, aldehydes or boronates, but most inhibit the mammalian proteasome more potently than that of *M. tuberculosis*.[83] Selectivity is not unprecedented however, as demonstrated by discovery of the oxathiazole-2-one compounds GL5 and HT1171 (Figure 6). These compounds are bactericidal against non-replicating *M. tuberculosis* treated with subinhibitory levels of nitric oxide[21] and have >1000-fold increased activity against mycobacterial over human proteasomes. Specificity is thought to be conferred by interaction of the drug with residues outside of the active site not conserved in mammalian proteasomes.[21]

Lon also makes for a promising target, as it has been implicated in biofilm formation, motility and stress tolerance, and mutants have been shown to have reduced colonization and virulence in *Salmonella enterica* serovar Typhimurium, *Actinobacillus pleuropneuoniae*, *Vibrio cholera* and *P. aeruginosa*.[84–87] In the only effort to date to identify bacterial Lon inhibitors, proteasome inhibitors were screened *in vitro* and the peptidyl boronate MG262 was identified.[18] However, this compound is still 2000-fold more potent against the 20S proteasome.[18] As with oxathiazole-2-one compounds, taking advantage of residues divergent in human homologs is likely to be required to develop a highly specific Lon inhibitor.

Specificity may also be achieved by moving outside of the catalytic active site to binding sites that perturb protease function, but are poorly conserved between human and bacteria orthologs. ADEPs aptly demonstrate the potential of this approach. Although they have not been directly tested on human ClpP (hClpP), ADEPs are non-toxic to human cells up to 25 μg ml^{-1}, suggesting that they have poor, if any, affinity for the human enzyme.[58] Structural comparison of *E. coli* (EcClpP) and hClpP supports this notion. Their backbone structure is largely conserved, with a root mean squared deviation of 0.63 Å;[88] however, inspection of the hydrophobic pocket used for ADEP binding shows that hClpP has several substitutions reducing its hydrophobicity (Asn55Pro, His60Tyr and His112Phe) along with a charge inversion (Glu56Lys) at the distal portion of the docking groove. It is quite possible that these changes will prevent ADEP binding in hClpP. It should be noted, however, that EcClpX, although not EcClpA, can activate hClpP.[88] In any case, searching for drugs binding less conserved regulatory sites may be the key to finding highly specific antibacterials.

SIGNAL PEPTIDASES

In addition to the AAA+-dependent proteases, a few additional peptidases are proven antibiotic targets. Among them are the bacterial

SPs that are required for protein secretion. Bacterial proteins targeted for secretion to the periplasm (Gram negative) or the exterior of the cell are delivered to their destination via the efficient secretion networks such as Sec and TAT. SPs are required to cleave the targeting peptide following protein secretion and are therefore essential enzymes required for protein homeostasis. SPs come in two forms: type I enzymes that cleave peptides that target proteins moving across the bacterial cell membrane and type II enzymes that function primarily in the targeting of lipoproteins.[4] Type I SPs operate using an active site Ser, whereas type II enzymes are Asp proteinases. Both classes are targeted by natural product and synthetic inhibitors of different classes.

The first inhibitors of SP-I were β-lactam antibiotics reported 20 years ago. In particular, carbapenems with the 5S configuration (Figure 7a) efficiently covalently inactivate SP-I.[89,90] The natural product arylomycin lipopeptides (Figure 7a) inhibit SP-I by binding to the substrate binding and active sites (reviewed in Craney and Romesburg[91]), thereby blocking activity. A naturally occurring mutation of Ser to Pro in the substrate-binding pocket renders bacteria resistant to arylomycin. Subsequent efforts to overcome this effect through synthesis of arylomycin variants have been initiated.[92] A screen for synergizers by the Merck Frosst group of imipenem in MRSA identified two natural product SP-I inhibitors, the arylomycin-like actinocarbamycin and the cyclic peptide krisinomycin (Figure 7a).[93] The logic of synergy likely is the result of inhibition of secretion of β-lactamases that inactivate imipenem. Synthesis of actinocarbamycin generated M131 (Figure 7a) with improved *in vitro* inhibition of the MRSA SP-I, SpsB and imipenem synergy activity in murine models of MRSA infection. None of these SP-I inhibitors have completed pre-clinical efficacy studies. Given the challenge of single amino acid mutations such as a common Ser to Pro for the arylomycins, revisiting SP-I inhibitors as potentiators of β-lactam antibiotics in β-lactamase-producing bacteria may offer a more productive route for drug discovery. Such β-lactam antibiotic adjuvants[94] could show efficacy against mechanistically diverse Ser and metallo-β-lactamases.

SP-II is an aspartyl-protease required for proper secretion of lipoproteins and the cyclic depsipeptide antibiotic globomyin (Figure 7b) produced by *Streptomyces halstedii* inhibits its activity in a number of bacteria.[95] Structure activity relationship studies demonstrated that the hydroxyl of the Ser residue was critical for antibacterial activity.[96] The crystal structure of globomycin in complex with LspA, a SP-II from *P. aeruginosa*,[97] demonstrates that this Ser is in contact with the proposed catalytic Asp124 and Asp143 of the active site, and that the antibiotic occupies the substrate signal peptide-binding site. This structure should help to guide additional improvements to globomycin, to increase efficacy. The hybrid polyketide-peptide macrolactone antibiotic TA (also known as myxovirescin; Figure 7b) is also an inhibitor of SP-II.[98] This product of *Myxococcus xanthus* is rapidly bactericidal with no significant toxicity. The determination of the structure of the LspA•globomycin structure offers a route to understand the mechanism of myxovirescin by determining its co-structure with LspA. The strong activity of inhibitors of SP-II against Gram-negative pathogens, which are currently a significant medical challenge, is worthy of a renewed emphasis on discovery of additional agents that target the essential process of lipoprotein metabolism.

OTHER PROTEASE ANTIBACTERIAL TARGETS

Proteases have a number of key roles essential to bacterial physiology and pathogenesis. Transpeptidases such as the penicillin-binding proteins and sortases offer an energy-neutral means to generate peptide bonds on the outside of the cell, in the absence of labile high-energy co-substrates such as ATP. The penicillin-binding protein transpeptidases were among the first validated antibiotic targets, offering the main biochemical target of the β-lactam antibiotics.[99] Sortases graft secreted proteins to the bacterial cell wall in Gram-positive bacteria, many of which are essential for infection and thus are potential targets for antivirulence compounds.[100] For example, synthetic 3,6-disubstituted triazolothiadiazole inhibitors of *S. aureus* sortase have shown efficacy in a mouse model of infection.[101]

Indeed the role of proteases in bacterial virulence and infection is widespread across many species and here is an opportunity for antivirulence compounds that selectively target a narrow group of pathogens. The advantage of such compounds over antibiotics is the likely lessening of selective pressure that gives rise to resistance. On the other hand, the challenge of such molecules is that they are generally only effective before the establishment of an active infection and thus are required to be delivered either prophylactically or in conjunction with antibiotics. Nevertheless, prophylactic use can make great clinical sense under conditions of epidemics or outbreaks, or where there is potential for exposure to certain pathogens, for example, military personnel, natural disasters and so on. The *B. anthracis* lethal factor offers one such example.[5] This Zn^{2+}-protease is a critical component of the toxins that result in anthrax. Several efforts to develop inhibitors of lethal factor, some of which that have protective activity in rodent models of infection, have been reported (reviewed in Nestorovich and Bezrukov[102]). Another example where prophylaxis is warranted is infection by *Clostridium difficile* that often emerges in outbreaks in hospitals and in long-term care facilities. Critical to infection are the A and B toxins produced by pathogenic *C. difficile*. These toxins are composed of three domains: a receptor binding domain that targets the toxins to the host epithelial cell surface, a Cys protease domain that activates the toxin following endocytosis and a glucosytransferase domain that glucosylates Rho and Rac family GTPases, resulting in toxic downstream effects on the cell.[103] A screen for inhibitors of the Cys protease domain identified the organo-selenide ebselen.[104] Ebeselen efficiently blocked Cys protease activity of *C. difficile* toxins TcdA and TcdB *in vitro* and in cell culture, and also demonstrated protective activity in a mouse model of infection. The results from these examples of inhibition of virulence factors of *C. difficile* and *B. anthracis* augur well for validation of the general strategy of antivirulence compounds and targeting of protease in particular.

CONCLUSIONS

Proteases are among the largest family of enzymes in metabolism. They operate by a variety of chemical mechanisms and are vital to many aspects of bacterial cell life and pathogenicity. Nevertheless, in contrast to the proven efficacy of targeting proteases in other aspects of drug discovery, with the exception of the peptidoglycan biosynthetic transpeptidases sensitive to β-lactam antibiotics, there are no antibiotic protease inhibitors in the clinic. Although general mechanisms of chemical catalysis are conserved across kingdoms in protease superfamilies, there are sufficiently distinct substrates, active sites and structures to offer selectivity towards bacterial enzymes and limit toxic effects in the host. There are numerous examples of natural product inhibitors of bacterial AAA+ ATPase proteases and SPs to demonstrate that natural selection favors these enzymes as targets for antimicrobial agents. Furthermore, the importance of proteases in bacterial pathogenesis offers an untapped vista of targets for new

antivirulence drugs. Bacterial proteinases therefore are a great untapped frontier in the twenty-first century antibacterial drug discovery worthy of investigation and sustained effort.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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