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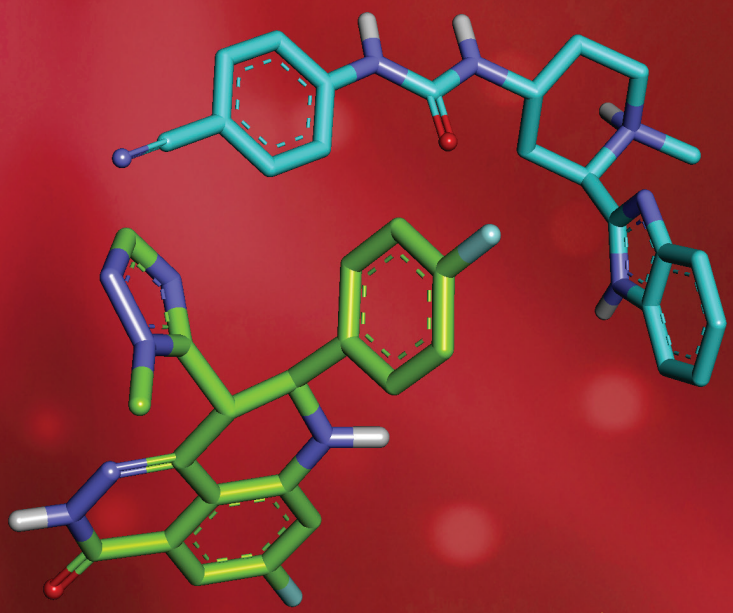
EDITOR-IN-CHIEF: JOANNE J. BRONSON

BRISTOL-MYERS SQUIBB, CAMBRIDGE, MASSACHUSETTS, U.S.A.



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CHAPTER 18

DEVELOPMENT OF SMALL MOLECULES TO MODULATE THE ACTIVITY OF THE ATP-DEPENDENT ClpP PROTEASE AS A NOVEL ANTIBACTERIAL AND ANTICANCER DRUG TARGET

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1. INTRODUCTION

Caseinolytic proteases (ClpPs) are complex serine proteases/endopeptidases regulated by chaperone proteins of the AAA+ superfamily (ATPases Associated with diverse cellular Activities) that are capable of cleaving peptides and proteins present in both bacterial and higher organisms.[1-4] ClpPs with their cognate ATPases are involved in the recognition and processing of many cellular macromolecules and, more generally, in protein homeostasis.[5,6] Consequently, ClpP, as a tightly regulated protease, is an interesting therapeutic target for a number of diseases, whether through inhibition or dysregulation (activation) of its activity.

Most attention to date has focused on bacterial ClpP, since it is highly conserved across many bacterial strains[7,8] and because of the largely unmet need for novel antibiotics to treat resistant pathogenic bacteria.[3,9-12] In many bacteria, ClpP in complex with its ATPase unfoldase AAA+ chaperones is responsible for much of the targeted protein degradation required to maintain protein homeostasis,[13-16] for example, bacterial ClpP targets proteins involved in chemotaxis, flagellar biogenesis, cell-division, metabolism, transcription regulation, damage repair, and housekeeping.[17-19] Other studies have shown that pathogenic bacteria with non-functional ClpP are avirulent and unable to cause infection.[20-24]

ClpP in humans is localized in the mitochondria and is responsible for quality control of mitochondrial proteins.[25,26] The metabolic cellular changes required in rapidly proliferating cancer cells lead to reactive oxygen species (ROS) that non-selectively damage biomolecules, causing mitochondrial dysfunction and cellular damage.[27,28] In response, human ClpP (HsClpP) is upregulated across many different types of cancer targeting mitochondrial proteins associated with cell proliferation.[19] Consequently, it is an emerging target for cancer chemotherapy.[29-33] Mutations in HsClpP are also implicated in Perrault Syndrome, which is characterized by sensorineural deficits and abnormal or missing ovaries in women.[4,34] HsClpP is also involved obesity and insulin resistance.[35,36]

Given the important regulatory role of ClpP, small molecules that are able to modulate ClpP activity are of interest as novel antibacterial and anticancer compounds. Herein, we describe the intriguing structural and mechanistic features of ClpP and the many efforts aimed at identifying different small molecules that inhibit or activate ClpP and/or interfere with its interaction with AAA+ chaperones.

2. ClpP PROTEASE: STRUCTURE AND MECHANISM OF ACTION

ClpP functions as a tetradecameric cylindrical serine protease composed of two stacked heptameric rings (~300 kDa for the tetradecameric complex) (Figure 1).[1,37-39] The heptameric rings are further stabilized by intersubunit electrostatic interactions near the rim of the two axial pores. The ClpP tetradecamer is then formed through interdigitation of ClpP handle domains of the two opposing rings, which are stabilized by conserved noncovalent contacts (Figure 1A). The ring-ring interface is flexible, allowing the ClpP barrel to compact and compress throughout its functional cycle, and to release degraded peptides through transient side pores.[32,40]

The catalytic sites of ClpP are compartmentalized within the proteolytic chamber of the tetradecamer and consist of conserved Ser-His-Asp catalytic triads present in each subunit (Figure 1C). ClpP has limited activity in degrading folded proteins, as they are too large to enter the proteolytic chamber through the ~10-20Å diameter axial openings.[41] However, the ClpP tetradecamer has 14 apical hydrophobic pockets that are the docking sites of the AAA+ chaperones. These pockets, which occur between neighbouring subunits, are highly conserved and are believed to interact with the hexameric ClpX,[42,43] ClpA or ClpC chaperone through a highly conserved IGF or IGF-like (I/L/V-G-F/L) motif in the ATPase.[44]

The hexameric AAA+ chaperones[45] recognize specific sequences in proteins and subsequently unfold the protein in an ATP-dependent manner. When bound to ClpP, the chaperones translocate the unfolded polypeptide into the ClpP barrel, resulting in the targeted degradation of proteins (Figure 2).[46,47] For example, *Mycobacterium tuberculosis* ClpC1 (MtClpC1) is the AAA+ chaperone that functions with ClpP1P2, forming the MtClpC1P1P2 complex to selectively degrade proteins. In some cases, such as *Bacillus subtilis* ClpP, an adaptor protein MecA, is required in addition to the AAA+ chaperone, ClpC, to confer proteolytic activity.[49] The ClpP-AAA+ complexes and adaptor proteins are able to target specific proteins.[50-52]

While ClpPs themselves are thought to be general proteases capable of cleaving many different sequences, and specific targeting is established by the AAA+ complexes, ClpPs from different species have different site cleavage preferences.[53,54] For example, the aminomethylcoumarin peptide Suc-Leu-Tyr-AMC is a good substrate for *Escherichia coli* ClpP (EcClpP), but

a poor substrate for MtClpP. Co-crystal structures of *Helicobacter pylori* ClpP (HpClpP) in the presence of the heptapeptide NVLGFTQ (PDB ID: 2ZL2) or tetrapeptide AAAA (PDB: 2ZL4)[55] suggest that peptide substrates are oriented by a hydrogen bond network between two adjacent β-strands, establishing a three-stranded, antiparallel β-sheet. Moreover, since the apo-forms of ClpP are oriented such that the catalytic triad residues (Asp-His-Ser) are not aligned in the active configuration, peptide binding is suggested to induce a conformational change of ClpP into an active form.

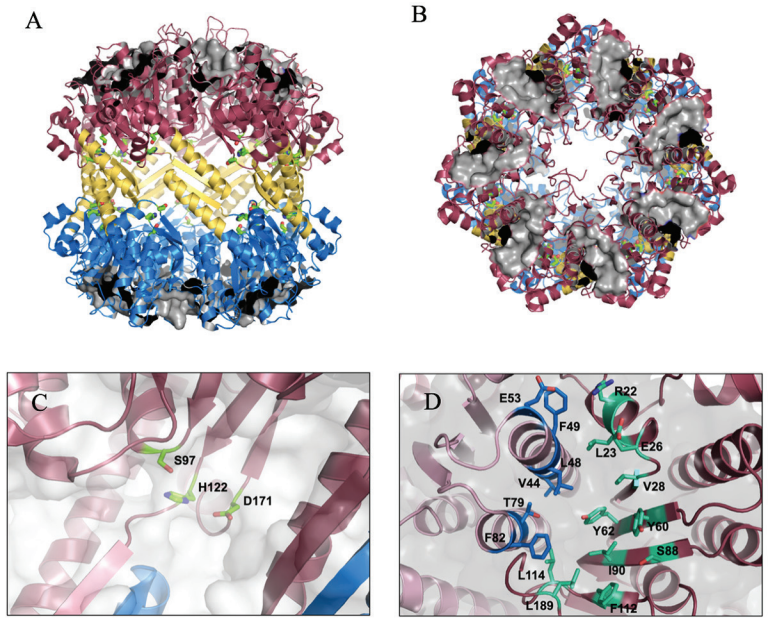

Figure 1 Cartoon representation of crystal structure of tetradecameric EcClpP (PDB: 1YG6). The two stacked heptameric rings are colored in red and blue with the handle region in yellow. **A**. The view of the ClpP barrel from its side. **B**. The view of ClpP looking down into its proteolytic chamber. Allosteric activating compound binding sites are shown as gray surfaces, while the catalytic Ser-His-Asp triads are shown as green sticks. **C**. Active site where proteolysis occurs, showing a properly aligned catalytic triad. **D**. Each allosteric site is formed by two adjacent ClpP subunits (shown in different shades of red for clarity). Key residues from each subunit in the interface that form the hydrophobic interaction surface are shown as colored sticks (blue and light green).

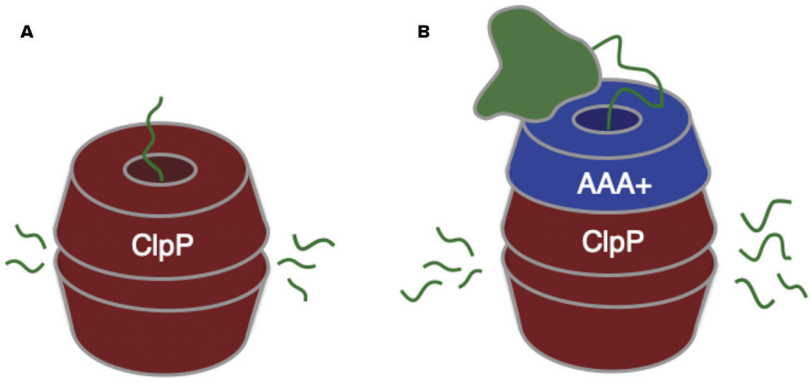

Figure 2 Mechanism of action of ClpP. **A**. ClpP assembles into a barrel-shaped tetradecameric complex with peptidase activity. **B**. When complexed with an AAA+ chaperone such as ClpX, the assembly is capable of unfolding protein substrates (shown in green) and translocating them into the ClpP proteolytic chamber where degradation occurs. The ClpXP complex has both protease and peptidase activity, with the former requiring ATP hydrolysis by ClpX for unfolding to occur prior to degradation.

3. INHIBITION OF ClpP PROTEASE

The active-site catalytic triad found within the core of ClpP represents the most direct site for interaction of small molecules with ClpP, and a variety of inhibitors have been developed for this purpose, including irreversible and reversible covalent inhibitors, as well as non-covalent inhibitors.

3.1 Peptidomimetic-Based Inhibitors

A co-crystal structure of EcClpP bound to the peptidic chloromethylketone[56] transition state inhibitor **1** revealed that both S97 and H122 catalytic triad residues are covalently attached to the inhibitor (Figure 3).[57] ClpP was unable to exhibit any peptidase or protease activity when it was incubated with **1**, and covalent modification occurred as evident by mass spectrometric detection of a covalently bound ClpP monomer.

Utilizing the peptide sequence specifically recognized by MtClpP, electrophilic C-terminal boronic acid tripeptides were generated as reversible covalent inhibitors (through boronate formation of the active site Ser).[54] Some of these Ac-X-Lys-boroMet peptide boronates inhibited the peptidase activity of MtClpP with K_i of <1 μM. Replacement of the boronic acid with an

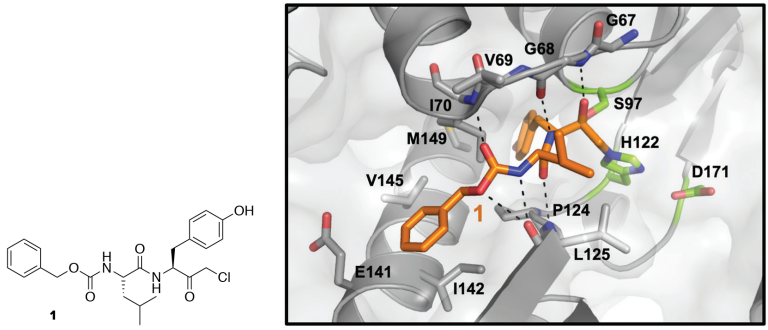

Figure 3 Cartoon representation of co-crystal structure of benzyloxycarbonyl leucyltyrosine chloromethylketone (Z-LY-CMK) **1** (orange sticks) with EcClpP (PDB: 2FZS) (*c.f.*, apo EcClpP [PDB: 1TYF, 1YG6]). The active site Ser97 forms a tetrahedral adduct with the carbonyl carbon of the peptide, while the imidazole nitrogen of His122 is covalently attached to the terminal α-methylene carbon of the ketone. The hemiketal oxygen atom of the inhibitor is located in the oxyanion hole formed by the amide NH groups of Gly68 and Met98, which form hydrogen bonds with the oxyanion in the tetrahedral complex. Hydrogen bonds between ClpP residues and **1** are shown as broken black lines. The catalytic triad residues are colored green, while other residues that interact with the small molecule are colored gray.

aldehyde led to inactive compounds, while replacement of the *N*-terminal acetyl group with a picolinoyl group enhanced the inhibitory activity of the peptide in most cases, with *N*-(Picolinoyl)-Trp-Lys-boroMet **2** identified as a very potent inhibitor with a K_i = 0.18 μM (Figure 4). Compound **2** exhibits MICs in the low μM range for *M. tuberculosis* but did not show any activity against either *E. coli* or *Staphylococcus aureus* (up to 200 μM). Bortezomib **3** (human 26S proteasome inhibitor) was identified as a potent inhibitor of ClpP1P2 in *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG and *M. tuberculosis* H37Rv.[58] Follow-up studies identified **4** as a selective growth inhibitor for M. tuberculosis with 74-fold greater potency for ClpP1P2 over the human proteasome, and favorable ADME properties.[59]

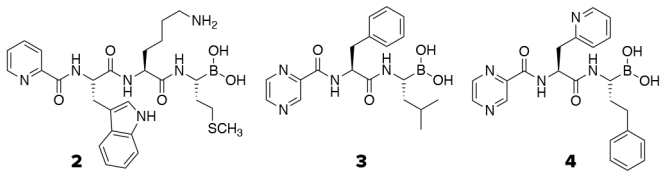

Figure 4 Boronic acid-peptide inhibitors of MtClpP1P2.

3.2 Other Covalent Inhibitors

The most well-studied group of covalent inhibitors are β-lactones which undergo ring-opening as "suicide inhibitors" to generate an *O*-acylated serine residue.[60,61,62] Racemic β-lactones **5** and **6** inhibit growth of *M. tuberculosis* and the non-pathogenic *M. smegmatis* through ClpP inhibition with MIC values of 10 and 45 μg/mL, respectively (Figure 5).[63] Compounds with disubstituted β-carbons or with fused rings attached to the β-lactone ring did not inhibit the growth of the bacteria. Structure-activity relationship (SAR) studies of the racemic β-lactone series established analogs **7** and **8** as able to inhibit the protease moderately with EC_{50} values of 3.4 and 5.4 μM, respectively, while **9** fully inhibited activity (EC_{50} = 4.5 μM).[64] Compound **7** did not affect growth of commensal bacteria found in the intestinal tract (*E. coli* and *Enterococcus faecalis*) and skin (*Staphylococcus epidermidis* and *Pseudomonas aeruginosa*) at 1 mM. However, human HaCat keratinocytes were shown to be sensitive to both **7** and **9**, indicating a need for further optimization.

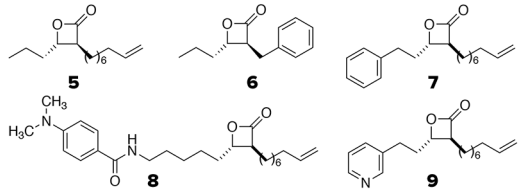

Figure 5 β-Lactone covalent inhibitors of ClpP.

It is known that ClpP, as an active tetradecamer, is held together by a network of hydrogen bonds that keeps the catalytic triad (Ser98, His123, Asp172 in SaClpP) in place. Asp170 and Arg171 also interact across the heptamer-heptamer interface to help align the triad in close proximity. Active-site mutations (L150A, N151Q, T169A, among others) were identified that caused the SaClpP to revert into a catalytically inactive heptamer.[65,66] The observation that mutation of residues in the active site can lead to changes in the conformation of the protein that prevent active tetradecamer formation suggest that small molecules could similarly lead to enzyme inhibition by preventing active tetradecamer formation (deoligomerization). Diisopropylfluorophosphate (DFP) **10**, β-sultam **11**, and β-lactone **12** cause complete inhibition and deoligomerization of SaClpP into the heptameric state and modification of the active site by 57%, 63%, and 35%, respectively, as determined by mass spectrometry (Figure 6). An X-ray co-crystal

structure of ClpP with **10** revealed the active-site serine residue (Ser96) to be covalently modified (Figure 7).[65] In the β-lactone series, modelling studies suggested the (*S*,*S*)-*trans* stereoisomeric series is the enantiomerically active form, while β-sultam inhibitor **11** led to subsequent elimination of the active site serine to a dehydroalanine. Thus, common serine protease inhibitors of ClpP may cause inhibition not only through direct active site inhibition but also by deoligomerization.

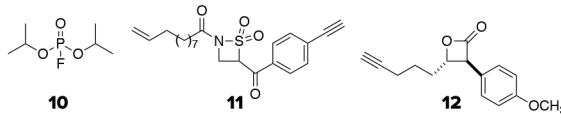

Figure 6 Small-molecule inhibitors of SaClpP oligomerization.

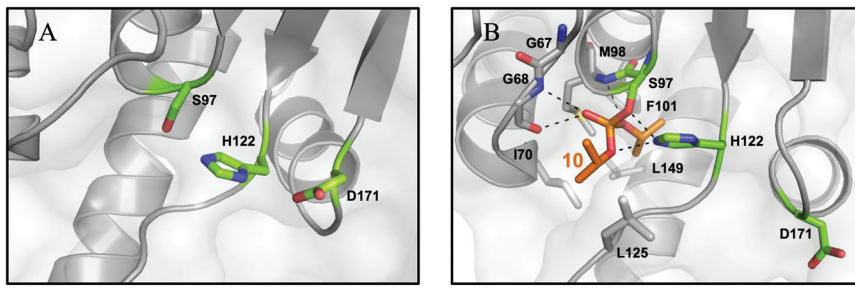

Figure 7 Cartoon representations of crystal structures of BsClpP in activator-bound form and bound with inhibitor **10** (*c.f.*, apo BsClpP [PDB: 3KTG, 3KTH, 3TT6]). A) Activator-bound BsClpP (with ADEP1 ligand **31a** [see Figure 13]; PDB: 3KTI) showing a properly aligned catalytic triad. B) BsClpP co-crystallized with DFP inhibitor **10** (orange sticks) (PDB: 3TT7). DFP is covalently bound to the catalytic serine residue. Hydrogen bonds between ClpP residues and **10** are shown as broken black lines.

An unbiased high-throughput screen (HTS) established five aryl esters and one acyl triazole as covalent inhibitors of *S. aureus* ClpP (SaClpP) at concentrations ranging from 0.3-1.3 μM (Figure 8).[67] Compound **13** is more effective at inhibiting SaClpP activity than β-lactone compounds at 1 μM and does not result in the inhibition of human mitochondrial HsClpP at 1 and 10 μM concentration. Acylation of the active-site serine was confirmed by mass spectrometry studies (only **14** achieved full acylation, with **13** being 50% acylated), while the active-site serine mutant S98A did not bind. Interestingly, active-site acylation by the aryl esters leads to deoligomerization of the tetradecamer into inactive heptamers, which is an attractive dual mode of inhibition that increases the longevity of the deactivation. SAR studies revealed the need for an electron deficient aryl

ester to promote acylation, while increased substitution around the ester increased stability but decreased ClpP reactivity. In addition, **15** (the (*R*)-enantiomer) resulted in partial acylation of ClpP and deoligomerization into heptamers, whereas **16** (the (*S*)-enantiomer) resulted in the complete acylation of all 14 ClpP active sites but did not cause deoligomerization of the tetradecamer. The difference in behavior is postulated to arise because the α-methyl group of the (*R*)-**15** interferes with the active-site histidine of the catalytic triad, which is believed to mediate a hydrogen bonded network involved in the deoligomerization process. Analogs of **14** were also developed that selectively inhibit HsClpP (and not SaClpP) with sub-micromolar IC_{50} values (**17**-**19**).[33]

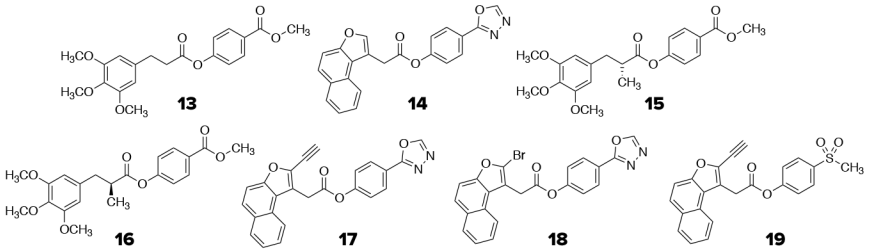

Figure 8 Aryl ester covalent inhibitors of ClpP.

A screen of a library of serine protease inhibitors identified six compounds that were able to inhibit EcClpP with IC_{50} values ranging from 8.2 to 49.5 μM.[68] SAR studies established α-amino diphenyl phosphonates **20** and **21** as the most potent inhibitors of ClpP with IC_{50} values of 0.5 μM each (Figure 9). However, only **20** was able to demonstrate significant bacterial growth reduction under nitric oxide stress conditions. Benzamidine **21** was suggested to be a more favorable lead compound as it displays as potent ClpP inhibition as **20** with a lack of inhibition against chymotrypsin and select eukaryotic cell lines.

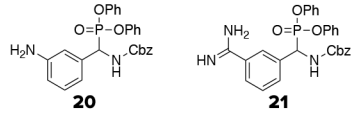

Figure 9 Phosphonate ester covalent inhibitors of ClpP.

3.3 Non-Covalent Inhibitors

Non-covalent inhibitor **22** was identified by HTS (Figure 10).[69] X-ray structural determination of co-crystals of **22** with SaClpP revealed binding near the active site between α-helix E and strand β9 in a non-substrate-like mode and a reorganization of the binding pocket side-chains (Figure 11). SAR optimization of **22** determined that the oxazole-thiophene moiety, which engages a hydrophobic pocket, could not be replaced with phenyl or oxazole rings, but that replacement of the *N*-isopropyl group with groups that promote potential π-stacking interactions with His142 resulted in compounds showing increased inhibition. Analogs **23** and **24** exhibited the greatest potency, inhibiting ClpP with IC_{50} values of 1.5 and 0.9 μM, respectively (Figure 10). Intracellular target engagement was demonstrated by a diazirine-based photoprobe analog of **24**. Interestingly, this series of compounds does not result in deoligomerization and were rendered ineffective upon ClpX binding to the protease. This was attributed to ClpX binding mediating a change in the conformation of ClpP, releasing the compounds from the binding pocket.

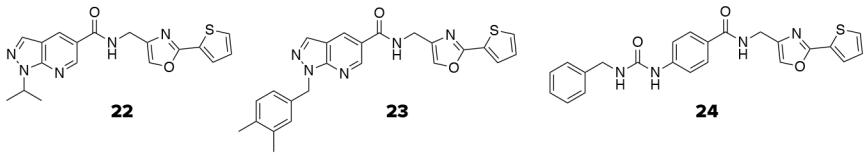

Figure 10 Non-covalent oxazole inhibitors of ClpP.

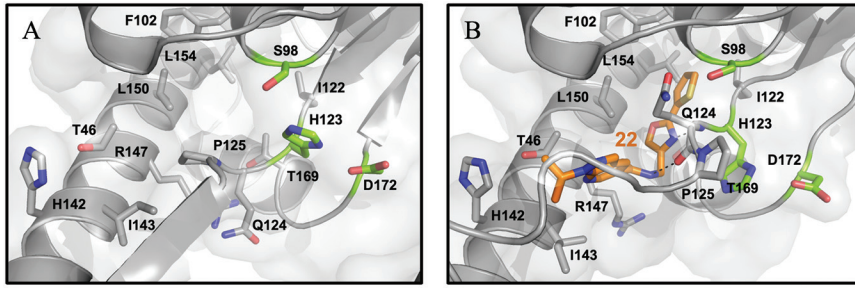

Figure 11 Cartoon representations of crystal structures of SaClpP in apo form and bound with **22** (*c.f.*, apo SaClpP [PDB: 3QWD, 4EMM, 3ST9, 3STA, 3V5E]). A) The catalytic site of apo *S. aureus* ClpP showing a properly aligned catalytic triad (PDB: 3V5E). B) SaClpP co-crystallized with **22** (orange sticks) showing non-covalent inhibition by binding near the catalytic sites (PDB: 5DL1). Binding of **22** displaces Ile143 and Arg147 due to steric interactions and induces a 180° flip of Pro125 in the β9 strand, causing His123 to be reoriented away from the nucleophilic Ser98 residue of the catalytic triad. Gln124 is also rearranged to a position where it forms two hydrogen bonds between the peptide backbone and **22**.

A variety of other molecules have been identified as ClpP inhibitors. Alkylidene rhodanine **25** is a non-competitive inhibitor perturbing the expression of virulence factors in a ClpP-dependent manner and reducing the ability of *S. aureus* to adhere and invade host cells (Figure 12).[70] Compounds **26**-**28** inhibit *Plasmodium falciparum* ClpP and inhibit *P. falciparum* growth, the parasite that causes malaria, in the 25-75 μM range with minimal cytotoxicity against HeLa cells suggesting a high therapeutic window to treat malaria (Figure 12).[71] SAR elaboration led to pyrimidone **29** as the highest potency compound (EC_{50} value of 9 μM). An *in-silico* screening approach led to the identification of pyrroles such as **30** as antimycobacterial (MIC = 10.0 μm) and inhibitors of MtClpP1P2 peptidase activity.

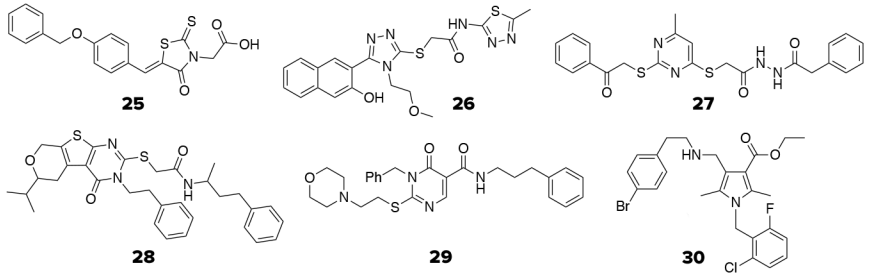

Figure 12 Non-covalent oxazole inhibitors of ClpP.

4. ACTIVATION OF ClpP PROTEASE

Another method used to cause bacterial cell death involves dysregulating or activating ClpP activity. By itself, ClpP is only able to degrade short polypeptides, since its entrance pore to the catalytic chamber is too small for folded proteins to enter.[73] It is only when ClpX or other chaperones bind to ClpP that unfolded peptide can enter the proteolytic chamber. Some small molecules are able to activate its peptidase activity in an ATPase independent manner by mimicking ClpX binding and causing a conformational change that opens the entrance pore. This results in uncontrolled and nonspecific degradation of essential housekeeping proteins. Although the mechanism by which ClpP drug-based activation causes cell death is not completely understood, it is known that activated ClpP degrades FtsZ, an essential cytoskeleton protein involved in cell division.[74,75]

4.1 Acyldepsipeptide Activators of ClpP

The most well-studied activators for ClpP are the acyldepsipeptides (ADEPs). The first ADEPs, called the "A54556 complex" or **31a**-**f**, were isolated from *Streptomyces hawaiiensis* (Figure 13).[76] The main component, **31a** or "factor A," was later renamed as ADEP1 and was able to demonstrate exceptional antibacterial activity against several strains of Gram-positive bacteria.[77] The closely related enopeptins A and B (**31g** and **31h**) show antimicrobial activity against Gram-positive bacteria (e.g., a methicillin resistant *S. aureus*) and Gram-negative mutant bacteria having defective cell membranes.[78,79] The ADEPs comprise a 16-membered ring depsipeptide linked via a phenylalanine group to a hydrophobic "tail." The macrocyclic lactone bond is formed between the proline (or 4-methylproline) and serine residues. Solution-phase total syntheses of the enopeptins[80] and the A54556 family[81] both used macrolactamization reactions, a strategy that has been used for most ADEP analog syntheses. More recently, an improved protocol for ADEP synthesis was developed using a lanthanide mediated macrolactonization approach.[82,83] This protocol allows for more convenient access to ADEP analogs using solid-supported synthesis of a seco acid and a solution-phase macrolactonization with Shiina's reagent and $Dy(OTf)_3$ (30-100 mol%).

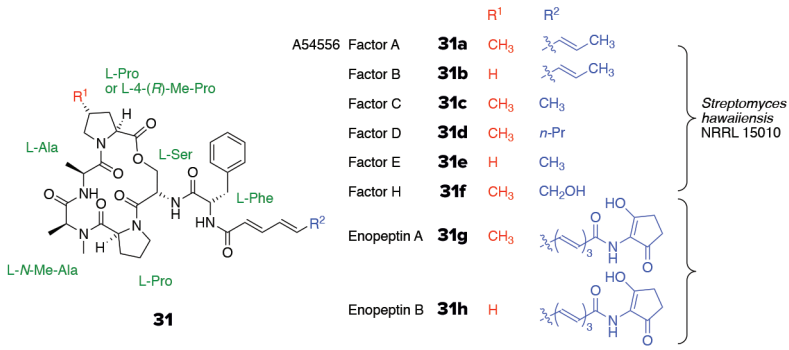

Figure 13 ADEP natural product activators of ClpP and the related enopeptins.

Of the natural products, **31d** is the most active compound against both Gram-positive bacteria (MIC < 0.0625 μg/mL versus 2 μg/mL for vancomycin against *Streptococcus pneumoniae* [ATCC 46919]) and Gram-negative bacteria (MIC < 0.0625 μg/mL versus 64 μg/mL for kanamycin A against *Neisseria meningitidis* [H44/76]), even without the use of an external membrane permeabilizing agent.[76,78,81] SAR, chemical biology, bioanalytical, and structural biology studies on ADEP analogs of general structure **32** have

been conducted on a range of organisms and their ClpPs (human, bacterial, and mycobacterial), using wild-type, ClpP deletion, and resistant cell lines and clinical isolates (Figure 14). The ADEPs compete directly with ClpX binding, resulting in the conversion of ClpP into an unspecific proteolytic enzyme[39] capable of degrading unfolded or poorly stable polypeptides.[84]

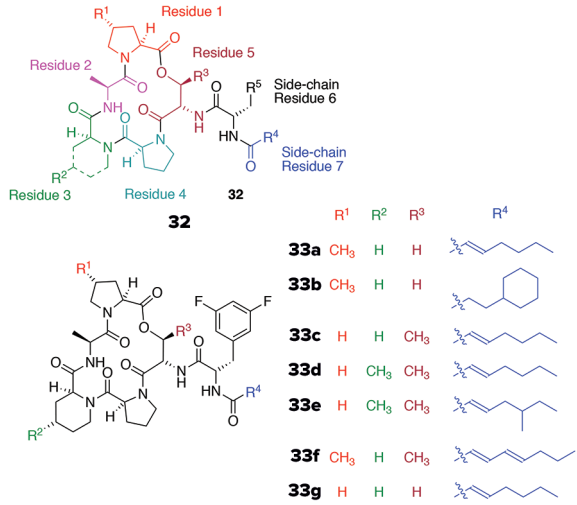

Figure 14 Synthetic ADEP activators of ClpP.

The initial report showed that ADEP analogs **32** are active against a range of Gram-positive bacteria including multidrug-resistant clinical isolates, and are also efficacious in studies of *E. faecalis* and *S. pneumoniae* infections in mice and rats.[77] Extensive SAR studies have revealed the requirement for L-proline or (*R*)-4-methyl-L-proline at residue position 1 and a pendant L-phenylalanine or analog at residue position 6. 3,5-Difluorophenyl analogs in particular show significantly increased potency,[77,83,85] while heteroaromatic or cyclic aliphatic side-chains at this position,[83,85] or a D-phenylalanine[77] show no activity. Additionally, rigidification of residue position 3 with an L-pipecolic acid[77] or 4-methyl-L-pipecolic acid group leads to strongly enhanced activity for the series of compounds **33a**-**g**.[83] For example, the L-pipecolic acid derived analog **33a** shows substantially enhanced antibacterial activity relative to **31a** (e.g., MIC = 0.05 versus 6.3 μg/mL for methicillin-resistant *S. aureus* NRS 119 [MRSA]) and improved efficacy relative to Linezolid.[77] Similar behavior was exhibited by ADEP **33b**,

and, additionally, both **33a** and **33b** were shown to be much more active than **31a** against penicillin-resistant strains of *S. pneumoniae* and vancomycin-resistant strains of *Enterococcus faecium*.[77] Conversely, introduction of a D-pipecolic acid,[86] a glycine or a *N*-Me glycine[85] at position 3 abolishes activity, while a L-proline analog is much less active.[83]

Attempts to increase ADEP stability *in vitro* have shown that replacement of the macrolactone ester by amides (either NH or NMe) results in dramatic reductions in antibacterial activity against Gram-positive and negative bacteria.[83,87] Rigidified ADEP analogs incorporating an *allo*-threonine substitution for serine at residue 5, particularly **33c** and **33d**, lead to enhanced ClpP binding, activation, and antibacterial activity.[88,89] Rigidification has been correlated with slower rates of deuterium exchange (by ^{1}H NMR) in ADEPs.[88] The side-chain residue 7 generally requires a *trans*-alkene (or diene) side chain and must be lipophilic.[83,85] Substitution of the pendant side-chain to mimic the isoleucine of the (I/L)GF loop motif of the AAA+ proteins gave inactive compounds, but addition of a simple methyl branch (e.g., R^4 = 1-(2-methylbutyl) or 1-isopentyl) increased activity for analogs containing an N-Me-Ala or pipecolic acid at residue 3 (e.g., **33e**).[90] Generally analogs with more rigidifying amino acids have greater potency, with one of the most active identified as ADEP **33f**, against numerous Gram-positive organisms including an MRSA clinical isolate, *S. pneumoniae*, *B. subtilis*, and *Listeria innocua* as well as more potent activity against the vancomycin-resistant *E. faecalis* V583 (VRE) than previously reported ADEP analogs (MIC = 0.0039 μg/mL).[83] ADEP **33f** also showed potent activity against the Gram-negative organisms, *N. meningitidis* H44/76 (MIC = 0.0313 μg/mL) and the chloramphenicol-resistant strain, *Neisseria gonorrhoeae* N.279 (MIC = 0.0156 μg/mL), but was inactive against *E. coli* MC4100 and *P. aeruginosa* PA01.[83]

X-ray crystallographic analysis of ADEP analogs alone reveal a conformation in which the pendant NH group of Phe (residue 6) H-bonds with the carbonyl group of Pro (residue 1), and the NH group of Ala (residue 2) H-bonds with the carbonyl group of Phe (residue 6).[81,85] The presence of the internal H-bonds "cloaks" the H-bond donors and acceptors of the ADEPs, thus facilitating membrane permeability. Analysis of the known ADEP-bound ClpP X-ray co-crystal structures shows that the latter H-bond is retained such that the ring conformation does not change significantly, whereas the former H-bond is absent, resulting in a reorientation of the side-chain residues.

Crystallographic analysis of ADEPs **31a** and **33b** bound to ClpP[32,37,39,83,91] and **33e** to ClpP1P2[92] reveal that binding to the hydrophobic allosteric pockets between two adjacent subunits, induces conformational changes in the *N*-terminal region, an enlargement of the axial pore, as well as in some cases a switching into an extended conformation that aligns the catalytic triad (Figure 15).[93-95] These changes mimic those proposed to occur when IGF loops of ClpX bind at the hydrophobic pockets of the ClpP apical surfaces.[39,44,96]

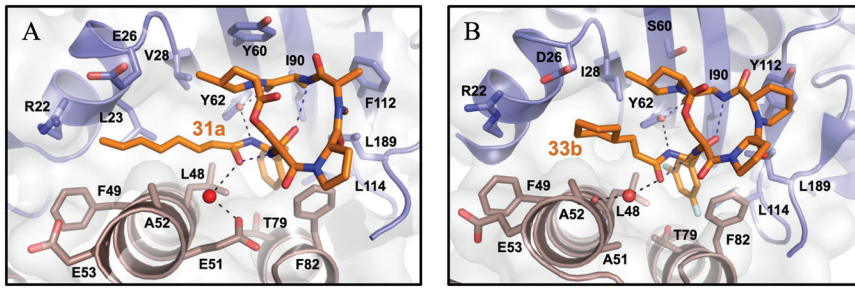

Figure 15 Cartoon representations of bacterial ClpPs bound to different ADEP activators (*c.f.*, apo EcClpP [PDB: 1TYF, 1YG6] and apo BsClpP [PDB: 3KTG, 3KTH, 3TT6]). A) EcClpP co-crystallized with ADEP **31a** (orange sticks) (PDB: 3MT6). B) *B. subtilis* ClpP co-crystallized with ADEP **33b** (orange sticks) (PDB: 3KTJ). A conserved water molecule (red sphere) mediates a hydrogen bond between ADEP and a ClpP residue. Hydrogen bonds between ClpP and ADEP are shown as broken black lines, while intramolecular hydrogen bonds that stabilize ADEP conformation are shown as broken blue lines.

Interestingly, in mycobacteria, ADEPs prevent the binding of ClpC1 to ClpP1P2 resulting in inhibition of protein degradation, but in the presence of additional *Z*-Leu-Leu activation occurs.[97] Analogs **33d** prolonged survival in MRSA infected mice,[89] while a combination of **33a** and rifampicin treated *S. aureus* biofilms in a mouse model of a chronic infection.[98] **33a** co-administered with ampicillin displays bactericidal activity against a strain of stationary-phase vancomycin-resistant *E. faecalis* and mature VRE biofilms that far surpassed the results generated with clinically used antibiotic combinations, such as ampicillin-gentamicin and ampicillin-daptomycin, while increasing *in vivo* efficacy in a murine peritoneal septicemia model (*E. faecalis* V583 [VRE]).[91] In general, the issue of resistance to ADEPs is a concern, such that co-administration of other agents is warranted, particularly for organisms such as *M. tuberculosis* where efflux pump inhibitors increase their effectiveness.[99]

ADEP **31a** has been shown to down-regulate cyclin D1, CDK4 and PCNA expression and inhibit the MAPK-ERK pathway in human renal cancer cells, leading to G1 phase cell cycle arrest and growth inhibition.[100] Not surprisingly, ADEPs adopt a similar binding interaction with mitochondrial HsClpP as in bacterial ClpPs with its 3,5-difluorophenylalanine residue making essential π-stacking interactions with the hydrophobic pocket of the allosteric site (Figure 16A).[32]

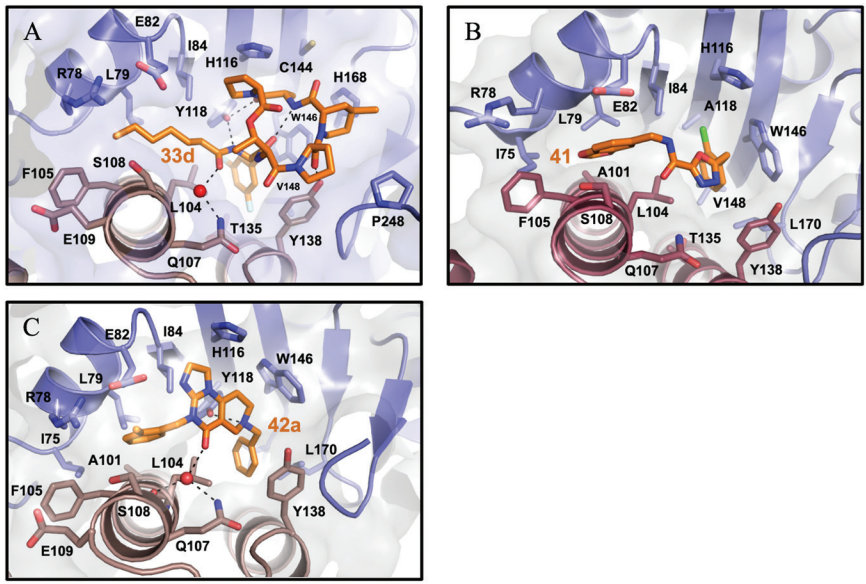

Figure 16 Cartoon representations of HsClpP bound to different activators (*c.f.*, apo HsClpP [PDB: 1TG6]). A) HsClpP bound co-crystallized with ADEP **33d** (orange sticks) (PDB: 6BBA). It can be seen that **33d** is localized in the hydrophobic pocket between two subunits of HsClpP. B) HsClpP Y118A mutant co-crystallized with compound **41** (orange sticks) (PDB: 6H23). Like the ADEPs, **41** binds in the same hydrophobic pocket between two interacting subunits. C) HsClpP co-crystallized with imipridone **42a** (orange sticks) (PDB: 6DL7). A bridging water molecule that mediates hydrogen bonding between **42a** and a Gln residue is also found as in Figure 16A.

4.2 Other Small-Molecule Activators of ClpP

A screen established **34** and **35** to activate *E. coli* ClpP (Figure 17).[101] SAR studies identified **37** to activate ClpP slightly more than the parent compound, while **36** was able to activate ClpP to the same extent as ADEP **31a**. The paraherquamide-related indolinone natural product sclerotiamide **38** was identified from a screen of bacterial and fungal metabolites and

extracts as an activator of recombinant EcClpP.[102] Notoamide B, which is an analog of **38** lacking the C-10 hydroxyl group, has no activity.

Truncation studies on the ADEPs determined that the side-chain, and not the macrocycle, was the minimum structural feature required to retain ClpP activation and antibacterial activity.[103] The most active analog **39** displayed modest antibacterial activity against *B. subtilis* (MIC = 8 μg/mL). It is interesting to note that a simple IGF-loop peptide binds ClpP only very weakly (K_d > 200 μM).[104] Interestingly, the same types of difluorinated truncated analogs have, in excess, been shown to be potentiators of ADEP activity.[105] This is believed to occur through a mechanism of efflux inhibition in a similar manner to that demonstrated for reserpine and verapamil.[99]

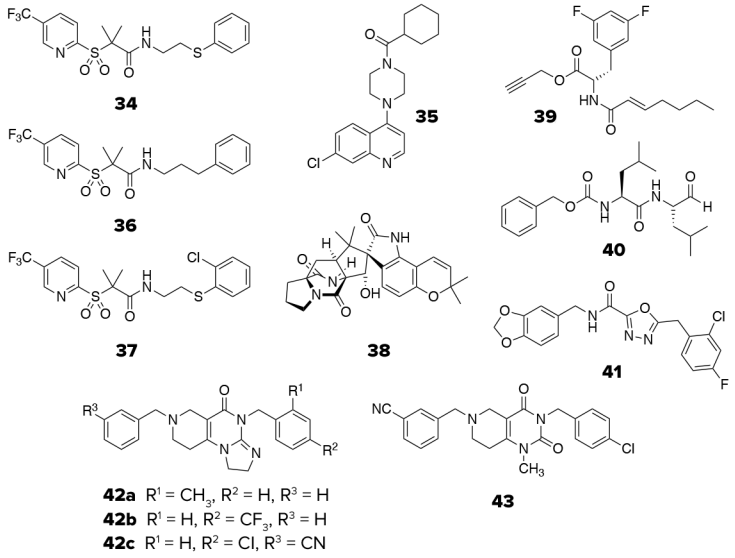

Figure 17 Small-molecule ClpP activators.

Paradoxically, dipeptide aldehyde substrate analogs such as Z-Leu-leucinal **40**, which would be expected to act as inhibitors, activate ClpP1P2 (K_d = 0.24 mM and Hill coefficient = 5-7, while *Z*-Leu-Leu also activates with a K_d = 2.2 mM).[106] This occurs through a mechanism involving dissociation of ClpP1 or ClpP2 tetradecamers, followed by association to form an active ClpP1P2 tetradecameric complex. In addition, the chaperone ClpC1 was shown to activate ClpP1P2 (and not ClpP1 or ClpP2) and only in the presence of **40** for the hydrolysis of proteins. A later study identified Bz-

Leu-Leu and Z-Leu-Leu as activators of *M. tuberculosis* MtClpP1P2, with the ClpC1P1P2 and ClpXP1P2 displaying 2-3-fold increased activity. An X-ray co-crystal structure showed the Bz–Leu-Leu ligand adopting different orientations in the ClpP1 and ClpP2 active sites (PDB: 5DZK).[107] Studies have shown that 20% modification of the catalytic serine residues of LmClpP2 in *Listeria monocytogenes* by a chloromethylketone inhibitor leads to increased proteolysis, whereas at 50% modification full inhibition occurs.[108]

Oxadiazole **41** is an activator of HsClpP *in vitro*.[109] X-ray co-crystallographic studies of HsClpP protease showed a similar mode of binding of **41** as for ADEPs, stabilizing the tetradecameric conformation for ClpP by binding to the hydrophobic pockets linking two monomers (Figure 16B). Substrate specificity is imparted by the interaction of the halogenated benzyl ring of **41** through π-stacking with the hydrophobic pocket. This pocket is unique for HsClpP consisting of Tyr118, Tyr138, and particularly Trp146 which is characteristic for HsClpP.

Imipridone (ONC201) **42a**, which is being evaluated in clinical trials against acute myeloid leukemia and other cancers, and **42b** (ONC212) have recently been shown to activate ClpP and kill malignant cells in a ClpP-dependent manner.[110] A co-crystal structure of **42a** with HsClpP reveal an analogous binding mode of **42a** as for ADEP **33d** and a concomitant widening of the axial pore diameter (Figure 16C). Anti-tumor effects were demonstrated using a xenograft mouse model. In a parallel study, analogs such as **42c** and **43** were shown to be more potent than **42a**.

5. TARGETING OF AAA+ ClpP UNFOLDASES

Another approach to dysregulate the ClpP proteolytic system as a whole is to target the AAA+ chaperones. This approach is much less well studied, in part because structural data for the ClpP-AAA+ complexes is not available.

5.1 ClpC1 Chaperone Proteins

The anti-inflammatory cyclic heptapeptide natural product cyclomarin **44** has been shown to kill *M. tuberculosis* (MIC = 0.1 μM) by targeting the ClpC1 subunit (Figure 18).[112] It does not compete with ATP for active site binding and is believed to enhance the ATPase activity of ClpC1 by binding to an allosteric site of the chaperone.[113] The related cyclic heptapeptide

rufomycin I **45** is bactericidal for *M. tuberculosis* (MIC = 0.02 μM) and *Mycobacterium abscessus* (MIC = 0.4 μM). It binds to ClpC1 (K_d ~ 100 nM), decreasing ClpC1P1P2 proteolysis of casein while having no effect on the ATPase activity of ClpC1.[114] A HTS of Actinomyces extracts identified the macrocyclic tridecadepsipeptide natural product ecumicin **46**, to be a selective inhibitor of *M. tuberculosis* (minimum bactericidal concentration, MBC = 0.34 μM) over *S. aureus*, *E. coli*, *Candida albicans*, and mammalian cell growth. Ecumicin inhibits ClpP degradation of casein, while selectively activating the ATPase activity of ClpC1 over other AAA+ analogs (ClpX, ClpB, ClpA, and mouse proteasome).[115] Ecumicin completely inhibited growth of *M. tuberculosis* in mice (32 mg/kg) suggesting that it is a viable therapeutic lead. The highly basic cyclic lasso peptide lassomycin **47** exhibits an MIC of 0.8-3 μg/mL against various strains of *M. tuberculosis*, including

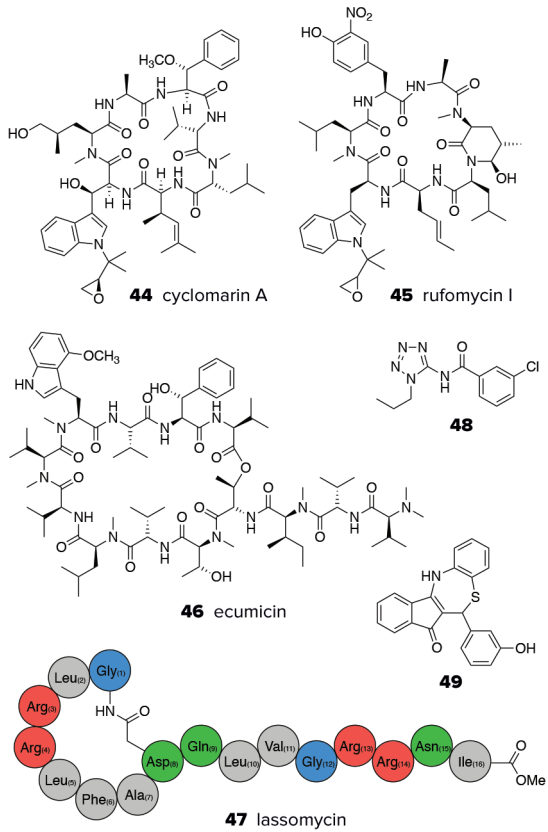

Figure 18 Structures of molecules that target ClpC1 and ClpX.

extremely drug-resistant isolates.[116] It is believed to bind to the acidic region of the ClpC1 ATPase complex, and like ecumicin it stimulates ATPase activity without stimulating ClpP1P2-catalyzed protein breakdown. Both lassomycin and ecumicin, upon binding to ClpC1, induce conformational changes that prevent ClpC1 from associating with ClpP1P2.

5.2 ClpX Chaperone Proteins

Aminotetrazole **48** is an inhibitor of ClpXP and also sensitizes *Bacillus anthracis* and *S. aureus* toward penicillin, cathelicidin, and daptomycin (Figure 18).[117] A separate HTS led to the discovery of a series of structurally related compounds, including **49**, which inhibit ClpX ATPase activity (IC_{50} = 0.8 μM) but not ClpP activity.[118] Size exclusion chromatography revealed a unique mode of action of **49** involving dissociation of the ClpX hexamer. SAR studies revealed a requirement for a hydroxyl group in the *meta* position of the pendant aryl ring to achieve complete inhibition, and that substitution is tolerated in the "western" aromatic ring, but not in the "northern" aromatic ring.

6. CONCLUSIONS

The role of ClpP in maintaining protein homeostasis in bacterial cells and in the human mitochondria by degrading unwanted proteins that are aggregated, damaged, or no longer needed, has established it as a potential target for therapeutic intervention. More generally, it represents a fascinating target for medicinal chemists and chemical biologists, with relatively little known about ClpP kinetics and thermodynamics, issues of selectivity, and the intriguing symmetry mismatch between the tetradecameric ClpP in its interaction with the hexameric AAA+ chaperones.[119,120] While structural biology studies have been essential in the understanding of ClpP, a deeper understanding would be established from a high-resolution structure of ClpP bound to its unfoldase chaperone.

Given the complexity of the ClpP-AAA+ systems, it is not surprising that small-molecule complexes of ClpP have been sought to serve as a surrogate model for elucidating the molecular mechanism of ClpP function. These small molecules have been found to both inhibit and activate ClpP, and in some cases, both, by engaging the active sites or apical binding sites. A diverse range of such molecules have been identified, including traditional "drug-like" small molecules, β-lactones, aryl esters, boronic acids, cyclic

depsipeptides, and peptides. Inhibition or promotion of ClpP activity can lead to build-up or uncontrolled degradation of essential proteins, resulting in downstream effects, including cell-death, and in the case of bacteria, reduced infectivity and virulence. The majority of studies to date have focused on potential anti-infective applications of ClpP inhibition or activation against human pathogens such as mycobacteria (tuberculosis), *Plasmodium* (malaria), and *S. aureus*. Compounds have been identified that target resistant strains and clinical isolates, but the development of resistance to these agents remains a potential problem. Resistance could arise by bacteria mutating the apical site, the active site, or by deleting the *clpP* gene.[98] HsClpP is only found in the mitochondria, which allows for selective agents to be discovered. Additionally, since HsClpP is upregulated in many cancer cells, it constitutes a novel target for the development of novel anticancer drugs. However, such compounds need to have the proper physiochemical properties to cross the plasma membrane as well as the two mitochondrial membranes.

ClpP is particularly interesting given the significance of small molecules that target protein-protein interactions (PPIs). Specifically, small-molecule mimicry of PPIs to achieve activation, as occurs for the ADEPs, is arguably a more challenging goal than the inhibition of the protease or PPIs and is an approach that should be more broadly considered. Whether the observations that have been made in the context of ClpP have relevance to other self-compartmentalized proteases,[121] such as Lon or the proteasome, is another intriguing aspect that is worthy of exploration.

Acknowledgments

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