Chemical Modulation of Human Mitochondrial ClpP: Potential Application in Cancer Therapeutics

Keith S. Wong† and Walid A. Houry*†,‡

†Department of Biochemistry, University of Toronto, Toronto, Ontario M5G 1M1, Canada
‡Department of Chemistry, University of Toronto, Toronto, Ontario M5S 3H6, Canada

ABSTRACT: The human ClpP proteolytic complex (HsClpP) is a serine protease located in the mitochondrial matrix and participates in the maintenance of the mitochondrial proteome among other cellular functions. HsClpP typically forms a multimeric complex with the AAA+ protein unfoldase HsClpX. Notably, compared to that of normal, healthy cells, the expression of HsClpP in many types of solid and nonsolid cancers is found to be upregulated. While the exact role of HsClpP in tumorigenesis is not clear, certain types of cancers are highly dependent on the protease for cell proliferation and metastasis. In light of these observations, recent research has focused on the discovery and characterization of small organic molecules that can target and modulate HsClpP activity. These include compounds that inhibit HsClpP’s proteolytic activity via covalent modification of its catalytic Ser residue as well as those that activate and dysregulate HsClpP by displacing HsClpX to negate its regulatory role. Importantly, several of these compounds have been shown to induce HsClpP-dependent apoptotic cell death in a variety of cancerous cells. This review provides an overview of these research efforts and highlights the various types of small molecule modulators of HsClpP activity with respect to their potential use as cancer therapeutics.

1. INTRODUCTION

1.1. HsClpXP in Human Mitochondria. The mitochondrion is a vital organelle that serves as the cell’s center of energy metabolism and carries out many other important functions. Its activities are sustained by the proper maintenance of the mitochondrial proteome, which is achieved via a number of molecular mechanisms. One such mechanism employs molecular chaperones for the folding of mitochondrial proteins and proteases for the prompt removal of proteins that are damaged and/or misfolded. Among the proteases utilized in this regard is the HsClpXP complex that is found in the mitochondrial matrix. It consists of two proteins: the AAA+ protein unfoldase, HsClpX, and the serine protease, HsClpP. “AAA” stands for ATPase associated with diverse cellular activities, which is a diverse superfamily of ATPases that utilize the highly conserved AAA+ domain for ATP binding and hydrolysis. Proteins in this superfamily use the energy generated from ATP hydrolysis for various types of molecular remodeling activities. Serine proteases refer to a large superfamily of proteases that catalyze the hydrolysis of peptide bonds of their substrate proteins via the highly conserved catalytic Ser-His-Asp triad, for which the molecular mechanism is well characterized.

HsClpX and HsClpP are encoded by the nuclear genes CLPX (NCBI GeneID 10845) located on chromosome 15 and CLPP (NCBI GeneID 8192) located on chromosome 19. Their domain architecture is shown in Figure 1A. HsClpX is 633 amino acid residues in length and consists primarily of the AAA+ domain preceded by a zinc-binding domain (ZBD) for substrate protein recognition. At the N-terminus of HsClpX is a mitochondrial targeting sequence (MTS) that is required for its translocation into the mitochondrial matrix. HsClpP is 277 amino acid residues in length and consists almost entirely of the serine protease domain. Like HsClpX, a MTS is positioned...
HsClpX and HsClpP are differentially expressed in different tissues. HsClpX is strongly expressed in skeletal and heart muscles but less so in liver, brain, placenta, lung, kidney, and pancreas. Similarly, HsClpP is expressed at high levels in skeletal muscles, at intermediate levels in heart, liver, and pancreas, and at low levels in brain, placenta, lung, and kidney.

The functional oligomeric state of the HsClpXP complex consists of 14 HsClpP subunits forming a cylinder of two heptameric rings (Figure 1Bi) that is capped at each end by six HsClpP subunits forming the hexameric ring typical of AAA+ ATPases. In this configuration, the catalytic residues of HsClpP (Ser153, His178, and Asp227) (Figure 1Bii) are enclosed within the lumen of the ClpP cylinder to prevent unspecific proteolytic activities, while HsClpX serves as the gatekeeper that recognizes only specific proteins and targets them for degradation (Figure 1C).

The interaction between the HsClpX and HsClpP oligomers is stabilized by the highly dynamic docking interactions of the ATPase’s IGF loops (L439-G440-F441 within the E436−G450 region of HsClpX) with the hydrophobic pockets formed by neighboring HsClpP subunits at the ClpX−ClpP interface.

The degradation of a substrate protein by ClpXP, in general, occurs in a coordinated, stepwise manner as illustrated in Figure 1C. First, the substrate is recognized and bound by ClpX, followed by its unfolding by ClpX in an ATP-dependent manner. The unfolded substrate is then translocated through ClpX into ClpP’s catalytic chamber where it is degraded into small peptides. The peptides then exit HsClpP via its side pores. Subsequently, HsClpXP can start a new round of proteolysis upon the binding of a new substrate.

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1.2. Biological Functions of HsClpXP.

1.2.1. Cellular Functions of HsClpXP. The primary function of HsClpXP is to facilitate the rapid turnover of specific substrate proteins that are misfolded, thereby preventing their accumulation as protein aggregates that cause impairment of normal cellular function. Indeed, the level of HsClpXP expression is known to increase during a mitochondrial unfolded protein response (UPRmt) in response to the accumulation of misfolded mitochondrial proteins, mitochondrial frataxin deficiency, and respiratory deficiency caused by mutations in mitochondrial DNA (mtDNA). Importantly, the loss of HsClpXP at the N-terminus of HsClpP for mitochondrial translocation. HsClpX and HsClpP are differentially expressed in different tissues. HsClpX is strongly expressed in skeletal and heart muscles but less so in liver, brain, placenta, lung, kidney, and pancreas. Similarly, HsClpP is expressed at high levels in skeletal muscles, at intermediate levels in heart, liver, and pancreas, and at low levels in brain, placenta, lung, and kidney.

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function causes the accumulation of misfolded, nonfunctional proteins that disrupt normal mitochondrial activities. For example, Seo et al. reported that knocking down ClpP resulted in the accumulation of misfolded SDHB (subunit B of Complex II) and loss of Complex II activity, which in turn disrupts oxidative phosphorylation (OXPHOS), impairs ATP biosynthesis, and increases the level of cellular oxidative stress.17 Similarly, Deepa et al. showed that the loss of ClpP in muscle cells impairs the expression of proteins involved in UPRmt and attenuates the activities of respiratory complexes.18

In addition to protein quality control, evidence that HsClpXP has regulatory roles in multiple biological pathways is accumulating. For example, it may participate in the negative feedback mechanism for regulating heme biosynthesis by the degradation of the mitochondrial S-aminolevulinate synthase ALAS1 and the erythroid-specific ALAS2, both of which mediate the first step in protoporphyrin IX biosynthesis.19,20 HsClpXP may also affect mitophagy via facilitating the turnover of phosphatase and tensin homologue-induced kinase 1 (PINK1) that in turn modulates the recruitment of the E3 ubiquitin ligase Parkin to the mitochondrial surface.21 Furthermore, HsClpXP is implicated in modulating mitochondrial fission—fusion dynamics via its interaction and turnover of dynamin-related protein 1 (Drp1).18 Mitochondrial translation may also be affected by HsClpXP as it can recognize and degrade nitric oxide-associated protein 1 (NOA1) that regulates that process.22 In depolarized mitochondria, HsClpXP aids in the quenching of ROS production by the turnover of specific respiratory proteins, such as NDUFV1 and NDUFV2 that constitute part of the peripheral arm of Complex I, which in turn alters OXPHOS.23 HsClpX also carries out biological functions independent of HsClpP. For example, HsClpX can act as a molecular chaperone for ALAS2 during its maturation process without the participation of HsClpP.24 HsClpX may also have a role in maintaining the mitochondrial genome by stabilizing mitochondrial transcription factor A (TFAM).25 In addition, HsClpX may function in promoting apoptosis via direct interaction with the E3 ubiquitin-protein ligase XIAP (X-linked inhibitor of apoptosis proteins), which prohibits XIAP from inhibiting caspasases via the same mechanism of other known IAP-binding proteins.26 Furthermore, HsClpX alone may stimulate UPRmt by inducing the upregulation of the transcriptional regulator CHOP (C/EBP homologous protein) during myogenesis.14

1.2.2. Role of HsClpP in Cancer. The exact function of HsClpP in cancer pathology is not well understood, although the expression of HsClpP has been shown to be generally upregulated in solid cancers of multiple organs or tissues, such as lung, stomach, liver, thyroid, bladder, breast, ovary, prostate, testis, and the central nervous system.16,17,27 The level of HsClpP expression is also increased significantly in the non-solid acute myeloid leukemia.28 Furthermore, immunohistochemistry (IHC) analysis of patient tissue samples covering 17 cancer types has shown moderate to high levels of HsClpP expression in malignant cells compared to normal ones.29

Recent experimental evidence also suggests that HsClpP expression is required for the proliferation and metastasis of specific types of cancer. For example, levels of proliferation and colony formation of the prostate cancer cells PC3 have been shown to decrease significantly when HsClpP expression is prohibited.17 Furthermore, the ability of PC3 cells to invade normal tissues and metastasize in a xenografted mouse model is largely compromised if HsClpP is depleted.17 Nevertheless, depleted HsClpP expression has minimal effects on the proliferation of the MCF7 breast cancer cells,17 which highlights the importance of HsClpP expression in cancer pathology being highly dependent on cell type.

Similar to solid cancers, depletion of HsClpP has been shown to reduce the rate of growth and viability of multiple lines of leukemic cells that show elevated levels of expression of the protease.26 Notably, the loss of HsClpP leads to decreased OXPHOS and increased ROS production resulting from a structurally and functionally compromised Complex II.25 An interesting finding is that, while the level of UPRmt is elevated consistently across leukemic cell lines with a high level of HsClpP expression, depleting the protease produces no observable impact on either the expression of UPRmt markers or mitochondrial morphology.26 This is in contrast to the findings in immortalized muscle cells19 and fibroblasts,27 which reflects a variation in the potential role of HsClpP in UPRmt among different types of cancer.

On the basis of the current experimental evidence and clinical data, the increase in the level of HsClpP expression does not appear to be a direct contributor to oncogenesis given that it is essential only for the proliferation and pathology of particular cancer types. Alternatively, the level of HsClpP expression may be increased as a consequence of intracellular conditions during the oncogenic process that are shared among the different types of cancer.

As discussed under section 1.2.1, HsClpP is known to provide stress relief from the accumulation of ROS by mediating the turnover of specific subunits of OXPHOS complexes. In cancer cells, increases in the level of intracellular ROS is important for facilitating and sustaining oncogenesis.30 Thus, increasing the level of expression of HsClpP may serve as a coping mechanism for these cells to grow and proliferate under increased intracellular ROS with increased risks of protein misfolding and aggregation. In support of this notion, heat shock proteins (HSPs) and molecular chaperones are known to be overexpressed in cancer cells to protect against the deleterious effects of accumulated oncogenic mutations and death by ROS-induced apoptosis.31 Importantly, these proteins may serve as potential anticancer drug targets by circumventing the cancer cell’s ability to cope with elevated ROS levels.32 By the same premise, the potential of HsClpP as an anticancer therapeutic target has been explored, leading to the discovery and development of small molecule compounds that can specifically target and modulate the proteolytic activity of HsClpP.

2. CHEMICAL MODULATORS OF HSCLP APP ACTIVITY

In the following sections, we will showcase recent research and development of small organic molecules that can specifically target HsClpP and modulate its proteolytic activity and discuss their anticancer therapeutic potentials. Notably, these molecules constitute but a minor portion of a larger library of ClpP-modulating compounds that were originally designed as potential antibiotics against various bacterial species and other pathogenic organisms (e.g., Plasmodium falciparum, the causative agent of malaria in humans), which has been described in detail elsewhere.30,33,34 Here, we will discuss the effect of some of these ClpP-modulating compounds in the context of targeting different cancers.

2.1. Inhibitors of HsClpP. Chemical inhibition of the proteolytic activity of HsClpP is perhaps the most direct
therapeutic strategy against cancer cells that depend heavily on the protease to proliferate. Several specific inhibitors that can target HsClpP have been developed and optimized against specific types of cancers. In general, these compounds disable HsClpP tetradecamer activity by covalently modifying the 14 catalytic Ser153 residues located within its lumen (Figure 2A).

2.1.1. β-Lactones. β-Lactones were first developed as potential antibiotics against Staphylococcus aureus that suppress its pathogenesis via inhibition of its ClpP protease (SaClpP). Notably, one analogue that was developed, A2-32-01 (Figure 2B), has shown therapeutic potential against acute myeloid leukemia. While the specific mechanism of HsClpP inhibition by A2-32-01 has not been described in detail, it is believed to follow the general mechanism of β-lactones, where the drug covalently modifies the catalytic Ser153 via one of two possible reactions (Figure 2C) previously proposed.

The first evidence for the cancer therapeutic potential of A2-32-01 was illustrated by its cytotoxicity in leukemic cell lines such as OCI-AML2, TEX, and K562, although it had no effect on the acute promyelocytic leukemia HL-60 (Table 1). Notably, the level of HsClpP expression is highly elevated in OCI-AML2, TEX, and K562 in comparison to the level in HL-60. In addition, A2-32-01 is also cytotoxic in the 143B osteosarcoma cell line but not for the mitochondrion-depleted 143B Rho (0) cells (Table 1). Together, the cytotoxicity of A2-32-01 has been implicated to manifest in the mitochondria and is likely dependent on high levels of HsClpP expression.

The therapeutic potential of A2-32-01 was further illustrated in its cytotoxicity against primary leukemic cells expressing high levels of HsClpP (Table 1). Importantly, the drug had negligible effects on the viability of normal primary hematopoietic cells (Table 1), which illustrates specific targeting of cancer cells over normal ones. Furthermore, using a xenografted immunodeficient mouse model, A2-32-01 was shown to suppress the proliferation of OCI-AML2 and primary human leukemic cells in vivo, with no observable toxic side effects on the animals (Table 1).

2.1.2. Phenyl Esters. Phenyl esters were developed as a new class of HsClpP inhibitors that aim to improve upon both drug potency and chemical stability relative to β-lactones. Among the phenyl esters that were first developed to have shown inhibition of HsClpP’s peptidase activity is AV-167 (Figure 2D), which originates from a counter screen to identify potential ClpP-inhibiting antibiotics against S. aureus. Characterization of the mechanism of inhibition by mass spectrometric methods revealed that AV-167 shares a common reaction with other known active phenyl esters, in which the hydroxyl group of ClpP’s catalytic Ser residue performs a nucleophilic attack on the drug’s carboxyl group, resulting in the displacement of the drug’s phenyl moiety that is subsequently converted into its phenol equivalent (Figure 2D).
Table 1. Biological Activities of Small Molecule Compounds Targeting HsClpP in Various Cell Lines

<table>
<thead>
<tr>
<th>compound</th>
<th>molecular effect</th>
<th>cell line</th>
<th>disease model</th>
<th>biological activity</th>
<th>ref</th>
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<tbody>
<tr>
<td>A2-32-01</td>
<td>inhibition</td>
<td>TEX</td>
<td>acute myeloid leukemia</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 25 μM</td>
<td>28</td>
</tr>
<tr>
<td>OCI-AML2</td>
<td></td>
<td>OCI-AML2</td>
<td>acute myeloid leukemia</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 25 μM; impairs complex II and HsClpP activities and delays growth of cells engrafted in SCID mice by ~50%; no observable liver, muscle, or renal toxicity</td>
<td>28</td>
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<tr>
<td>K562</td>
<td></td>
<td>HL-60</td>
<td>chronic myeloid leukemia</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 25 μM</td>
<td>28</td>
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<tr>
<td>HL-60</td>
<td></td>
<td>143B</td>
<td>acute promyelocytic leukemia</td>
<td>no effect on cell viability</td>
<td>28</td>
</tr>
<tr>
<td>143B</td>
<td></td>
<td>143B Rho (0) mitochondria depletion control for 143B</td>
<td>no effect on cell viability</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>human primary AML cells</td>
<td></td>
<td>human primary AML cells</td>
<td>acute myeloid leukemia</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ranges from ~25 to ~125 μM, with cells expressing high HsClpP levels being the most sensitive (R&lt;sup&gt;2&lt;/sup&gt; = 0.9264); impairs proliferation of cells engrafted in NOD-SCID mice by ~60−70%; no host toxicity observed</td>
<td>28</td>
</tr>
<tr>
<td>human primary normal hematopoietic cells</td>
<td></td>
<td>normal/noncancerous cells</td>
<td>minimal to no effect on cell viability</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>TG42</td>
<td>inhibition</td>
<td>Huh7</td>
<td>heptocyte-derived carcinoma</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 20 μM based on apoptotic cell count 42 h post-treatment; cell migration decreased by ~50% at 30 μM drug</td>
<td>39</td>
</tr>
<tr>
<td>TG53</td>
<td>inhibition</td>
<td>Huh7</td>
<td>heptocyte-derived carcinoma</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 30 μM based on apoptotic cell count 42 h post-treatment; cell migration decreased by ~50% at 30 μM drug</td>
<td>39</td>
</tr>
<tr>
<td>ADEP-28</td>
<td>activation</td>
<td>HEK293 T-REx</td>
<td>N/A</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 0.36 μM</td>
<td>45</td>
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<td></td>
<td></td>
<td>HEK293 T-REx CLPP&lt;sup&gt;−/-&lt;/sup&gt;</td>
<td>ClpP deletion control for HER293 T-REx</td>
<td>no effect on cell viability</td>
<td>45</td>
</tr>
<tr>
<td>ADEP-41</td>
<td>activation</td>
<td>HEK293 T-REx</td>
<td>N/A</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 0.49 μM; intrinsic, caspase-dependent apoptosis induced in ~10% of cells after a 72 h exposure at 10 μM</td>
<td>45</td>
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<td></td>
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<td>HEK293 T-REx CLPP&lt;sup&gt;−/-&lt;/sup&gt;</td>
<td>ClpP deletion control for HER293 T-REx</td>
<td>no effect on cell viability; no apoptosis observed</td>
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<td>HeLa</td>
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<td>HeLa</td>
<td>endocervical carcinoma</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 0.54 μM</td>
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<td>HeLa T-REx</td>
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<td>HeLa T-REx</td>
<td>endocervical carcinoma</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 0.48 μM</td>
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<td>U2OS</td>
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<td>U2OS</td>
<td>osteosarcoma</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 0.58 μM</td>
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<td>SH-SYSY</td>
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<td>SH-SYSY</td>
<td>neuroblastoma</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 0.86 μM</td>
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<td>ONC201</td>
<td>activation</td>
<td>OCI-AML2</td>
<td>acute myeloid leukemia</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 2.4 μM; reduced leukemic burden in engrafted SCID mice by ~3−5-fold with drug treatment twice daily at 100 mg/kg for 13 days</td>
<td>48</td>
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<tr>
<td>TEX</td>
<td></td>
<td>OCI-AML3</td>
<td>acute myeloid leukemia</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 0.7 μM</td>
<td>48</td>
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<td>Z-138</td>
<td></td>
<td>mantle cell lymphoma</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 1.2 μM</td>
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<td></td>
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<tr>
<td>human primary AML cells</td>
<td></td>
<td>human primary AML cells</td>
<td>acute myeloid leukemia</td>
<td>~10% cell viability after drug treatment at 5 μM for 72 h</td>
<td>48</td>
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<tr>
<td>HEK293 T-REx</td>
<td></td>
<td>HEK293 T-REx CLPP&lt;sup&gt;−/-&lt;/sup&gt;</td>
<td>N/A</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 1 μM</td>
<td>48</td>
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<tr>
<td>SUM159</td>
<td></td>
<td>pleomorphic breast carcinoma</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 0.3 μM</td>
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<td></td>
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<tr>
<td>ONC212</td>
<td>activation</td>
<td>OCI-AML2</td>
<td>acute myeloid leukemia</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 76 nM</td>
<td>48</td>
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<tr>
<td>TEX</td>
<td></td>
<td>OCI-AML3</td>
<td>acute myeloid leukemia</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 71 nM</td>
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<td>Z-138</td>
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<td>mantle cell lymphoma</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 60 nM</td>
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<tr>
<td>human primary AML cells</td>
<td></td>
<td>human primary AML cells</td>
<td>acute myeloid leukemia</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 49 nM; prolonged median survival (49 vs 55 days) of NSG mice engrafted with cells overexpressing HsClpP with drug treatment at 50 mg/kg per day every other day</td>
<td>48</td>
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<td>~6% cell viability after drug treatment at 200 nM for 72 h; significantly prolonged median survival (36 vs 82 days) of NSG mice engrafted with leukemia-initiating cells (LICs) pretreated with 250 nM drug for 72 h</td>
<td>48</td>
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Notably, AV-167 was shown to inhibit the peptidase activity of HsClpP against fluorogenic peptidyl substrates and HsClpP proteolytic activity against model protein substrates when in complex with ClpX from Escherichia coli (EcClpX). AV-167 was found to be equipotent against SaClpP and HsClpP. Hence, structural analogues of AV-167 were synthesized and characterized. Among them, TG42, TG43, and TG53 (Figure 2D) carry an activated ester and a substituent functional group at position 2 of their respective naphthofuran moieties (Figure 2D, indicated by asterisks). Importantly, these analogues were shown to be significantly more potent than AV-167 in the inhibition of HsClpP’s peptidase and proteolytic activities. Furthermore, they displayed improved selectivity for HsClpP, as illustrated by their preferred association with HsClpP present in the lysate of E. coli cells expressing the recombinant form of the protease and inability to inhibit SaClpP’s activity. However, activity-based protein profiling (ABPP) analysis of TG42 in intact Jurkat and Huh7 cells revealed that the compound strongly interacts with endogenously expressed HsClpP as well as many other proteins, which may reflect limited selectivity of TG42 to distinguish HsClpP from other human proteins or the restricted access of the compound into the mitochondrial matrix.

To illustrate their anticancer potential, HuH7 cells were treated with TG42 or TG53 and their effects on cell proliferation and migration were assessed. Notably, drug-treated cells displayed concentration-dependent cell death as early as 42–48 h post-treatment caused by apoptosis (Table 1). Furthermore, HuH7 cell migration was impaired after incubation with either TG42 or TG53 for 16 h (Table 1). Nevertheless, the causal relationship between HsClpP inhibition and the observed phenotypes remains undetermined.

2.2. Activators of HsClpP. Unlike the other major mitochondrial AAA+ proteases (i.e., LonP1, i-AAA, and m-AAA), the ATPase and proteolytic functions of the HsClpXP complex are segregated to HsClpX and HsClpP, respectively. In addition, substrate degradation by HsClpP is dependent on the unfoldase activity of HsClpX under normal circumstances (Figure 3Ai). These characteristics of HsClpXP thus create a unique window for chemical interference via disabling the gatekeeping function of HsClpX. Indeed, small molecules such as activators of ClpP protease (ACPs), acyldepsipeptides (ADEPs), and related compounds have been shown to dysregulate the function of bacterial ClpXP complexes by physically displacing ClpX from ClpP while keeping ClpP in its activated state (Figure 3B–D). Importantly, the dysregulated activation of bacterial ClpP by these compounds induces potent bactericidal effects against various pathogenic microbes, including S. aureus, Streptococcus pneumoniae, Neisseria meningitidis, Neisseria gonorrhoeae, Bacillus subtilis, Enterococcus faecalis, and Listeria innocua, which illustrates the promising potential of these compounds as antibiotics.

Recently, analogues of ADEP that can specifically target HsClpP have been identified. Notably, these analogues have been shown to induce apoptotic cell death in immortalized human cancer cell lines with high potency. Other compounds that dysregulate HsClpP in the same manner have also been identified and characterized and shown to have anticancer properties. These new findings thus pave the

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<tr>
<th>compound</th>
<th>molecular effect on HsClpP</th>
<th>cell line</th>
<th>cell line</th>
<th>disease model</th>
<th>IC₅₀ (μM)</th>
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<td>TR-57</td>
<td>activation</td>
<td>HEK293 T-REX</td>
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<td>CLPP−/− ClpP deletion control for HEK293 T-REX</td>
<td>≤0.1</td>
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<td>HEK293 T-REX</td>
<td>HEK293 T-REX</td>
<td>pleomorphic breast carcinoma</td>
<td>IC₅₀ ~ 0.01</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HEK293 T-REX</td>
<td>HEK293 T-REX</td>
<td>non-malignant</td>
<td>no effect on cell viability below 1 μM drug treatment</td>
<td>48</td>
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way to exploring the potential of pharmacological dysregulation of HsClpP in cancer therapeutics.

2.2.1. Acyldepsipeptides. 2.2.1.1. General Molecular Mechanism of ClpP Dysregulation by ADEPs. The molecular mechanism of ClpP dysregulation by ADEP has been characterized in detail. The first step involves the binding of ADEPs to the hydrophobic pockets between neighboring ClpP subunits that normally accommodate the docking of ClpX’s IGF loops.49 Given that ADEP has a higher affinity for ClpP than does ClpX, ADEP outcompetes the IGF loops of ClpX for binding to ClpP.10 Because the dynamic docking of IGF loops to ClpP’s hydrophobic pockets is crucial in maintaining the ClpX–ClpP interaction, the tight binding of ADEP effectively disrupts the binding of ClpX, leading to the rapid dissociation of the ClpXP complex.10 Furthermore, the binding of ADEP induces structural effects in ClpP that simulates the ClpX–ClpP interaction and forces the protease to maintain its activated state, which is characterized by the structuring of its axial loop50 and a widened axial pore49 that allows unrestricted access of peptides, molten globules, and loops in folded proteins to ClpP’s proteolytic chamber, resulting in their dysregulated degradation.51

2.2.1.2. HsClpP Dysregulation by ADEP Causes Apoptotic Cell Death. In a recent identification and characterization of ADEP analogues showing high specificity for HsClpP, ADEP-28 and ADEP-41 (Figure 3B) were found to activate both the peptidase and protease activities of HsClpP with great potency.45 In addition, the presence of ADEP at low concentrations was shown to induce dissociation of HsClpX.45 Biologically, both ADEP-28 and ADEP-41 induced cytotoxicity in HEK293 T-REx cells that express endogenous HsClpP with submicromolar IC50 values45 (Table 1). Notably, the expression of HsClpP was found to be essential for ADEP-induced cytotoxicity, as illustrated by HEK293 T-REx CLPP−/− cells showing a high tolerance for the drug45 (Table 1). The severity of the cytotoxicity also increased in proportion to the intracellular level of HsClpP,45 which implicates greater ADEP sensitivity as the level of HsClpP expression is increased.

To illustrate the anticancer potential of ADEP, ADEP-41 was shown to induce cytotoxicity in various cell lines of...
cancerous origins, such as HeLa (endocervical carcinoma), U2OS (osteosarcoma), and undifferentiated SH-SY5Y (neuroblastoma), with IC50 values measured in the submicromolar range45 (Table 1). The underlying cell death mechanism was subsequently determined to follow the intrinsic, caspase-dependent apoptotic pathway, as clearly indicated by ADEP-treated cells showing characteristic phenotypes that include an increase in the number of DNA strand breaks, mitochondrial fragmentation, loss of OXPHOS, and activation of caspase-3 as well as caspase-9.45

2.2.1.3. Structural Evidence Points to a Highly Dynamic ADEP–HsClpP Complex. To decipher the molecular mechanism that governs the activation of HsClpP by ADEP, the atomic structure of the protease in complex with ADEP−28 was determined by X-ray crystallography at 2.8 Å resolution.45 Similar to other ADEP–ClpP complexes, ADEP−28 binds noncovalently to a highly complementary hydrophobic pocket that is formed between two neighboring HsClpP subunits45 (Figure 4). Binding of ADEP−28 induces widening of its axial pore (compare the pore size of apo HsClpP in Figure 4Ai and that of ADEP-HsClpP in Figure 4Ai) and the structuring of HsClpP’s axial loops (Figure 4Bi, Ci) that facilitates the unregulated entry and degradation of substrate proteins or peptides.45

An unexpected finding of this study is that the ADEP−28-bound HsClpP tetradecamer adopts a compact conformation, which decreases the height of the ADEP−28–HsClpP cylinder by ~8 Å (compare the apo form shown in Figure 4Bi and the ADEP−28–HsClpP complex shown in Figure 4Bi) and brings the catalytic Ser153 residues from two apposing subunits ~7 Å closer (Figure 4Bi, ii). This compaction is also characterized by the rotation of one ring relative to another (Figure 4Bi, ii; indicated with the tilted green dotted line). While previously determined ADEP–ClpP structures adopting the extended conformation have led to the hypothesis that ADEP binding constrains ClpP in its active, extended conformation,52 the structure of the ADEP−28–HsClpP complex in the compact conformation provides the first structural basis

Figure 4. HsClpP is activated by different compounds via a common molecular mechanism. (A) Top views of the tetradecamer of (i) apo HsClpP, (ii) ADEP−28-bound HsClpP [Protein Data Bank (PDB) entry 6BBA],45 (iii) the D9-bound HsClpP Y118A mutant (PDB entry 6H23),46 and (iv) ONC201-bound HsClpP (PDB entry 6DL7).48 The bound activator molecules are shown as colored spheres. (B) Side views of the tetradecamer of (i) apo HsClpP, (ii) ADEP−28-bound HsClpP, (iii) the D9-bound HsClpP Y118A mutant, and (iv) ONC201-bound HsClpP. HsClpP is colored gray, and Ser153 residues are colored blue (top ring) or red (bottom ring). The bound activator compounds are shown as colored sticks. For each structure, both the distance between the Ser153 residues from two apposing subunits and the height of the HsClpP tetradecamer are shown. For ADEP−28-bound HsClpP, the axial loops (indicated with asterisks in panel ii) were excluded in determining its height, given that they are unresolved in all of the other structures. For all three activator-bound HsClpPs, the turning of the top HsClpP heptameric ring relative to the bottom one is shown via the tilted dotted green line linking the two Ser153 residues, in contrast to the vertical dotted green line shown in apo HsClpP. (C) Structure and orientation of (i) ADEP−28, (ii) D9, and (iii) ONC201 when bound in the hydrophobic binding pocket at the interface of two neighboring HsClpP subunits. The activators are shown as sticks with colors distinguishing the different elements. The two HsClpP subunits are represented in ribbon format with a surface contour. The axial loop in ADEP−28-bound HsClpP is illustrated. For D9-bound HsClpP Y118A and ONC201-bound HsClpP, the expected position of the unresolved axial loop is indicated by a dotted circle. For D9, the two possible orientations of the bound activator are distinguished by the red- and pink-colored molecules. (D) Overlap of ADEP−28-, D9-, and ONC201-bound HsClpP tetradecamers in the hydrophobic pocket of HsClpP.
demonstrating that the ADEP–ClpP complex is in fact structurally dynamic and is able to sample through multiple conformational states over its catalytic cycle.45

2.2.2. Compound D9. In addition to ADEP, other structurally distinct compounds have been found to induce dysregulated activation of HsClpP. In a high-throughput screen to identify inhibitors of SaClpP, 70 compounds of ∼140000 were identified as being able to activate the protease’s activity against fluorogenic peptides.46 The SaClpP-activating compounds were further investigated for their abilities to activate ClpP from other species, of which compound D9 (Figure 3C) was the only candidate able to activate HsClpP.46 Importantly, D9 showed species specificity as it preferentially activates the proteolytic activity of HsClpP while having minimal effects on ClpP of bacterial origins.46

2.2.2.1. D9 Shares the Same HsClpP Activation Mechanism as ADEP. Biochemical characterization of the D9–HsClpP interaction and activation of the protease revealed that, similar to the case for ADEP, binding of D9 induces stabilization of HsClpP’s active oligomeric state.46 Furthermore, D9 exhibited a high affinity for HsClpP that is illustrated by the compound’s effective displacement of EcClpX from preformed EcClpX–HsClpP complexes that are catalytically active.46

With regard to structural characterization of the D9–HsClpP complex, while the compound failed to co-crystallize with wild-type (WT) HsClpP, co-crystals of D9 and the Y118A proteolytically activated mutant of HsClpP were obtained and the structure of the D9–HsClpP Y118A complex was determined at 3.2 Å resolution.46 Similar to the case for ADEP, the noncovalent binding of D9 induces HsClpP activation by widening of the protease’s axial pore (Figure 4Aii), although no structuring of the axial loops was observed (Figure 4Bii,Cii).46 Furthermore, the D9–HsClpP complex exhibited the same compact conformation (Figure 4Bii) as observed in the ADEP-28–HsClpP complex (Figure 4Bi), thus reaffirming the hypothesis that the drug-bound activated state of ClpP is structurally dynamic.46,47

Notably, D9 binds to the identical hydrophobic pockets formed between neighboring HsClpP subunits as ADEP, with two plausible but similar conformations (Figure 4Ci; different D9 conformations shown in different colors).46 Similar to the case for ADEP, the D9–HsClpP interaction is highly complementary; such structural modifications to the two terminal moieties of D9 almost invariably decrease the compound’s potency.46

The biological effect of D9’s interaction with HsClpP has not been reported.46 If D9 is specific to only HsClpP, it is expected to induce apoptotic cell death in a manner similar to that of ADEP, given their identical mechanism in HsClpP activation.

2.2.3. Imipridones. The imipridones constitute a third class of structurally distinct HsClpP-activating compounds that have been characterized to date. These include ONC201 and its derivatives ONC212 and the TR compounds (Figure 3D). ONC201 was initially identified in an in vitro screen of 747 compounds that have been approved for clinical use or trials in treating malignant and nonmalignant tumors.48

2.2.3.1. Imipridones Dysregulate HsClpP via the Same Mechanism as ADEP and D9. Similar to ADEP and D9, imipridones activate HsClpP’s activity in a dose-dependent manner against model peptidyl or protein substrates.46,47 Direct interaction of ONC201 with HsClpP was shown via isothermal titration calorimetry (ITC) with purified proteins and using a cellular thermal shift assay (CESTA) in cells.48 Affinity chromatography using immobilized drug molecules showed that the TR compounds have greater affinity for HsClpP than ONC201.47

The structure of the ONC201–HsClpP complex was determined at 2 Å resolution.48 Similar to ADEP and D9, ONC201 binds noncovalently to the highly complementary hydrophobic pockets formed between neighboring HsClpP subunits (Figure 4Aiv).48 Notably, binding of ONC201 induces widening of HsClpP’s axial pore (Figure 4Aiv), while the complex adopts a compact conformation (Figure 4Biv) that highly resembles that of ADEP and D9 binding.46 As with D9, the axial loops in the ONC201–HsClpP complex are not resolved in the crystal structure (Figure 4Biv,Cii).

Structural comparison of ADEP, D9, and ONC201 in complex with HsClpP reveals that all three activators share considerable overlap when docked at HsClpP’s binding pocket. As shown in Figure 4D, the two opposing hydrophobic elements of the activators (i.e., the difluorophenyl moiety and the hydrophobic tail of ADEP-28; the two polar ring-bearing moieties of D9 and ONC201) are buried in HsClpP, while the main part of the molecule (i.e., the cyclic peptidyl ring of ADEP-28; the mid section of D9 and ONC201) rests horizontally on or close to HsClpP’s surface.

2.2.3.2. Biological Effects of HsClpP Dysregulation by Imipridones. The biological effects of imipridones were studied in detail at the cellular and organismal levels. Both ONC201 and ONC212 were shown to be cytotoxic to both leukemic and lymphoma cell lines as well as primary acute myeloid leukemic (AML) cells but have minimal adverse effects on normal bone marrow mononuclear cells (BM-NMC)48 (Table 1). Likewise, the TR compounds were found to be highly cytotoxic against the triple negative breast cancer (TNBC) cells SUM153.47 (Table 1). With aDDEP, the cytotoxicity of imipridones is ClpP-dependent and is illustrated by the CLPP/−/− null mutation conferring resistance to both ONC201 and ONC212 in HEK293 T-REx cells48 (Table 1). Similarly, HsClpP knockdown (KD) produces a similar resistance to ONC201 and TR-57 in SUM153 cells.47 Furthermore, primary AML cells expressing high levels of HsClpP are more sensitive to ONC20148 (Table 1), which further validates the role of HsClpP in facilitating the cytotoxic effects of the drugs.

The biomolecular basis for the HsClpP-dependent cytotoxicity of imipridones is proposed to be rooted in OXPHOS impairment associated with specific respiratory chain subunits. First, identification and profiling of HsClpP-interacting proteins by BioID revealed that treating cells with ONC201 significantly reduced the level of expression of subunits of Complex I, Complex II, and Complex IV, resulting in a decrease in both basal oxygen consumption rate (OCR) and spare reserve capacity (i.e., difference between basal and maximal OCR), while mitochondrial ROS production was increased.46 Furthermore, structural damage to both the mitochondrial cristae and the matrix was observed in drug-treated cells.46 The imipridone-induced mitochondrial stress may also activate the integrated stress response (ISR) and subsequent cellular events, as evidenced by drug-treated cells showing an increased level of expression of the ISR-activating ATF4 (activating transcription factor 4) and CHOP and the downregulation of TFAM and TUFM (elongation factor Tu,
mitochondrial), thereby inhibiting de novo mitochondrial protein biosynthesis.\textsuperscript{47}

2.2.3.3. Imipridones Show ClpP-Dependent Anticancer Properties. Evidence in support of the anticancer properties of imipridones in animal models has been published. As illustrated via a mouse model of xenografted luciferase-labeled Z-138 cells, orally administered ONC212 reduced significantly the tumor burden of mice grafted with Z-138 cells over-expressing HsClpP, while the drug had no effect on cells overexpressing an inactive HsClpP D190A mutant\textsuperscript{48} (Table 1). Furthermore,\textit{ex vivo} ONC212 treatment of leukemia-initiating cells (LiCs) derived from primary AML cells prior to their engraftment to NOD-SCID gamma (NSG) mice effectively inhibited their engraftment capacity and prolonged considerably the survival of recipient mice (36 vs 82 days of median survival)\textsuperscript{48} (Table 1).

3. CLOSING REMARKS

Recent research efforts in the chemical modulation of HsClpP’s proteolytic activity have led to the discovery of multiple classes of compounds that can target the protease with various degree of selectivity. Many of these compounds are shown to induce apoptotic cell death in cancer-derived cell lines and/or primary cells, which highlights their potential use in anticancer therapeutics. Notably, while the pharmacological inhibition of HsClpP may prove to be a sufficient strategy against cancer cells that rely heavily on the protease for survival and proliferation, e.g., prostate cancer\textsuperscript{17} and AML,\textsuperscript{28} it may fall short for cancer cells that are less dependent on HsClpP, e.g., breast cancer.\textsuperscript{17}

Compounds such as ADEP, D9, and imipridones activate the HsClpP’s proteolytic activity via a common mechanism of mimicking the biomolecular effects of HsClpX association in maintaining HsClpP in its active state while preventing its interaction with HsClpX and thereby negating the AAA+ unfoldase’s regulatory role. This dysregulation of HsClpP in turn induces apoptotic cell death in a variety of cancer cells. A major advantage of this approach is that cell death can be induced as long as HsClpP is expressed, regardless of the cell’s requirement of the protease for viability. Furthermore, the cytotoxicity of both ADEP and the imipridones increases in proportion to the cellular HsClpP level, which confers a certain degree of cellular selectivity in targeting cancer cells, many of which have upregulated expression of HsClpP.

Overall, while the pharmacological dysregulation of HsClpP shows great promise as a novel anticancer therapeutic approach, research is ongoing to optimize these drugs with respect to both their targeting specificity for HsClpP and their selectivity for cancer cells without damaging normal healthy ones, while maintaining other characteristics of the drugs that are crucial for their delivery to the targeted cells such as chemical stability, solubility, and cell permeability. Experimental efforts in this regard have often yielded mixed results, such that typically, chemical modifications may improve certain characteristics (e.g., drug potency) while diminishing others (e.g., aqueous solubility). Alternatively, efforts can be directed at designing new drug formulation, such that optimization of compounds can be focused on refining the characteristics related to potency (e.g., target specificity and affinity), while issues related to its compatibility with physiological environments (e.g., aqueous solubility and delivery) can be solved through proper formulation.

# REFERENCES


