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COMMUNICATION

Selective Activation of Human Caseinolytic Protease P (ClpP)

Matthias Stahl,[1,2] Vadim S. Korotkov,[1] Dóra Balogh,[1] Leonhard M. Kick,[1] Malte Gersch,[1,3] Axel Pahl,[1,4] Pavel Kielkowski,[1] Klaus Richter,[1] Sabine Schneider,*[,1] and Stephan A. Sieber*[,1]

Abstract: *Caseinolytic protease P (ClpP) is the proteolytic component of the ClpXP protein degradation complex. Eukaryotic ClpP was recently found to act as a sensor in the mitochondria-specific unfolded protein response (UPR^{mt}). However, its detailed function and dedicated regulation remain largely unexplored. Here we present a small molecule (**D9**) that acts as a potent and species-selective activator of human ClpP (hClpP) by mimicking the natural chaperone ClpX. Structure-activity relationship studies highlight the importance of a halogenated benzyl motif within **D9** that interacts with a unique aromatic amino acid network in hClpP. Mutational and structural studies suggest that this YYW motif tightly controls hClpP activity and regulates substrate turnover by interaction with cognate ligands. Moreover, this signature motif is unique to ClpP from higher organisms and does not exist in bacterial homologs allowing a species-selective analysis. Thus, **D9** represents a versatile tool to analyze mechanistic features of hClpP.*

Mitochondria are sophisticated energy-production compartments in many eukaryotic cells which rely on an accurate stress sensing system termed mitochondrial unfolded protein response (UPR^{mt}).[1] Caseinolytic protease P (ClpP) was recently linked to the induction of UPR^{mt} in mitochondria of *Caenorhabditis elegans* through the release of ClpP product peptides evoking a nuclear, transcriptional response switching on an organelle-specific heat-shock program.[1,2] However, it is currently unclear, if these findings also apply to human cells and customized chemical-biology approaches are needed in order to analyze the function of human ClpP (hClpP) more precisely.[3]

hClpP assembles into heptameric rings bearing hydrophobic interaction patches which line up the apical pores and enable binding to the hClpX chaperone via its tailored LGF loops.[4,5] The chaperone is responsible for substrate recognition, unfolding and translocation.[5] hClpX binding stabilizes a tetradecameric state of hClpP, which is otherwise found to form inactive heptamers *in vitro*.[6] Different conformational intermediates are also observed for bacterial ClpPs which either exist in an active extended, inactive compact or inactive compressed conformation.[7-9] Key features of these states are the orientation of a central E-helix which forms crucial contacts across the heptamer-heptamer

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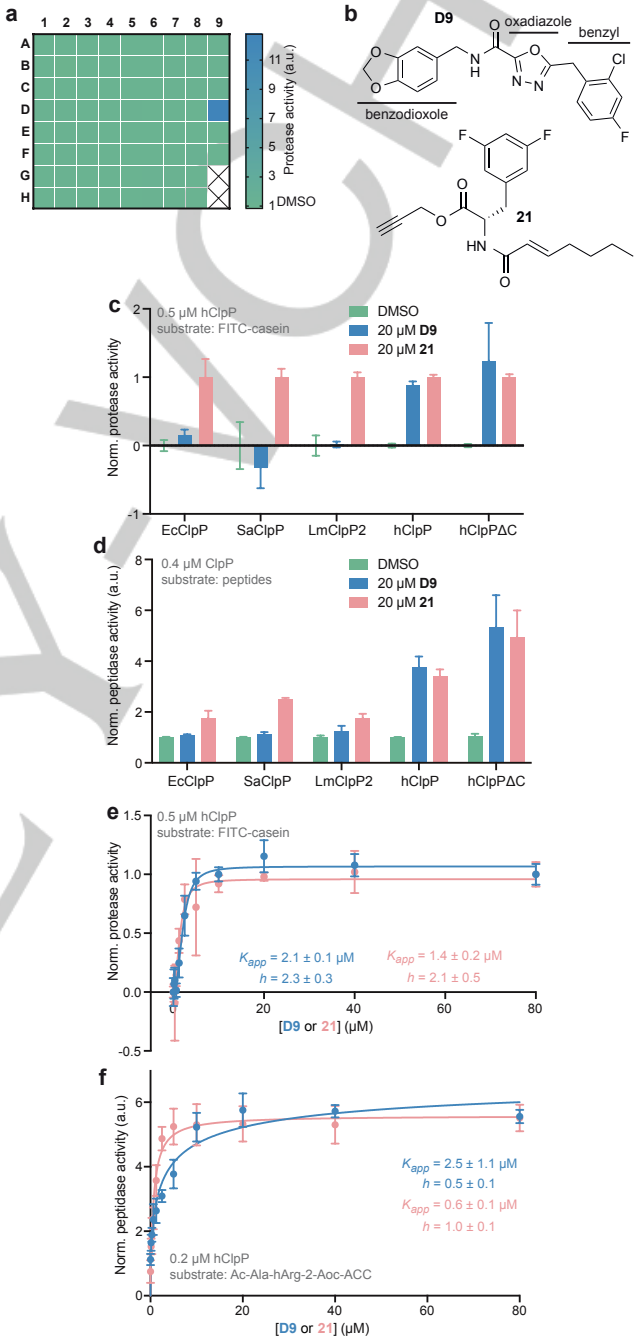

Figure 1: Discovery of **D9**. (a) Screen for proteolysis activity of hClpP with a set of 70 different putative small molecule activators. See also Figure S1. (b) Structures of **D9** and the previously introduced ClpP activator **21**. (c) Protease activities of ClpPs from *E. coli* (EcClpP), *S. aureus* (SaClpP), *L. monocytogenes* (LmClpP2), *H. sapiens* (hClpP), and the C-terminally truncated variant hClpPΔC. Degradation of the model substrate FITC-casein was recorded in the presence of 20 µM **D9**, **21**, or DMSO. DMSO replicate values were set to 0 and **21**-mediated activation was set to 1 for each species (n = 6). Shown are mean values and the standard deviation. (d) Same experimental setup as in c, but with fluorogenic peptides as substrates. Each experiment was normalized to the mean of the DMSO control (DMSO = 1; n = 6). Shown are mean values and the standard deviation. (e) Protease activity of hClpP for different concentrations of **D9**

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and **21**. Fit lines arise from allosteric sigmoidal curve fits. Apparent dissociation constants (K_{app}) and Hill parameters (h) are given. Activity at 80 µM compound was set to 1 and the DMSO control fixed to 0 (n = 6). Shown are mean values and the standard deviation. (f) Same analysis as in e but probing the peptidolytic activity of hClpP with a peptide as substrate. Shown are mean values normalized to the DMSO control and the standard deviation (DMSO = 1, n = 6, for DMSO control of **21**: n = 3).

interface in the extended tetradecameric state and is directly linked to the active site. In compressed or compact forms this helix kinks and thereby misaligns the catalytic triad. Binding of ClpX to the hydrophobic pockets results in conformational selection of the active, extended complex, however, the exact interplay between chaperone binding and the catalytic hotspots remains to be elucidated. [10,11] In addition, also small molecules were shown to exert conformational control. Acyldepsipeptides (ADEPs) enlarge ClpP's entry pores and trigger proteolytic activity by binding into the hydrophobic pockets.[12,13] A different class of molecules, activators of self-compartmentalizing proteases (ACPs), also address the same pockets and thereby efficiently activate ClpP from *Escherichia coli* (EcClpP).[14]

In recent years, a multitude of naturally occurring hClpP mutations were detected, mainly in cancer tissue samples[15] and in patients suffering from Perrault syndrome.[16-18] Dissecting the molecular details of hClpP function, i.e. conformational activation, will lead to a better understanding of mitochondrial protein homeostasis and disease mechanisms.

In an effort to identify chemical biology tools that would facilitate further studies, we looked for new small-molecule activators of hClpP via a two-tiered strategy: First, we investigated data from a recent high-throughput screen (HTS) with about 140,000 compounds originally designed to identify inhibitors of SaClpP peptidase activity.[19] As turnover of a peptidic fluorogenic substrate was recorded, we drew our attention to molecules that enhanced fluorescence from which we selected 70 promising compounds (see Supporting Information for criteria and details of the selection process). To dissect whether species-selective activators were among these hits, the compounds were profiled in proteolysis assays against a panel of ClpPs originating from *E. coli* (EcClpP), *Listeria monocytogenes* (LmClpP2), *H. sapiens* (hClpP), and *S. aureus* (SaClpP). In this assay, activation of ClpP for digestion of the fluorescently labeled protein substrate FITC-casein was measured (Figure 1a and Figure S1). Only one out of 70 compounds (**D9**, Figure 1b, c) showed significant stimulation of hClpP turnover, whereas the activity of no other ClpP variant was enhanced by any of the molecules tested (of note, repetition of peptidolytic SaClpP stimulation also failed suggesting a serendipitous discovery of **D9**). Similar results, i.e. a selective activation of hClpP, were also obtained in peptidase assays with an improved generation of ClpP substrates (Figure 1d).[20] Deletion of the hClpP C-terminal extension (hClpPΔC) retained activation. Remarkably, the selectivity of **D9** for human ClpP is reflected by its unique structural composition deviating from previously reported bacterial activators including ADEPs, ACPs, and ADEP fragments (e.g. **21**, Figure 1b).[14,21,22]

With a selective hClpP activator at hand, we more closely analyzed **D9** in dose-down protease and peptidase assays (Figure 1e, f). **D9** enhanced both proteolysis and peptidolysis in a concentration-dependent manner comparable to the ADEP fragment **21**.

Chaperones and many previously known small molecule activators address the same hydrophobic pockets at the apical site of the ClpP cylinder to exert conformational control over ClpP.[23] To investigate whether **D9** falls into the same category, we used analytical ultracentrifugation to monitor the oligomeric state of hClpP at various concentrations of **D9** (Figure 2a). As expected, in the absence of compound hClpP exclusively formed heptamers.[6] Minute amounts of **D9** (i.e. 0.18 µM to 1.07 µM $hClpP_7$) were already sufficient to induce a shift of the equilibrium towards the tetradecameric species (Figure 2b and Figure S2a,

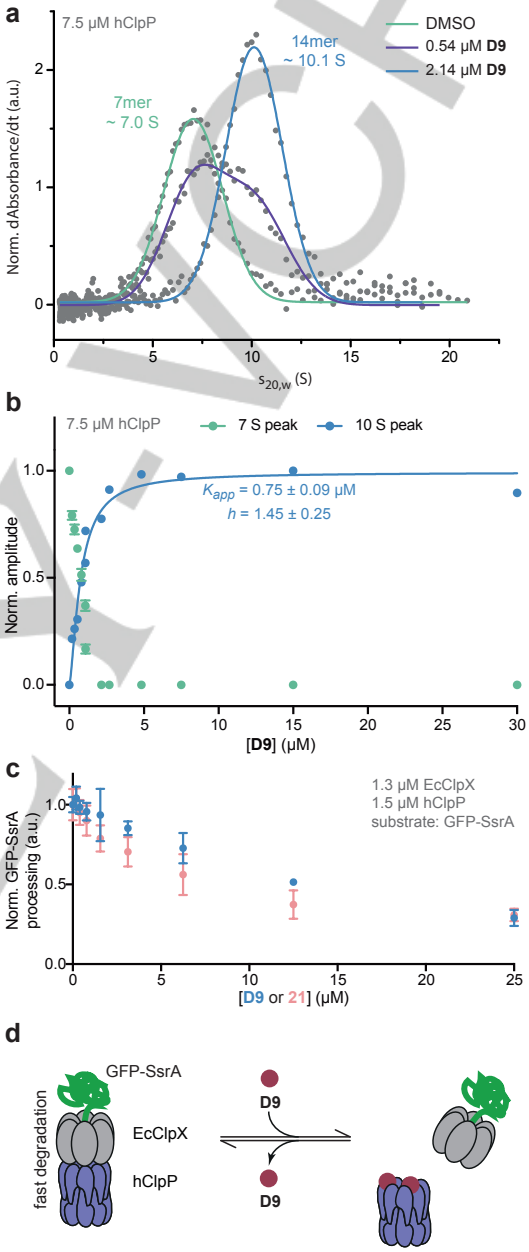

Figure 2: Macromolecular effects of **D9** on hClpP. (a) Density of sedimentation coefficients ($s_{20,w}$) from analytical ultracentrifugation runs with **D9** and hClpP. Each experiment was performed with a different ratio of compound to protein. Fit curves are based on a bigaussian model. 7 S peak: hClpP 7mer, 10 S peak: hClpP 14mer (n = 1 for each concentration, refer to the Supporting Information for the complete dataset consisting of 13 **D9** concentrations, Figure S2a, b). (b) Amplitudes of bigaussian fitting from panel a were normalized to a range between 0 and 1 and plotted against **D9** concentration. Increasing abundance of the 10 S peak was fitted with an allosteric sigmoidal model. (n = 1 for each concentration, see panel a, error bars represent standard errors from curve fitting) (c) Processing rates of GFP-SsrA by a complex of EcClpX and hClpP at different concentrations of **D9** and **21** (normalized to DMSO). Controls with single enzymes can be found in Figure S2c. Shown are mean values, error

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bars represent the standard deviations (n = 6). (d) Proposed model mechanism of **D9**-mediated deregulation of a ClpXP complex.

b). This transition fits a sigmoidal Hill model with an apparent dissociation constant K_{app} of 0.75 ± 0.09 μM and h = 1.45 ± 0.25 ($hClpP_1$ concentration: 7.5 μM) suggesting a highly sensitive binding site that exhibits conformational control over complex assembly. An obvious candidate for such a binding site are the hydrophobic pockets lining ClpP's pores and needed for coupling

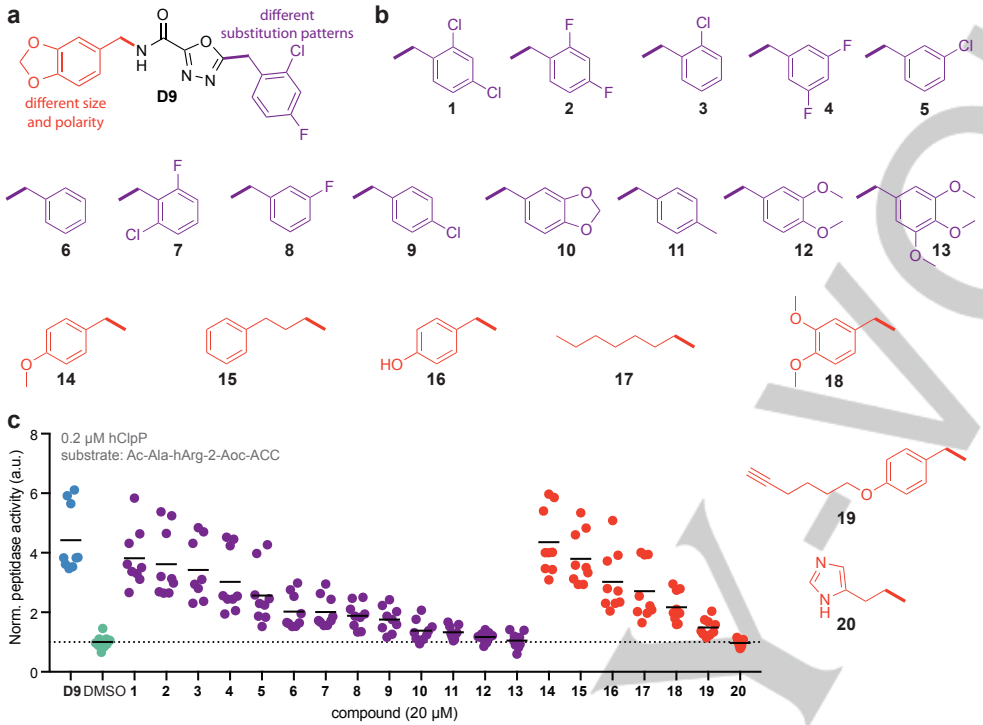

Figure 3: Structure-activity relationships with **D9** derivatives. (a) Base structure of **D9**. (b) The benzodioxole (red) and benzyl (purple) moieties were modified. (c) All compounds were subjected to peptidase activity tests with hClpP (normalized to DMSO = 1, n = 9, n = 8 for compound **3**). Black lines denote means.

to LGF loops of ClpX.[24] To clarify whether **D9**, like other activators, addresses these binding sites, we reconstituted the whole proteolytic system. ClpXP proteolytic activity is usually determined by the digest of green fluorescent protein (GFP) tagged with a short SsrA recognition sequence.[4] However, as hClpX is incapable of detecting and processing SsrA tags, we utilized ClpX from *E. coli* (EcClpX), which is known to bind hClpP and form proteolytic active complexes with GFP-SsrA.[4] ClpX unfolds and translocates tagged substrates to ClpP with a processing rate depending on ClpP binding.[25] As expected, the reconstituted ClpXP system exhibited profound GFP-SsrA degradation rates (Figure 2c and Figure S2c). However, upon addition of increasing concentrations of **D9**, we observed decreasing processing rates indicating a displacement of EcClpX from hClpP. The assumption is supported by similar results which we gained by using ADEP fragment **21** (Figure 2c and Figure S2c). Taken together, these results point towards a **D9** mediated conformational control of hClpP oligomerization through binding to hClpP's hydrophobic pockets (Figure 2d).

To investigate the structure-activity relationship (SAR) of **D9**, we obtained two sets of in total 20 custom synthesized or commercially available derivatives (Figure 3a, b). The first set (**1**-**13**) comprises variations of the benzyl substitution pattern of **D9**. Exchange of fluorine by chlorine or vice versa yielded homosubstituted analogs of **D9** (**1** and **2**) which retained a slightly reduced peptidase activation potential (Figure 3c). However, switching the two fluorine substituents to a 3,5 position (**4**), e.g. as observed in **ADEP2**, decreased activity further. A 2,6-configuration of chlorine and fluorine (**7**) showed almost no activity. Derivatives with only one halogen substituent at various positions (**3, 5, 8, 9**) showed reduced activation potential. An unsubstituted benzyl group (**6**) as well as a benzyl group bearing electron-donating methoxy (**12**, **13**), methylenedioxy (**10**) and methyl (**11**) groups showed no activation effect. These results indicate that halogen substitutions at the benzyl moiety of **D9** are essentially required to boost hClpP activity. As it was previously reported that simplified variants of ADEPs retain their activation potential as long as they keep the phenylalanine recognition motif,[22] we probed the benzodioxole part of **D9** for tolerating structural modifications in a second set of analogs (**14**-**20**). For example, a change to a methoxy-substituted benzyl (**14**) and extension of the carbon linker (**15**) were tolerated, however, introduction of hydroxyl (**16**), alkyl (**17**), dimethoxy (**18**), alkyne (**19**), or imidazole (**20**) groups either reduced or abolished activity suggesting that both parts of **D9** underlie constraints for stimulating hClpP peptidolysis.

We next sought to structurally investigate the unprecedented selectivity of **D9** for hClpP. Co-crystallization of wild-type hClpP with **D9** was unsuccessful. However, a recently reported gain-of-function mutation in SaClpP (Y63A) attracted our attention due to a higher tetradecamer stability and corresponding chance for forming crystals.[11] We cloned and purified the respective hClpP Y118A mutant which showed enhanced peptidase activity in absence of **D9** and even higher activity in presence of **D9** suggesting that this activation mutation can be transferred to the human ortholog (Figure S7). The mutant enzyme was crystallized in complex with the compound and its structure determined to 3.2 Å resolution (Figure 4). **D9** is localized in the hydrophobic pocket between two monomers on the apical sides of the hClpP barrel, crucial for the interaction with hClpX, which confirms **D9** as

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a novel chaperone mimic (Figure 4a, b and Figure S3a). In comparison with wild-type hClpP (PDB code 1TG6[26]) the **D9**-bound enzyme adopts a compact conformation as known e.g. for SaClpP as well as a recent structure of hClpP co-crystallized with an ADEP analog (Figure S3b, S4).[8,27] The height of the hClpP tetradecamer barrel is reduced by ca. 10 Å compared to the wild-type caused by a shortened E-helix at the interface between the two heptameric rings (Figure 4c). Further characteristics of this structure are a widened pore, a misaligned catalytic triad as well as a flipped Q179 residue which blocks the S1 pocket and simultaneously coordinates the active site serine (Figure 4a, d, and Figure S3e). This structural composition with an activator bound to an inactive complex, also observed in a recent ADEP structure,[27] is surprising and may reflect a snapshot of a proteolytic cycle sampling different conformational states. A closer inspection of the binding pocket revealed that, in line with SAR data, **D9** requires both the halogenated benzyl as well as the benzodioxol part for binding. Albeit **D9** can be modelled into the electron density in two alternate conformations (Figure 4b), the benzyl moiety slots into the same hydrophobic pocket as exploited by the phenylalanine moiety of ADEPs (PDB codes 6BBA,[27] 3KTI,[28] and 5VZ2 (unpublished)) and also the benzodioxole extends into a nearby groove known to accommodate hydrophobic tails (Figure 4b, e, and Figure S3a). Interestingly, the hydrophobic pocket of wild-type hClpP is lined up by a characteristic set of aromatic residues consisting of tyrosine 118, tyrosine 138, and tryptophan 146 (YYW motif). These aromatic residues are in position for π-stacking to coordinate the substituted phenyl ring. While aromatic residues at positions 118 and 138 are also present in bacterial orthologs, tryptophan 146 is a residue characteristic for hClpP which likely serves as a selectivity filter (Figure S3c, d, and Figure S6). In comparison to the **ADEP** structure,[27] the benzyl ring of **D9** is slightly shifted most likely due to the Y118A mutation which enlarges the pocket size (Figure 4e). To gain further insights via biochemical experiments, a set of hClpP point mutants located at the binding site was generated. All mutants were subject to peptidase assays in absence and presence of **D9** (Figure S7). Interestingly, double mutants of the YYW motif that either mimic the corresponding *E. coli* (Y138F, W146I) or *S. aureus* (Y138H, W146I) sites show lower maximum activities when treated with **D9** supporting this region as an important switch for species selectivity. The experimentally obtained binding mode of **D9** was further verified by docking.[29] After validation of the docking method by redocking **ADEP2** in a BsClpP co-crystal structure based on PDB code 3KTJ, **D9** was docked to the wild-type apo-hClpP crystal structure (Figure S5a-d). The highest ranked docking solution showed that in agreement with crystallographic data the **D9** benzyl ring is localized in a deep hydrophobic cavity at the interface of two monomers. Of note, all three residues including Y118, which is missing in the co-crystal structure, contribute to binding in the wild-type enzyme (Figure S5e).

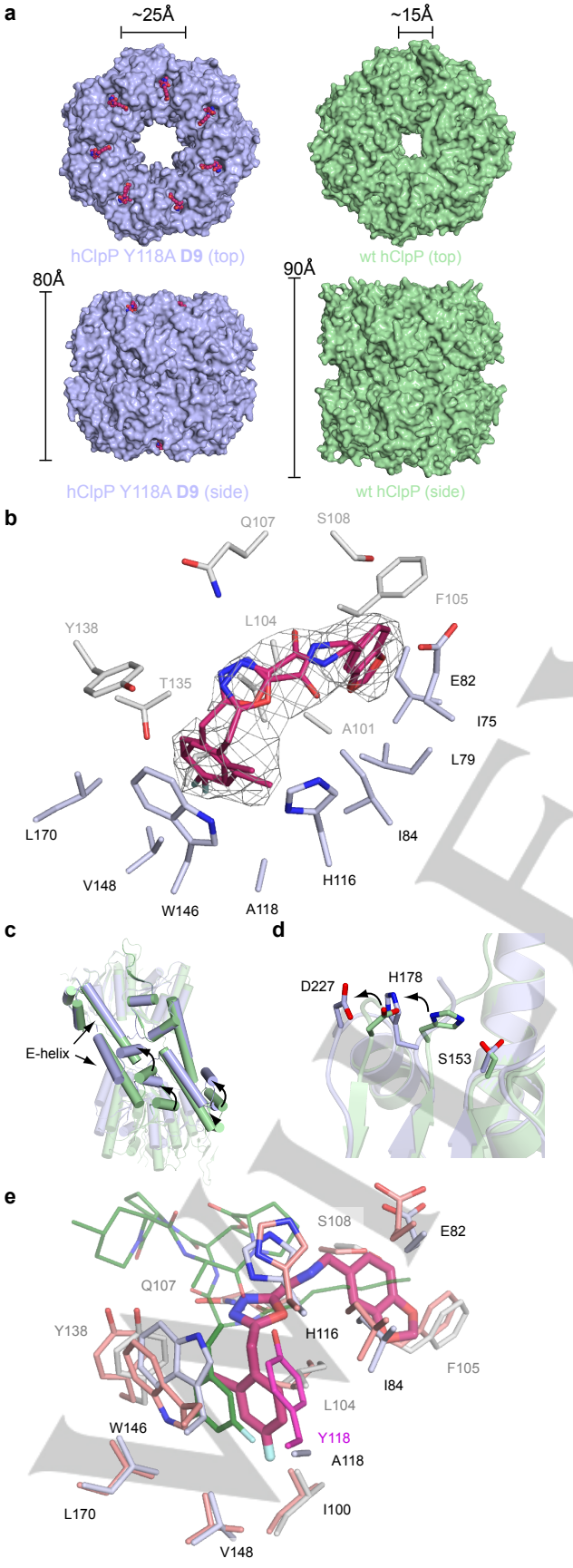

Figure 4: Crystal structure of hClpP Y118A in complex with **D9**. (a) Surface representation of the hClpP Y118A tetradecamer (light blue) with the bound **D9** molecules around the pore shown as space-fill model (red). Top and side view of the hClpP Y118A-**D9** complex and wild-type hClpP (PDB code 1TG6) highlighting the differences in their overall dimensions. (b) Simulated-annealing omit F_o-DF_c electron density map of **D9** contoured at 2.5 σ and surrounding residues. The two modelled alternative conformations of **D9** (red) and residues of the different protein monomers building the binding cleft (grey and light-blue) are shown as stick model. (c) Superposition and ribbon representation of the monomeric structures of wild-type hClpP (green) and hClpP Y118A-**D9** complex (grey). The shortening of the E-helix results in structural compaction of the tetradecamer at the ring-interface (arrows). (d) Overlay of the active sites from wild-type (green) and Y118A-**D9** hClpP showing the misalignment in the mutant structure. (e) Superposition of wild-type hClpP-**ADEP28** (light pink, PDB code 6BBA) and hClpP Y118A-**D9** (grey) complexes. **ADEP28** and **D9** are shown as

green and red stick models, respectively. For clarity only one conformation of **D9** is displayed. Y118 of wild-type hClpP is highlighted in pink.

In conclusion, we identified **D9** as a potent activator for hClpP which, in sharp contrast to previous stimulators, is inactive on bacterial homologs. In a combination of biochemical, SAR, crystallography and docking studies, we unraveled the **D9** binding pocket which is lined up by an aromatic core structural motif. Further evidence such as competitive binding with the chaperone, induction of an oligomeric shift and pore widening confirm **D9** similar to ADEPs as a mimic of ClpX. The unique YYW network within the binding pocket is an important feature that controls hClpP proteolytic activity as well as **D9** binding, presumably via π-stacking interactions triggered by electronic effects of crucial electron-withdrawing halogen substituents. Ligands binding to this motif can modulate the degree of activity and for example induce a stable formation of the active 14mer. Remarkably, this is already induced by binding of one molecule to the heptamer suggesting a native protective mechanism: hClpP is only activated upon a tight and close binding of ClpX. **D9** is synthetically easily accessible and as a simple mimic of ClpX function can thus be used as a chemical tool to dissect ClpX-driven global conformational changes. Especially in the context of the growing importance of the mitochondrial degradome regarding to aging and disease, we here provide a valuable tool for further in depth studies.[30]

Keywords: Human ClpP • Proteolysis • Activation • Crystallography

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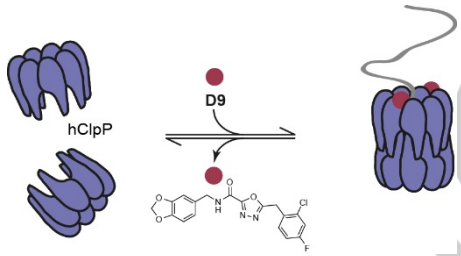
Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

A species-selective small molecule activator (**D9**) of human ClpP was identified. Crystallography and docking studies revealed binding into a tailored aromatic pocket characteristic for ClpPs of higher organisms.



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Selective Activation of Human Caseinolytic Protease P (ClpP)