# The Role of ClpP Protease in Bacterial Pathogenesis and Human Diseases

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ABSTRACT: In prokaryotic cells and eukaryotic organelles, the ClpP protease plays an important role in proteostasis. The disruption of the ClpP function has been shown to influence the infectivity and virulence of a number of bacterial pathogens. More recently, ClpP has been found to be involved in various forms of carcinomas and in Perrault syndrome, which is an inherited condition characterized by hearing loss in males and females and by ovarian abnormalities in females. Hence, targeting ClpP is a potentially viable, attractive option for the treatment of different ailments. Herein, the biochemical and cellular activities of ClpP are discussed along with the mechanisms by which ClpP affects bacterial pathogenesis and various human diseases. In addition, a comprehensive overview is given of the new classes of compounds in development that target ClpP. Many of these compounds are currently primarily aimed at treating bacterial infections. Some of these compounds inhibit ClpP activity, while others activate the protease and lead to its dysregulation. The ClpP activators are remarkable examples of small molecules that inhibit protein−protein interactions but also result in a gain of function.
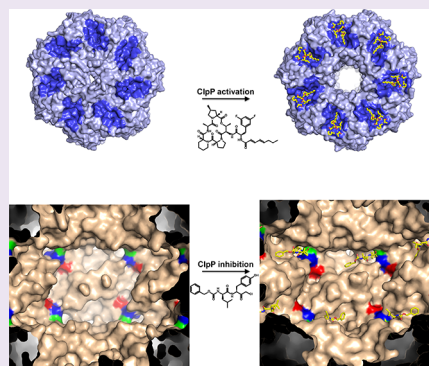
I. INTRODUCTION AND OVERVIEW OF CLPP FUNCTION

Proteolysis is an essential cellular activity that mediates protein turnover and the removal of undesired proteins from the intracellular environment. The conductors of this function, the proteolytic machineries, allow for the cell to maintain proteostasis and to adapt to changing environments by providing the ability to alter protein levels. The best known of these cellular machines is perhaps the eukaryotic 26S proteasome along with a number of bacterial proteases such as ClpP, Lon, HslUV, and FtsH. In bacteria, ClpP and Lon perform the majority of the cellular proteolytic activities with estimates suggesting them to be responsible for 80% of cellular proteolysis.[1] Over the past few years, with the discovery of ClpP-targeting antibacterial compounds and the increased awareness of its functional importance in the cell, ClpP has become a focal point for drug development research.[2−6]

ClpP stands for caseinolytic protease P and is a highly conserved self-compartmentalizing processive serine protease. It is well-characterized in multiple species where it is found to be involved in the proteolysis of damaged and misfolded proteins, ribosome-stalled proteins, as well as regulatory proteins. In *Escherichia coli*,[7] in a setup utilizing a proteolytically inactive variant of ClpP as a trap, over 60 proteins were identified as potential substrates of this protease. The identified proteins include those involved in metabolism, cell division, transcription regulation, and damage repair (Figure 1A). A similar repertoire of candidate substrates has been found for ClpP in *Bacillus subtilis* indirectly through use of 2D-PAGE in

wild type and *clpP*-lacking mutant cells[8] (Figure 1B). In these and other bacteria, ClpP has been found to be important for the degradation of proteins involved in nutrient starvation, stationary phase adaptation, heat-stress response, cell-cycle progression, biofilm formation, cell motility, nutrition, and metabolism.[9,10] Though it may target different proteins in different organisms, identification of its substrates in model bacteria show that ClpP is responsible for the regulation of a significant portion of the bacterial proteome. Having a wide range of impact on the proteome, it is not surprising that in a number of bacterial pathogens, ClpP function plays a critical role in infectivity and virulence.[11−16]

ClpP is also present in human cells, localized to the mitochondrial matrix. Within this compartment, ClpP is required for protein homeostasis,[17] where it is involved in the degradation and regulation of several enzymes of the electron transport chain and other cellular metabolic pathways (Figure 1C).[17−19] It is also linked with an unfolded protein response.[20] The loss of ClpP activity is linked to infertility and sensorineural hearing loss.[17,21] Furthermore, overexpression of ClpP has been linked to different carcinomas.[18,22]
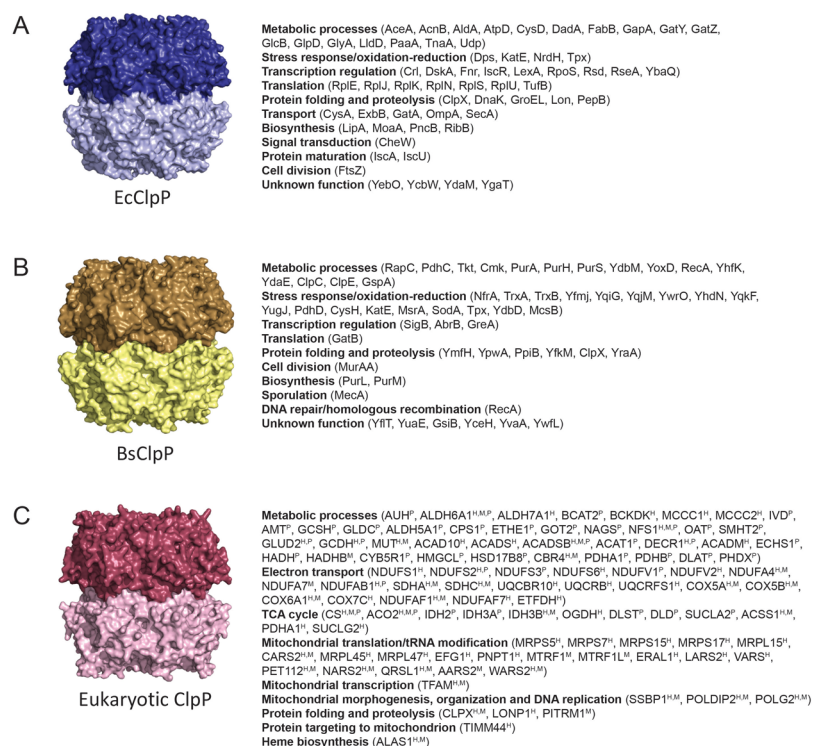
Given the role of ClpP in different diseases, the protease is considered a viable target for drug development. Currently, excellent progress has been made in developing antibacterial compounds targeting ClpP. Below, we describe the structure

A

Metabolic processes (AceA, AcnB, AldA, AtpD, CysD, DadA, FabB, GapA, GatY, GatZ, GlcB, GlpD, GlyA, LldD, PaaA, TnaA, Udp)
Stress response/oxidation-reduction (Dps, KatE, NrdH, Tpx)
Transcription regulation (Crl, DskA, Fnr, IscR, LexA, RpoS, Rsd, RseA, YbaQ)
Translation (RplE, RplJ, RplK, RplN, RplS, RplU, TufB)
Protein folding and proteolysis (ClpX, DnaK, GroEL, Lon, PepB)
Transport (CysA, ExbB, GatA, OmpA, SecA)
Biosynthesis (LipA, MoaA, PncB, RibB)
Signal transduction (CheW)
Protein maturation (IscA, IscU)
Cell division (FtsZ)
Unknown function (YebO, YcbW, YdaM, YgaT)

EcClpP

B

Metabolic processes (RapC, PdhC, Tkt, Cmk, PurA, PurH, PurS, YdbM, YoxD, RecA, YhfK, YdaE, ClpC, ClpE, GspA)
Stress response/oxidation-reduction (NfrA, TrxA, TrxB, Yfmj, YqiG, YqjM, YwrO, YhdN, YqkF, YugJ, PdhD, CysH, KatE, MsrA, SodA, Tpx, YdbD, McsB)
Transcription regulation (SigB, AbrB, GreA)
Translation (GatB)
Protein folding and proteolysis (YmfH, YpwA, PpiB, YfkM, ClpX, YraA)
Cell division (MurAA)
Biosynthesis (PurL, PurM)
Sporulation (MecA)
DNA repair/homologous recombination (RecA)
Unknown function (YflT, YuaE, GsiB, YceH, YvaA, YwfL)

BsClpP

C

Metabolic processes (AUH[P], ALDH6A1[H,M,P], ALDH7A1[H], BCAT2[P], BCKDK[H], MCCC1[H], MCCC2[H], IVD[P], AMT[P], GCSH[P], GLDC[P], ALDH5A1[P], CPS1[P], ETHE1[P], GOT2[P], NAGS[P], NFS1[H,M,P], OAT[P], SMHT2[P], GLUD2[H,P], GCDH[H,P], MUT[H,M], ACAD10[H], ACADS[H], ACADSB[H,M], ACAT1[P], DECR1[H,P], ACADM[H], ECHS1[P], HADH[P], HADHB[M], CYB5R1[P], HMGCL[P], HSD17B8[P], CBR4[H,M], PDHA1[P], PDHB[P], DLAT[P], PHDX[P])
Electron transport (NDUFS1[H], NDUFS2[H,P], NDUFS3[H], NDUFS6[H], NDUFV1[P], NDUFV2[H], NDUFA4[H,M], NDUFA7[M], NDUFAB1[H,P], SDHA[H,M], SDHC[H,M], UQCBR10[H], UQCRB[H], UQCRFS1[H], COX5A[H,M], COX5B[H,M], COX6A1[H,M], COX7C[H], NDUFAF1[H,M], NDUFAF7[H], ETFDH[H])
TCA cycle (CS[H,M,P], ACO2[H,M,P], IDH2[P], IDH3A[P], IDH3B[H,M], OGDH[H], DLST[P], DLD[P], SUCLA2[P], ACSS1[H,M], PDHA1[H], SUCLG2[H])
Mitochondrial translation/tRNA modification (MRPS5[H], MRPS7[H], MRPS15[H], MRPS17[H], MRPL15[H], CARS2[H,M], MRPL45[H], MRPL47[H], EFG1[H], PNPT1[H], MTRF1[M], MTRF1L[M], ERAL1[H], LARS2[H], VARS[H], PET112[H,M], NARS2[H,M], QRSL1[H,M], AARS2[M], WARS2[H,M])
Mitochondrial transcription (TFAM[H,M])
Mitochondrial morphogenesis, organization and DNA replication (SSBP1[H,M], POLDIP2[H], POLG2[H,M])
Protein folding and proteolysis (CLPX[H,M], LONP1[H], PITRM1[M])
Protein targeting to mitochondrion (TIMM44[H])
Heme biosynthesis (ALAS1[H,M])

Eukaryotic ClpP

Figure 1. ClpP substrates in different organisms. Potential ClpP substrates identified in (A) *E. coli*,[7] (B) *B. subtilis*,[8] and (C) eukaryotes. ClpP substrates in eukaryotes were identified in a human embryonic kidney cell line[18] (indicated by superscripted H), mouse embryonic fibroblasts[17] (indicated by superscripted M), and *Podospora ansperina* fungal model[19] (indicated by superscripted P); however, the names given are those of the human proteins. The proteins are grouped according to their function as defined by the gene ontology (GO) functional classifications. Eukaryotic ClpP is represented here by the HsClpP structure.

and function of ClpP and discuss the chemical biology and drug development efforts targeting this protease.

II. CLPP STRUCTURE AND REGULATION

ClpP typically forms a tetradecameric cylinder composed of two rings with heptameric symmetry. As shown in Figure 2A for *E. coli* ClpP (EcClpP), the ClpP protomer can be divided into three subdomains: an N-terminal loop segment, the head domain, and the handle domain.[23] The handle domain forms the interface linking the two heptameric rings into the functionally active tetradecamer[23] (Figure 2A,B). The two ClpP heptameric rings form a chamber within which 14 active sites comprised of the canonical serine−histidine−aspartic acid triad are found (Figure 2A,B).

The protease is often found as a homomeric assembly as evidenced by the EcClpP; however, multiple isoforms of ClpP in a single organism organized in homomeric or heteromeric assemblies of paralogous subunits are also present. Examples include *Mycobacteria tuberculosis* containing the ClpP1 and ClpP2 isoforms encoded on a single operon. Both isoforms form an active tetradecameric protease that is composed of a heptameric ring each of ClpP1 and ClpP2 paralogs.[24,25] *Pseudomonas aeruginosa* also has two ClpP paralogs; however, ClpP1 and ClpP2 form distinct tetradecameric complexes under separate regulatory controls as they perform different functions in the cell.[26] Many cyanobacteria and plastids of plants contain multiple isoforms of ClpP and ClpR, which is a proteolytically inactive variant of ClpP, that form different types of mixed tetradecameric complexes.[27]

ClpP by itself acts as a peptidase by degrading short peptides that can enter its proteolytic chamber through the narrow axial pores (Figure 2B,C). However, for efficient proteolysis of long polypeptides and proteins, ClpP typically forms a complex with an ATPase chaperone (named Clp ATPase) that unfolds target substrates and threads them into the ClpP chamber for degradation (Figure 2C).[28] These chaperones belong to the Hsp100 class of ATPases associated with diverse cellular activities or the AAA+ superfamily and act as a cap to the ClpP cylinder by binding one or both ends of the cylinder (Figure 2C). There are different types of such caps in different organisms; for example, in *E. coli*, both ClpX and ClpA act as ClpP caps.

The Clp ATPases form a hexameric complex containing a central pore through which the substrates are translocated into the protease chamber (Figure 2C). Though the mechanistic intricacies of the symmetrical mismatch between the hexameric Clp ATPases and the 7-fold symmetric ClpP protease are currently a matter of debate, the most commonly accepted model proposes the ATPases to induce a conformational change in the protease from a closed to an open, active conformer that is amenable to the translocation of large unfolded substrates into the ClpP proteolytic chamber for degradation.[29] While currently there is no high-resolution structure of a Clp ATPase in complex with ClpP protease, the interaction between the two complexes has been characterized biochemically. This association is highly dependent on the docking of the Clp ATPases through their IGF/L motif-containing loops onto the hydrophobic pockets of ClpP located on the apical surface of the heptameric rings (Figure 2B) with additional dynamic contacts being made by the N-terminal loops of ClpP with axial pore loops of the ATPase. The details
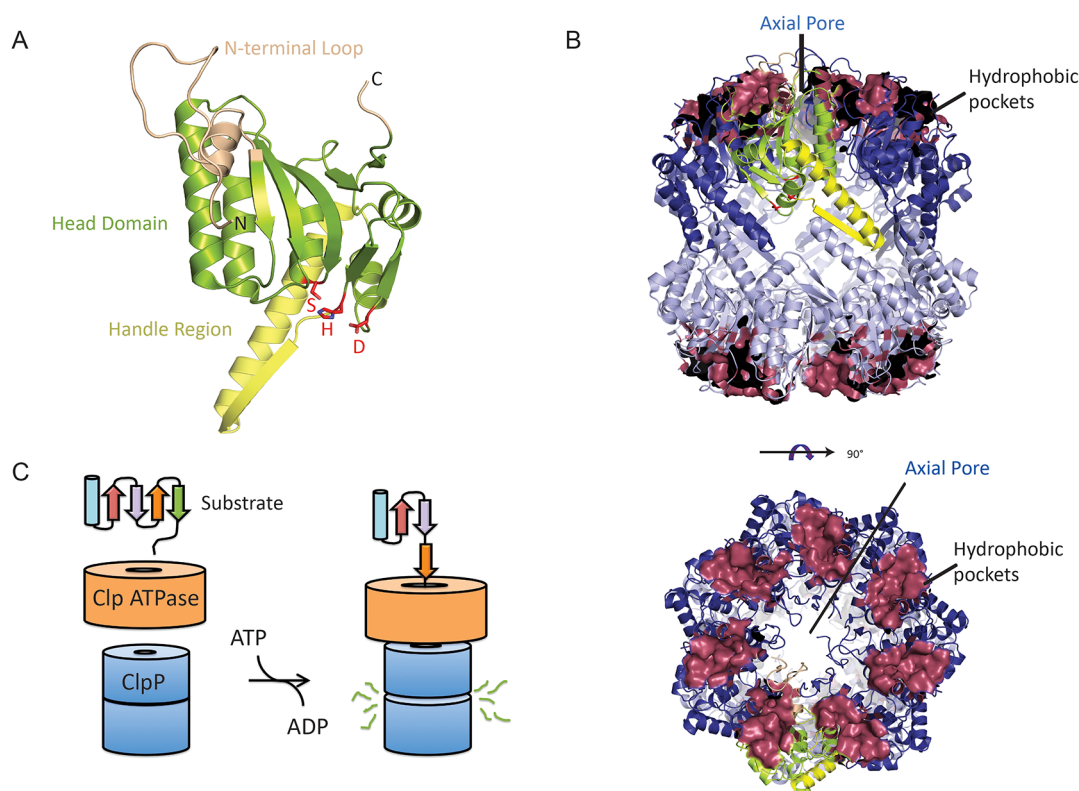
Figure 2. ClpP structure. Structure of EcClpP (PDB ID 1YG6) (A) protomer and (B) tetradecamer side view and top view. In A, the N-terminal axial loop region (beige), the head domain (green), and the handle region (yellow) are indicated. Residues of the catalytic triad are shown as stick models (red). In B, the tetradecamer is shown with the two heptameric rings in light and dark blue. The axial pore and hydrophobic patches (red) are highlighted. In C, a schematic of the ClpP proteolytic function is shown as the substrate is channelled through the Clp ATPase pore (ClpX) into the ClpP proteolytic chamber for degradation.

of the interaction between the protease and ATPases and their mechanism have been described in recent literature.[28]

The Clp ATPases act as selective filters as they target a specific set of substrates for degradation under different conditions and under different regulatory controls.[10] They often identify substrates by inherent or added degron tags that are either accessible or become exposed in sequences of partially unfolded proteins.[7] The selection of substrates identified for degradation varies depending on the Clp ATPases.[30] Thus, with multiple such ATPases often present in cells, the ATPases tightly control ClpP degradation activity.

Further impacting and fine-tuning the control of protein degradation by ClpP are adaptors that bind to the Clp ATPases and influence the substrate choice. Examples of such adaptors in *E. coli* include RssB, SspB, and UmuD that bind to ClpX, and ClpS that binds to ClpA. These adaptors modulate the substrate selectivity of the ATPases in response to relevant stresses and signals.[31,32] Other adaptors regulate the functional assembly of the Clp ATPases themselves such as the effect of the MecA adaptor on the ClpC ATPase in *B. subtilis*.[33] Adding to the complexity of ClpP regulation, antiadaptors have been identified that inhibit or regulate the activity of the adaptors themselves.[34,35] The different Clp ATPases, adaptors, and antiadaptors provide a means for the extensive regulation of ClpP-dependent protein degradation.

III. ROLE OF CLPP IN BACTERIAL PATHOGENESIS

The role of ClpP in infectious diseases was inferred more than two decades ago from investigations on the pathogenic bacteria *Salmonella typhimurium*, *Listeria monocytogenes*, and *Staph-*

ylococcus aureus.[11,12] In the first of these, a transposon-based mutagenesis screen identified 40 mutants of the Gram-negative *Salmonella typhimurium*, which causes food borne illness in the form of typhoid and gastroenteritis, to interfere with disease onset in a mouse model of typhoid fever.[11] The gene for ClpP was among these mutants. The role of ClpP in *S. typhimurium* pathogenesis was then further confirmed by Yamamoto and colleagues in a follow-up study.[36] Yamamoto's group demonstrated that, in contrast to wild type lines, cells lacking ClpP are unable to survive within the peritoneal macrophages, an important step in the establishment of *S. typhimurium* virulence.[36,37] Using global transcriptomic analysis, ClpP was shown to be a critical player in the regulation of genes on the *Salmonella* pathogenicity island 1 *via* the transcriptional factors RpoS and CsrA.[38]

For the identification of proteins involved in virulence associated with the Gram-positive *S. aureus*, transposon-based mutational analysis was used in the construction of a total of 1248 deletion strains.[12] Among gene mutants identified to attenuate or reduce the virulence of *S. aureus* in a murine model of bacteraemia was the ClpX ortholog.[12] Subsequently, the importance of ClpP itself in pathogenesis by *S. aureus* was soon established when mutants lacking either ClpX or ClpP were found to be deficient in virulence in a murine skin abscess model.[9] In addition to its impact on the regulation of several stress responses, the effect of ClpP on *S. aureus* virulence was found to be related to the secretion of the hemolytic factor α-hemolysin (encoded by the *hla* gene) and other effectors *via* the regulation of factors encoded by the *agr* locus including RNA III and the autoinducing peptide.[9] ClpP was also found to

regulate the levels of the heme-iron extracting Isd (iron-regulated surface determinants) proteins,[39] which are required for pathogenesis.

The third major, early example of a link between bacterial virulence and ClpP was found in *Listeria monocytogenes*, an intracellular Gram-positive pathogen that escapes the phagosomal compartment to replicate in the cytoplasm of various types of cells.[40] *L. monocytogenes* encodes two isoforms of ClpP, ClpP1 and ClpP2.[41] The functional significance of ClpP1 is not known, but mutants lacking ClpP2 were susceptible to the bactericidal activity of the macrophage host as these bacteria lacked their usual hemolytic abilities.[40,41] ClpP2 was found to be required for the expression of the Listeriolysin O virulence factor, which forms pores to allow for bacterial escape from the phagocytic vacuole after invasion of the host. Though ClpP2 mutants were taken up by the macrophages at similar levels to those of the wild type, the mutants suffered in their ability to escape and replicate to spread the infection.[40] Another factor, SvpA, implicated in the intracellular survival and escape of the pathogen from the phagosomes of bone marrow macrophages was observed to be under the regulatory control of ClpP2, its partner ClpC ATPase, and the adaptor MecA.[42]

The role of ClpP in the pathogenesis of *Streptococcus pneumonia* has also been demonstrated. *S. pneumonia*, a Gram-positive infectious agent involved in pneumonia, meningitis, and bacteremia, is found to transform from an asymptomatic to a virulent form due to morphological and genetic changes that may be induced by heat stress response mechanisms.[43] Mutant strains lacking *clpP* were found to lose their ability to colonize the nasopharynx, a niche environment for these bacteria in asymptomatic hosts, or to lose their ability to invade lung tissues in mouse models of septicemia and had poorer survival in murine macrophages.[43] Similarly, *Legionella pneumophila* Δ*clpP* mutants lacked the ability to escape the endosome−lysosome pathway in mammalian cells.[16] In *Pseudomonas aeruginosa*, ClpP was found to regulate the production of alginate, an exopolysaccharide that protects bacteria and that is associated with the onset of cystic fibrosis disease.[13] *Enterococcus faecalis* lacking ClpP or the Clp ATPases had attenuated virulence in moth infection models.[15]

Finally, unlike other bacteria, ClpP was found to be essential for the viability of *M. tuberculosis*, a pathogen linked with 1.7 million deaths in 2016.[14,44] The organism encodes ClpP1 and ClpP2 isoforms on a single operon, with the functional Clp protease comprised of a heptamer of each isomer forming a tetradecamer.[24] Both ClpP isomers, along with the ClpX and ClpC1 ATPases, were shown to be essential for the viability of the bacteria.[44] A functional ClpP1P2 complex was found to be required for viability and during infection by this pathogen.[14] Furthermore, the regulatory check imparted by ClpP1P2 through its degradation of the toxic substrate WhiB1 was identified to impart essentiality to the protease.[45]

All these organisms are recent examples adding to the growing list of pathogens shown to be impaired in their ability to infect or cause disease when lacking the ClpP protease system.

IV. ROLE OF CLPP IN *PLASMODIUM FALCIPARUM*

Plasmodium falciparum is the causative agent of malaria in humans. It is a unicellular eukaryotic parasite with a complex life cycle, whereby the organism adopts different cellular morphologies with multiple reproductive stages when it moves between the human host and the mosquito carrier. A unique feature of *P. falciparum* and other members of the Apicomplexa phylum to which it belongs is a plastid called the apicoplast. The organelle is bound by four membranes and is located in close proximity to a singular mitochondrion. The apicoplast is essential for the biosynthesis of fatty acids, isoprenoids, heme, and iron−sulfur clusters.[46] *P. falciparum* ClpP (PfClpP) is localized to the lumen of the apicoplast.[47] Expression analysis of PfClpP in *P. falciparum*'s asexual life cycle in human blood cells revealed that the protein is maximally expressed at the late trophozoite and early schizont stages.[47] These are the life stages in which *P. falciparum* multiplies and infects red blood cells.

Inhibition of PfClpP with a *β*-lactone compound resulted in significant growth reduction after 96 h of drug exposure.[47] Furthermore, although the treated parasites were still able to develop into viable merozoites that can subsequently infect fresh red blood cells after progressing through the first asexual cell cycle (0−48 h), the parasites failed to develop from early into late schizont during the second asexual cell cycle (48−96 h). The growth arrest observed in the second cell cycle was the result of failed cytokinesis necessary for the schizont to develop into mature merozoites, combined with failure in development and segregation of the apicoplast and disruption in the replication of apicoplast DNA.[47] Importantly, the growth arrest of drug-treated parasites rendered them incompetent in infecting new red blood cells, which resulted in a halt in parasitemia of the parasite cultures. Thus, the cellular function of PfClpP is essential for the replication and segregation of the apicoplast and cytokinesis of dividing *P. falciparum* cells.

V. ROLE OF CLPP IN HUMAN MITOCHONDRIA

ClpP is much less studied in human and other mammals. The mammalian ClpP is localized to the mitochondrial matrix and is an important part of the mitochondrial protein quality control system. Like its bacterial counterparts, ClpP physically and functionally associates with the AAA+ ATPase ClpX, which is also localized within the mitochondrial matrix. Given that ClpP is highly conserved across species, the primary sequence of human ClpP (HsClpP) shares a high degree of identity with ClpP from other mammals, bacteria, and other species.[48] The X-ray structure of HsClpP has been solved, and it closely resembles the structures of its bacterial orthologs.[49] Like the bacterial versions of ClpP, the active oligomeric state of HsClpP is that of a cylindrical tetradecamer, capped on each end by a hexameric HsClpX.[50] However, HsClpP exists mainly as a heptameric single-ring assembly, which is proteolytically inactive.[51] Transition from the inactive heptameric form to the active tetradecameric form is promoted by interaction with HsClpX.[51]

The primary function of mammalian ClpP is the maintenance of the mitochondrial proteome *via* degradation of oxidized and denatured proteins, which it performs in concert with ClpX.[52] This is necessary for proper mitochondrial function as many of the proteins that are potential substrates of human ClpP are directly involved in energy metabolism, mitochondrial translation, and amino acids and fatty acids metabolism, among other roles (Figure 1C).[17−19,22,53−55]

The loss of ClpP expression in various mammalian cell lines almost invariably results in mitochondrial respiratory deficiency. Reduced protein levels of both complex I and complex IV subunits have been noted in the myoblast cell line C2C12 and in heart cells of ClpP-deficient mice.[17,56] Similarly, many ClpP interactors are involved in mitochondrial translation (Figure 1C). Loss of ClpP leads to an accumulation of ERAL1 that

binds the 28S ribosomal subunit, attenuating the formation of mature mitochondrial ribosomes and decreasing the efficiency of translation initiation causing reduced protein synthesis.[17] In C2C12 cells lacking ClpP, the resultant increase in the presence of mitochondrial fission promoter DRP1 is linked to reduced mitochondrial size.[56] Furthermore, for both the C2C12 and mouse embryonic fibroblast cells, the absence of ClpP results in a decrease in the cell's doubling time, causing the cells to reach senescence earlier.[21]

The characterization of ClpP-associated phenotypes in both the mammalian cell and model organisms clearly illustrates the importance of ClpP in various mitochondrial functions that are vital to the cell and the health of the organism in general. Accordingly, ClpP has been shown to play vital roles in noninfectious human diseases. These can be grouped under two major categories: genetic disorders that arise from mutations that disrupt normal ClpP expression or its function, leading to mitochondrial dysfunction that impacts specific tissues and organs, and oncogenic disease in which ClpP is vital in sustaining the growth and metastasis of human cancer cells. Details of the roles of ClpP in these two categories of diseases are discussed below.

VI. ROLE OF HUMAN MITOCHONDRIAL CLPP IN PERRAULT SYNDROME

The physiological roles of ClpP have been investigated via the use of the *CLPP* null mice. A definitive phenotype of the *CLPP* null mice is the general infertility in both males and females.[21] Both the males' testes and the females' ovaries were shown to have reduced size.[17] Another prominent phenotype is sensorineural deafness. Gispert et al. reported that these animals become deficient in their motor startle response to sudden, loud acoustic stimuli at much earlier stages of their natural life span.[21] The *CLPP* null mice also displayed reduction in physical growth and motor activity, and adult *CLPP* null mice had both a lower body weight gain and a shorter stature.[17,21]

Notably, many of these phenotypes that are manifest in the *CLPP* null mice closely resemble the characteristic symptoms of Perrault syndrome in humans.[21] Perrault syndrome is a rare genetic disorder that is characterized by bilateral, sensorineural hearing loss in both male and female patients. Additionally, female patients also suffer from ovarian dysfunction that results in sterility or difficulty in conception. In contrast, male patients show normal fertility in all reported cases. Patients may also suffer from various neurological defects, such as developmental delay, intellectual disability, cerebella ataxia, as well as motor and sensory peripheral neuropathy.

Perrault syndrome is associated with the presence of biallelic mutations in any one of five genes. These are *HARS2*, *HSC17B4*, *LARS1*, *C10orf2*, and *CLPP*. On the basis of the known mutations in HsClpP implicated in Perrault syndrome, the primary effect of Perrault mutations in ClpP is likely to weaken its structural integrity, leading to an unstable ClpP with a shorter half-life.[57,58] This results in an overall reduction in cellular ClpP levels, leading to mitochondrial dysfunction and ultimately, the manifestation of disease. The physiological impact from the loss of ClpP on the entire organism reported to date appears to be localized to specific organs and tissues. Accordingly, the highest levels of ClpP expression are also detected in organs and tissues where the *CLPP* null phenotypes manifest. These include the testis, ovaries, heart, and skeletal muscles.[59] The higher levels of ClpP expression in these organs

and tissues are potentially reflective of their greater dependence on ClpP, possibly at different developmental stages or other currently unknown conditions, which could translate into a greater susceptibility of these organs and tissues to the loss of ClpP causing the observed disease phenotypes.

VII. ROLES OF HUMAN MITOCHONDRIAL CLPP IN THE VIABILITY, GROWTH, AND METASTASIS OF CANCER CELLS

A hallmark of many cancer cells is their accelerated and unregulated growth and proliferation. This typically requires significant alterations in energy metabolism that induce cellular oxidative stress *via* the increased generation of reactive oxygen species (ROS).[60] The increase in ROS causes oxidative damage to different biomolecules, leading to mitochondrial dysfunction and other cellular impairments.[52] To compensate, multiple mitochondrial chaperones and proteases are upregulated to preserve mitochondrial integrity and functions, while simultaneously suppressing the activation of apoptotic pathways that can be triggered by the increase in cellular ROS levels. ClpP is upregulated across various types of cancer. These include carcinomas in breast, prostate, colon, liver, uterus, ovary, thyroid, lung, bladder, stomach, Hodgkin's lymphoma, acute myeloid leukemia, as well as glioblastoma multiforme.[18,22] Also, ClpP expression was found to be 1.4 to 2.6 times higher in metastatic cells than nonmetastatic ones in non-small-cell lung cancer.[22]

Research on the cellular roles of ClpP has revealed that the protease is important in the pathology of several human cancers, including cell viability, proliferation, and metastasis, although the effect of ClpP and its importance is cell-type specific. For example, prostate adenocarcinoma PC3 cells are heavily dependent on ClpP for proliferation, and knockdown of ClpP results in cell cycle arrest and inhibition of colony formation.[22] Investigations of tumor cell invasion and metastasis in PC3 upon *CLPP* knockdown showed inhibition of cell migration in a wound closure assay.[22] With acute myeloid leukemia (AML), genetic knockdown in cell lines with high ClpP expression, such as the K562, TEX, and OCI-AML2 cell lines, results in the reduction of both cell growth and viability.[18] In contrast, knockdown of ClpP in the nonmetastatic breast adenocarcinoma MCF-7 cells had only a marginal effect on suppressing cell proliferation, even though ClpP was also highly expressed in these cells.[22]

Interestingly, the overexpression of ClpP in HeLa cells increased the cell's resistance against killing by cisplatin, a commonly used chemotherapeutic agent that damages DNA and induces apoptosis,[61] whereas knockdown of ClpP had the opposite effect.[62] The overexpression of a catalytically inactive ClpP mutant had no effect on protecting the cell from cisplatin or in delaying caspase activation, an indication of apoptosis. This highlights the importance of ClpP's proteolytic activity in the underlying mechanism, although the relevant substrate proteins targeted for degradation were not explicitly identified.[62] Nevertheless, the overexpression of ClpP induces an upregulation of the copper efflux pumps ATP7A and ATP7B, both of which have been shown to promote cisplatin release from the cell.[63] Profiling of caspase 7 and caspase 3 activation upon cisplatin treatment revealed that ClpP upregulation induces a delay in the accumulation of both caspases in their activated forms, suggesting that ClpP acts upstream of mitochondrial membrane permeabilization and caspase activation during cisplatin-induced cell killing.[62]
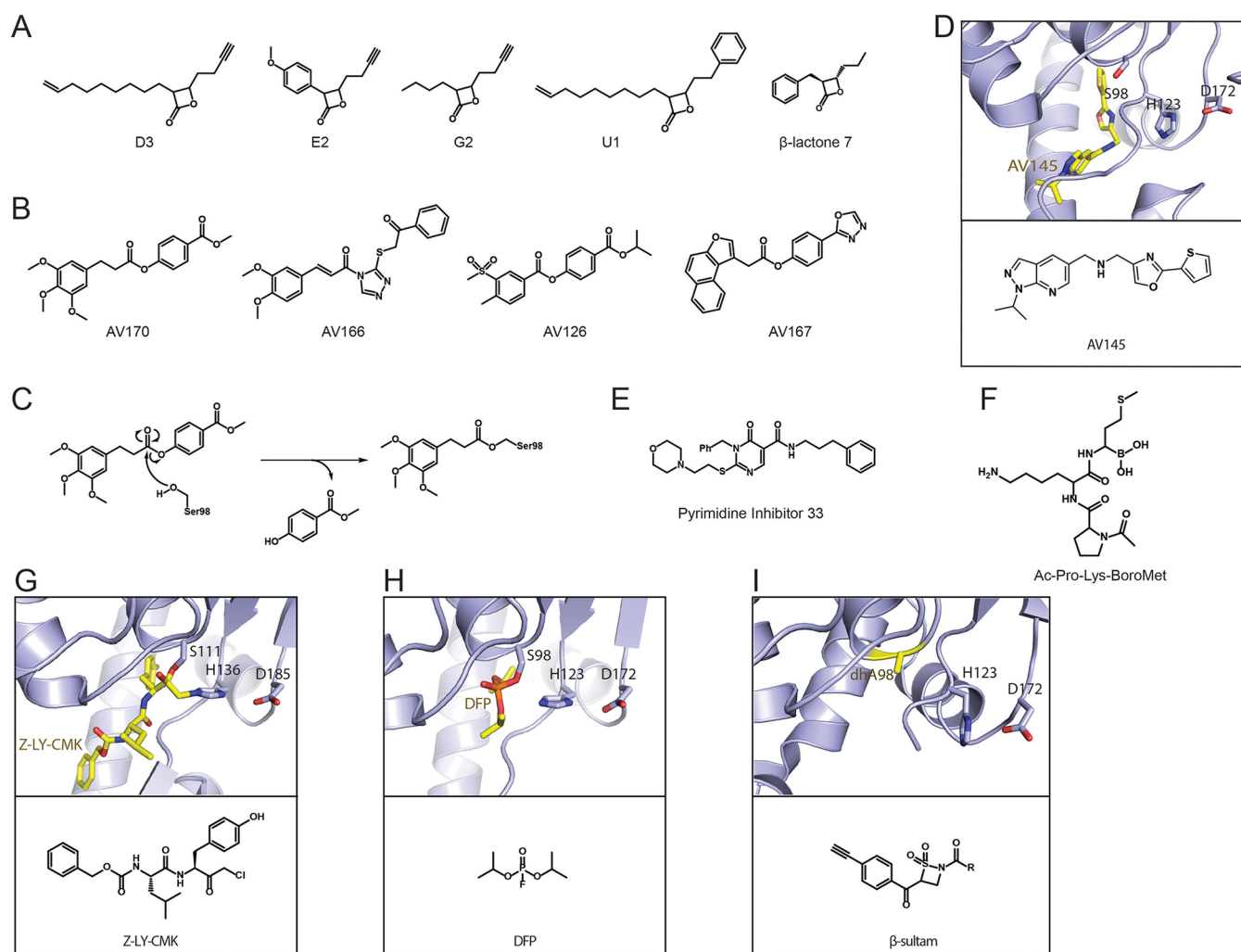
Figure 3. Inhibitors of ClpP. Shown are chemical structures of (A) β-lactone analogues and (B) phenyl ester analogues that inhibit ClpP. (C) A proposed mode of inhibitory action by β-lactone and phenyl ester compounds is illustrated for phenyl ester analogue AV170 on Ser98 in the active site of SaClpP. (D) The reversible inhibitor AV145 bound to SaClpP near the active site (PDB ID 5DL1). (E) The structure of a pyrimidine-containing inhibitor of PfClpP, named compound 33, is shown. (F) A peptide boronate Ac-Pro-Lys-BoroMet is drawn. Shown are the structures of the active sites of (G) EcClpP bound with Z-LY-CMK (PDB ID 2FZS), (H) BsClpP bound with DFP (PDB ID 3TT7), and (I) SaClpP with its serine modified to a dehydroalanine after β-sultam treatment (PDB ID 4MXI).

Taken together, ClpP plays a critical role in sustaining the growth and viability of specific types of cancer cells as well as in promoting their proliferation and metastasis.

VIII. DRUG-BASED MODULATION OF CLPP ACTIVITY—CLPP INHIBITORS

The specific inhibition of ClpP activity has primarily been achieved using β-lactone and the phenyl ester group of drugs. Several trans-β-lactone compounds were primarily identified to act against ClpP using a chemical proteomic strategy called activity-based protein profiling in which site-directed covalent probes were used to profile the activity of enzymes in proteomes.[64] The identified compounds were found to specifically label and irreversibly inhibit ClpP from several nonpathogenic and pathogenic bacterial strains.[3,65] Three compounds, D3, E2, and G2 (Figure 3A), were identified to selectively target and irreversibly inhibit ClpP of WT and methicillin-resistant S. aureus cells; D3 was the most potent. The addition of these compounds to S. aureus cells resulted in decreased expression of major virulence factors such as hemolysins, proteases, DNases, and lipases, which are

important players in countering the host response, tissue necrosis, and inflammation.

An optimized β-lactone inhibitor of S. aureus ClpP (SaClpP), U1 (Figure 3A), showed 3- to 5-fold increased inhibition compared to D3.[66,67] U1 inhibited ClpP from other bacteria as well. For example, it was found that U1 caused the down-regulation of important virulent factors such as listeriolysin O and phospholipases C in Listeria monocytogenes.[66] U1 also specifically inhibited the P. falciparum ClpP protease activity.[47] Furthermore, other β-lactone analogs were able to inhibit the ClpP1P2 peptidase activity in M. tuberculosis leading to cell death.[68] The most potent inhibitor identified in that study, β-lactone 7 (Figure 3A), contains a benzylic substituent on the α-carbon and an alkyl chain on the β-carbon and selectively inhibits ClpP2. β-Lactone inhibitors also showed activity against AML human cell lines expressing elevated levels of ClpP.[18]

Despite the successes observed in vitro, limitations of β-lactones reside in their generally low potency, poor selectivity, and relative instability as these labile electrophiles are quickly hydrolyzed in human plasma.[5,18,67] Consequently, other
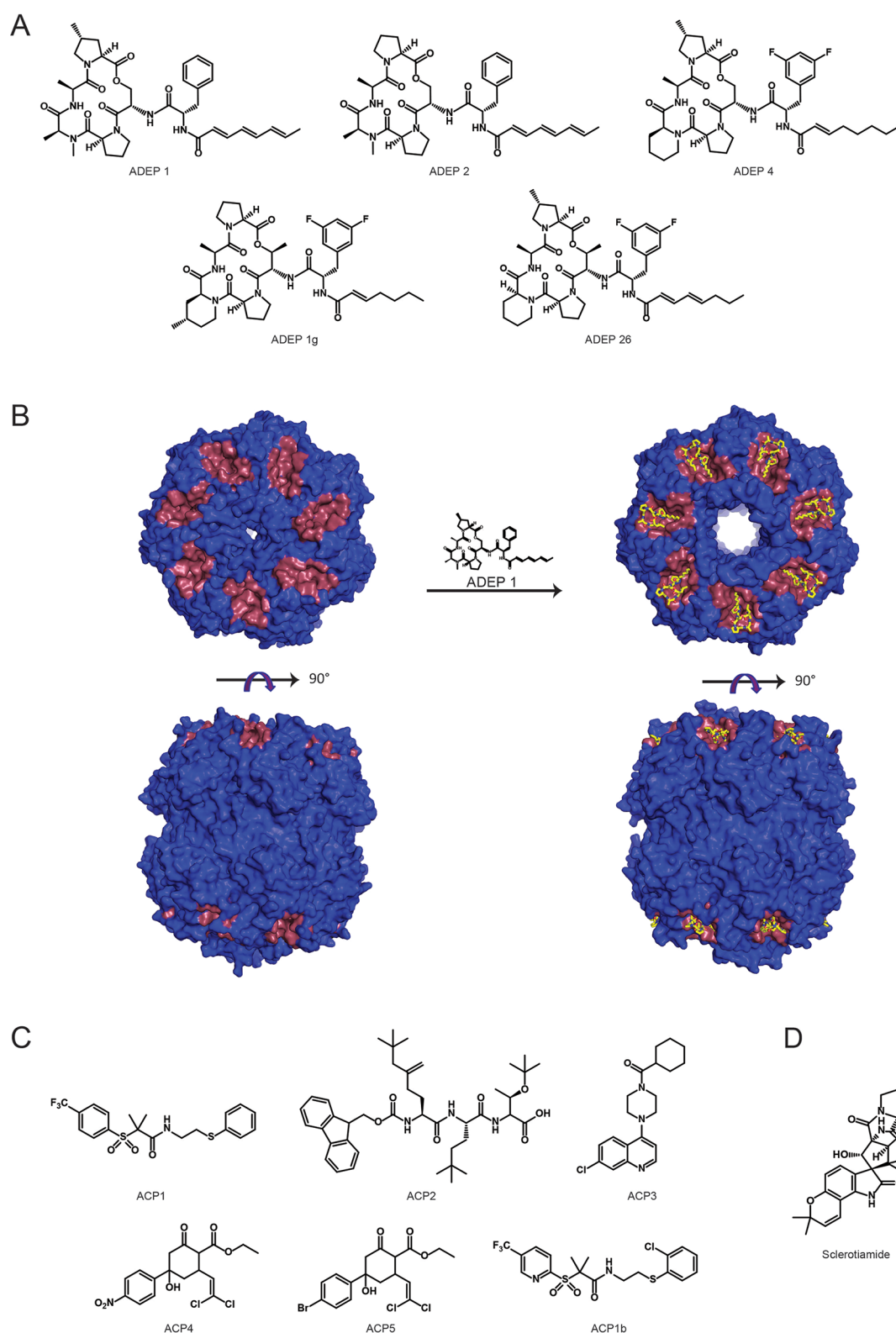
Figure 4. Activators of ClpP. (A) Shown are the chemical structures of ADEP compounds. (B) Top and side views of EcClpP unbound (PDB ID 1YG6) and bound with ADEP 1 (PDB ID 3MT6). ClpP is shown in a blue surface representation. The hydrophobic pockets are highlighted in purple with or without bound ADEP 1 shown as yellow sticks. (C) The structures of ACP1−5 and ACP1b are shown. (D) The structure of sclerotiamide I is shown.

chemical scaffolds are needed to obtain clinically viable compounds that target ClpP.

Using an unbiased high-throughput screen of more than 137 000 compounds, a novel class of phenyl esters has been found to inhibit ClpP.[5] From the screen, six potential hits were identified that contained activated ester or amide moieties (Figure 3B). These hits were then tested for inhibition of HsClpP and SaClpP. Most phenyl ester compounds did not

inhibit human ClpXP proteolysis but were selective for bacterial ClpP; their potency, inhibition kinetics, and plasma lifetime largely exceeded that of the β-lactones.[5] AV170 was found to be 4 times more effective for SaClpP in inhibiting its peptidase activity compared to β-lactones D3 and E2, followed by AV166 and AV126[5] (Figure 3B). Furthermore, AV167, which has a large naphthofuran moiety (Figure 3B), was found to be the only compound that reduced human mitochondrial ClpP peptidase activity at low concentrations.

Both β-lactones and phenyl esters inhibit ClpP through the covalent modification of the active site. For example, the active site Ser98 in SaClpP acts as a nucleophile which attacks these compounds and results in a trapped acyl-enzyme intermediate (Figure 3C). Accordingly, these inhibitors are also useful tools in further dissecting and understanding the ClpP catalytic mechanism.

A noncovalent inhibitor against *S. aureus* ClpP was identified in a high throughput screen.[69] The inhibitor (AV145) consists of three heterocycles, pyrazolopyridine and 2-(thiophen-2-yl)oxazole moieties (Figure 3D). A cocrystal structure demonstrated that AV145 bound to the handle region close to the active site of the protease, which locks SaClpP in a novel conformation (Figure 3D). However, the inhibitory effect of AV145 and its optimized derivatives was revoked due to ClpX binding to ClpP, and hence, the compounds had no pronounced effect on the bacteria itself.[69]

Another class of ClpP-inhibitor compounds effective against the *P. falciparum* protease was recently identified with the help of *in silico* screening.[70] The authors utilized the available structure of EcClpP in its active state to model the binding of a library of 450 000 compounds to the PfClpP protein. A select group from the screen showing potent *in vitro* inactivation of the PfClpP led to the identification and optimization of a series of candidates containing a pyrimidine ring. The most promising of these, referred to as compound 33, is shown in Figure 3E. The biochemical mechanism of inhibition is not known; however, the compound displayed the ability to interrupt the division and segregation of the apicoplast organelle leading to inhibition of parasite growth.[70] The relatively low cytotoxicity observed against HeLa cell lines bodes well for further optimization of the compound for clinical applications.

The essentiality of the *M. tuberculosis* ClpP1P2 proteins (MtbClpP1P2) for the tuberculosis pathogen has provided the opportunity for the design of drugs targeting it. Taking advantage of this and utilizing known peptide substrates for the protease, peptide boronates have been designed to inhibit the MtbClpP1P2 activity.[71] Figure 3F depicts one such compound. An active MtbClpP1 is known to be required for both peptidic and proteolytic activities of the protease, while MtbClpP2 is only essential for proteolysis. The di- and tripeptide boronates were made and were selective for MtbClpP1, yet they were able to inhibit both the peptidic and the proteolytic activities of the MtbClpP1P2. Furthermore, these compounds were selective for *M. tuberculosis*, with limited activity against the mammalian myeloma cells MM1.S and showed no growth inhibition of *E. coli* or *S. aureus*. It should be noted that peptide boronates have been designed to target the human proteasome and are being used in treating multiple myeloma.[72]

Three X-ray crystal structures of ClpP have also been solved with general serine protease inhibitors. The structure of EcClpP with benzyloxycarbonyl-leucyltyrosine chloromethyl ketone (EcClpP-Z-LY-CMK; Figure 3G)[73] and *B. subtilis* ClpP with diisopropylfluorophosphate (BsClpP-DFP; Figure 3H)[74] show

the binding of these inhibitors at the active site serine. Also, the action of a β-sultam inhibitor[75] was found to result in the conversion of the active site serine to dehydroalanine as observed by the solved X-ray structure of SaClpP treated with a β-sultam termed RKS07 (Figure 3I).

## IX. DRUG-BASED MODULATION OF CLPP ACTIVITY—CLPP DYSREGULATORS

Rather than inhibiting ClpP activity, a group of compounds of the acyldepsipeptide (ADEP) family (Figure 4A) were discovered to dysregulate (or activate) the function of the protease. Dysregulation of ClpP is achieved by ADEPs competing with and displacing the AAA+ ATPase ClpX in binding with ClpP, thereby bypassing ClpX's regulatory function. ADEP-binding also keeps ClpP in its active state and enables the protease to indiscriminately degrade susceptible proteins.[76]

ADEPs were first discovered by researchers at Eli Lilly and Company in 1985 and were isolated from *Streptomyces hawaiiensis* as eight factors that formed an antibiotic complex.[77] The antibiotic action of these factors was evidenced against Gram-positive *Staphylococcus* and *Streptococcus* strains. However, it was not until two decades later that the main components of the A54556 complex were structurally characterized (e.g. factor A (ADEP 1) and factor B (ADEP 2); Figure 4A) and identified to act specifically on ClpP.[2]

X-ray cocrystal structures have been solved for ADEP analogs bound to ClpP from a number of organisms.[25,78−80] ADEPs bind at hydrophobic pockets (H-pockets) on the top and bottom apical surface of ClpP away from the catalytic center (Figure 4B; also see Figure 2B). These H-pockets are located between adjacent monomers, surrounding the axial entrance pore; hence, a ClpP cylinder contains 14 such pockets. The pockets are sites where the Clp ATPases dock onto ClpP using loop regions containing a highly conserved IGF/L motif.[81] The interaction of the Clp ATPases is linked to the opening of the ClpP axial pore, providing substrates access to the proteolytic chamber.[79,82] Therefore, the association of the ADEPs at a similar location is thought to mimic the action of Clp ATPase binding and, at the same time, prevent the binding of the ATPase partner. ADEP binding opens up the ClpP axial pores and has an allosteric effect on ClpP structure[83] that is proposed to enhance the observed efficacy of protein degradation by ClpP without a need for Clp ATPases or associated adaptor proteins.[78,79]

The activation of ClpP function by ADEPs has been elucidated through observations of the antibiotic effect of the compounds on *E. coli*, *B. subtilis*, *S. aureus*, and *S. pneumoniae* among others.[2,84−86] In addition, Famulla and colleagues[87] presented data to indicate that, in *Mycobacteria*, the antibacterial activity of ADEPs is primarily due to their ability to prevent the binding between the ClpP complex and the Clp ATPase. *In vitro* assays have shown certain ADEP compounds to even have a similar activating influence on the proteolytic action of the human protease.[88]

The earliest ADEPs (ADEP 1 and 2; Figure 4A) were found to have limited antibacterial activity against Gram-positive pathogens, and Gram-negative bacteria were often not susceptible. In addition, they were inactive in mouse models due to poor solubility, fast systemic clearance, and the instability of the drug structure.[89] A series of manipulations of the ADEP structure have led to the identification of more stable and potent forms of the molecule. The main component
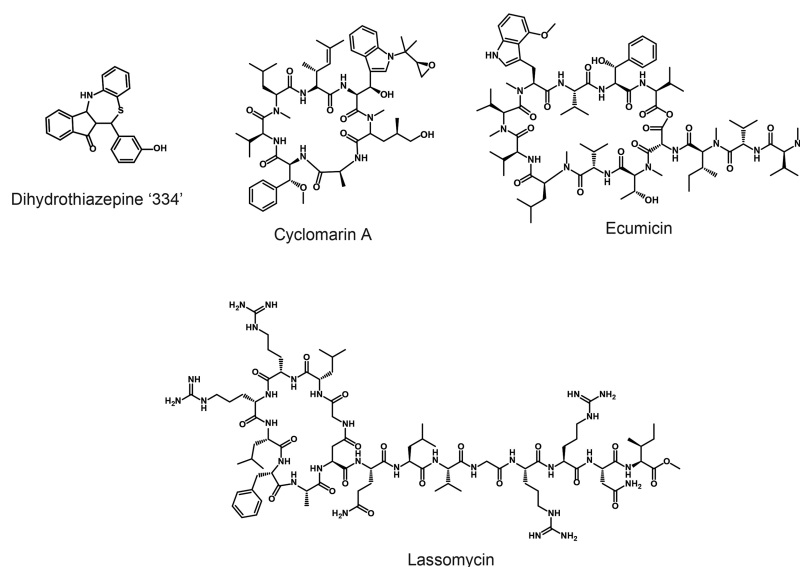
Figure 5. Structures of compounds targeting Clp ATPases. Shown are the structures of a dihydrothiazepine termed "334," cyclomarin A, lassomycin, and ecumicin.

of the ADEP consists of a peptidolactone macrocyclic core (a depsipeptide) coupled to an N-acylphenylalanine moiety *via* an exocyclic amide bond. The entropic cost of ClpP binding can be lowered through the inclusion of a ridged pipecolate moiety as shown in ADEP 4 (Figure 4A). In addition, when the polyunsaturated side chain of ADEP 2 was substituted with a heptenoyl moiety, along with a replacement of the phenylalanine with a 3,5-difluorophenylalanine residue, an enhancement of chemical stability and bioavailability was observed with a 160-fold increase of activity.[89] Later on, further improved antibacterial activity was gained by functionalization of the ADEP 4 macrocycle with methylated piperidine and conversion of the serine moiety into allo-threonine (ADEP 1g; Figure 4A), which resulted in a constrained conformation of the core.[90,91] The susceptibility of the depsipeptide ester linkage to hydrolysis remains a concern, yet its substitution to an amide or N-methyl amide linkage has not proven to be a viable option.[80,92] Furthermore, the ADEPs are nontrivial chemical synthesis targets, with the macrocyclic ring constituting the most challenging aspect of the molecule, a factor which has somewhat limited the development of extensive compound libraries and medicinal chemistry efforts.[93]

With improved stability, ADEPs have been found to exhibit potent activity against Gram-positive bacteria such as *Enterococci*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.[2,87,89,90,94] The effectiveness of ADEP 4, in combination with rifampicin, was shown against chronic biofilm infections by *Staphylococcus aureus* in mice.[95] ADEP 4 activity was further tested in mouse models against pathogenesis by *E. faecalis*, *S. aureus*, and *S. pneumonia* where toxicological and pharmacokinetic tests of the drug in mice and dogs showed moderate to high distribution and clearance with a 1−2 h half-life.[2]

Many of the ADEPs have bactericidal activity against Gram-positive bacteria, but their actions are limited in most Gram-negative bacteria due to their susceptibility to active efflux and/or limited penetration of the outer membrane.[2,80] Recent further optimization of the macrocyclic core residues and the N-acyl side chain by our group[80] led to a new ADEP derivative, termed ADEP 26 (Figure 4A), that not only displayed

enhanced activity against Gram-positive species, *S. aureus* and *Enterococcus faecalis*, but was also potent against two susceptible pathogenic Gram-negative species, *Neisseria meningitidis* and *Neisseria gonorrheae*.

To identify molecules with similar activities against ClpP as ADEPs but with different structural scaffolds, a high-throughput screening approach was used. Five structurally diverse activators, which we termed Activators of Self-Compartmentalizing Proteases (or ACPs; Figure 4C), were identified to have ADEP-like activities against EcClpP.[4] ACP1 (Figure 4C) was found to be the most effective and was further optimized, yielding ACP1b (Figure 4C), which showed good antibacterial properties. Bactericidal activity at low concentrations was observed with ACP1b against the Gram-positive *Streptococcus pneumonia* and *Staphylococcus aureus* along with six Gram-negative bacteria: *N. meningitidis*, *N. gonorrheae*, *H. influenzae*, *P. aeruginosa*, *L. monocytogenes*, and *E. coli*.[4] These ACP compounds are thought to possess a similar mechanism of ClpP activation to that of the ADEPs.

A third type of ClpP activator was identified in a recent study by Lavey and colleagues using a fluorescence-based protease assay to screen a library of about 450 structurally diverse fungal and bacterial secondary metabolites for the activation of EcClpP *in vitro*.[6] After multiple screenings, sclerotiamide, a nonpeptide-based natural product, was found to activate EcClpP (Figure 4D). Although the potency of sclerotiamide is much lower when compared to ADEPs and ACPs, its structure provides a unique three-dimensional bicyclo-[2.2.2]-diazoctane motif which has been recently reported to exhibit a range of biological activities.[96] Therefore, further optimization studies are required to improve potency through a structure–activity relationship for cellular target engagement and efficacy.

X. TARGETING CLP ATPASES

Other compounds are known to affect the activity of Clp ATPases rather than the ClpP protease and to have either antivirulence or antibacterial activity. The dihydrothiazepine "334" (Figure 5) compound and its derivatives act on ClpX, causing its deoligomerization.[97] The compound was shown to

reduce the levels of toxin production by *S. aureus* partially comparable to what is observed for *clpX* deletion mutants.

A group of compounds, the cyclomarin A,[98,99] lassomycin,[100] ecumicin[101] (Figure 5), and rufomycin analogs (structure unavailable)[102] target the ClpC1 ATPase in *M. tuberculosis*. While little has been described of rufomycin, cyclomarin A (cymA) was shown to bind the N-terminal domain of ClpC1 of *M. tuberculosis*.[98] In culture and in human macrophages, the compound was found to be bactericidal, even against multidrug resistant *M. tuberculosis* species. Though the mechanism of action has not been confirmed, structural analyses have led to the hypothesis of uncontrolled proteolysis engendered by the binding of the drug to ClpC1.[99]

Lassomycin was discovered encoded in the genome of the soil bacterium *Lentzea kentuckyensis*.[100] Ecumicin, on the other hand, was identified from a screen of over 65 000 actinomycete compounds.[101] Both compounds decouple the ATPase activity of ClpC1 from the proteolytic activity of ClpP1P2, which is proposed to be the cause for their antibacterial action on *M. tuberculosis* and other *Mycobacterium* species.

XI. CONCLUDING REMARKS

In summary, the role of ClpP in bacterial virulence is well established, and some potential physiological target substrates of the protease in several bacteria have been identified. In contrast, while there is good evidence linking the human ClpP to cancer and Perrault syndrome, understanding the physiological function and mechanism of action of human ClpP is still in its early stages.

Several compounds that modulate bacterial ClpP activity have been described. These compounds are aimed toward either inhibiting or activating ClpP. So far, no detailed studies of these compounds have been carried out on human ClpP. Nevertheless, it is reasonable to expect that several compounds that target bacterial ClpP can also affect the activity of human ClpP given the high sequence conservation of the protease. However, in targeting human ClpP, considerations have to be made to ensure that the compounds can transverse the cytoplasmic and mitochondrial membranes and reach the mitochondrial matrix. While there are several drug development programs by multiple groups targeting ClpP, no such compounds have yet reached the clinic. Therefore, there is still much work to be done for this goal to be ultimately achieved.

Finally, the Clp ATPase−ClpP protease complex constitutes a fascinating system that is particularly suitable for drug development either by targeting protein activities or protein−protein interactions. As such, the ADEP and ACP classes of compounds constitute remarkable examples of molecules which prevent protein−protein interaction but also lead to a gain-of-function causing ClpP protein activation and eventually cell death.

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ACKNOWLEDGMENTS

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