8 Recent Advances in Targeting Human Mitochondrial AAA+ Proteases to Develop Novel Cancer Therapeutics

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Abstract

The mitochondrion is a vital organelle that performs diverse cellular functions. In this regard, the cell has evolved various mechanisms dedicated to the maintenance of the mitochondrial proteome. Among them, AAA+ ATPase-associated proteases (AAA+ proteases) such as the Lon protease (LonP1), ClpXP complex, and the membrane-bound i-AAA, m-AAA and paraplegin facilitate the clearance of misfolded mitochondrial proteins to prevent the accumulation of cytotoxic protein aggregates. Furthermore, these proteases have additional regulatory functions in multiple biological processes that include amino acid metabolism, mitochondria DNA transcription, metabolite and cofactor biosynthesis, maturation and turnover of specific respiratory and metabolic proteins, and modulation of apoptosis, among others. In cancer cells, the increase in intracellular ROS levels promotes tumorigenic phenotypes and increases the frequency of protein oxidation and misfolding, which is compensated by the increased expression of specific AAA+ proteases as part of the adaptation mechanism. The targeting of AAA+ proteases has led to the discovery and development of novel anticancer compounds. Here, we provide an overview of the molecular characteristics and functions of the major mitochondrial AAA+ proteases and summarize recent research efforts in the development of compounds that target these proteases.

Keywords

Human mitochondria · Mitochondrial AAA+ proteases · Chemical modulators of AAA+ protease activity · Cancer therapeutics

8.1 Introduction

The mitochondrion is a vital organelle in the human cell with diverse biological functions such as energy metabolism, the biosynthesis of ATP and other important metabolites, signalling, cellular chemotaxis, development, and stress responses such as autophagy and apoptosis [73]. In this regard, the cell has evolved multiple mechanisms dedicated to maintaining mitochondrial health and function. These include the

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expression of various stress response proteins, the controlled biosynthesis and import of mitochondrial proteins, the assisted folding of these proteins and degradation of misfolded ones, the isolation of damaged mitochondria by modulating mitochondrial fission-fusion dynamics and their clearance from the cell via mitophagy [59].

The assisted folding of mitochondrial proteins and degradation of misfolded ones are mediated by specialized molecular chaperones and proteases that can be found in different mitochondrial compartments [59]. Among them, the ATPases Associated with diverse cellular Activities (AAA+) ATPase-coupled proteases (AAA+ proteases) mediate the active remodelling, unfolding and degradation of mitochondrial proteins using energy derived from ATP hydrolysis. Notable members of this class include the mitochondrial Lon protease (LonP1) and ClpXP complex that are found in the mitochondrial matrix, and the integral membrane proteases i-AAA, m-AAA and paraplegin that are localized to the inner mitochondrial membrane [59].

AAA+ proteases are important in facilitating the clearance of misfolded proteins from the mitochondria [59]. Accordingly, mutations in the AAA+ proteases that hinder or abolish their activities are associated with various developmental, neurosensory and metabolic disorders. These include CODAS syndrome (CODASS) due to mutations in LonP1 [27], Perrault syndrome 3 (PRLTS3) caused by mutations in ClpP [46], erythropoietic protoporphyria (EPP) that is caused by mutations in ClpX [101], optic atrophy 11 (OPA11) arising from mutations in i-AAA [43], spinocerebellar ataxia 28 (SCA28) [25] caused by mutations in m-AAA, and hereditary spastic paraplegia 7 (HSP7) due to mutations in paraplegin [75].

In addition to the aforementioned genetic diseases, AAA+ proteases are also important for the proliferation and metastasis of various human cancers. Notably, the increase in intracellular ROS level is commonly observed among cancer cells, as it promotes tumorigenesis and facilitates additional mutations leading to metastasis [81]. Consequently, a higher ROS level increases the frequency of mitochondrial protein oxidation and misfolding, which in turn induces an increase in the expression of AAA+ proteases as part of the cell's adaptation mechanism [81]. In this regard, the targeting of AAA+ proteases has been explored as a potential therapeutic strategy, which led to the discovery and development of various compounds with anti-cancer potential. This article provides an overview of the molecular characteristics and functions of the major mitochondrial AAA+ proteases, as well as, discusses recent research efforts aimed at the development of compounds targeting these proteases.

8.2 Mitochondrial Lon Protease (LonP1)

8.2.1 Molecular Characteristics of LonP1

LonP1 is a 959-residue long AAA+ serine protease found in the mitochondrial matrix and is encoded by the nuclear *LONP1* gene [79]. It has three distinct domains – the N-terminal domain, followed by the AAA+ ATPase domain and a C-terminal proteolytic domain (Fig. 8.1a). At the N-terminus of LonP1 is a 67-residue long mitochondrial targeting sequence (MTS) (Fig. 8.1a) that directs the localization of LonP1 to the mitochondrial matrix [99].

The atomic structure of human LonP1's proteolytic domain (Fig. 8.1b) has been solved to 2 Å resolution by X-ray crystallography (PDB: 2X36) [31]. The domain shares the same structural layout as the proteolytic domain of bacterial and archaeal Lon proteases with an active site that carries the conserved catalytic Ser-Lys dyad (S855-K898) (Fig. 8.1b; proteolytic site indicated with black dotted circle) that is non-canonical among serine proteases [31]. Structural analysis of the full-length LonP1 S855A proteolytic mutant by cryo-electron microscopy (cryo-EM) revealed the formation of a homo-hexamer when LonP1 is in complex with ADP (Fig. 8.1c, left) or AMP-PNP (Fig. 8.1c, right) [52]. In both cases, the AAA+ ATPase and proteolytic domains occupy the head region of the hexamer and provide the site for ATP hydrolysis and proteolysis

of substrate proteins, while the N-terminal domain occupies the hexamer's legs region [52]. The hexameric form is the most common oligomeric state adopted by proteins carrying the AAA+ ATPase domain [85].

The proteolytic activity of LonP1 is driven by a cycle of conformational changes resulting from the binding and hydrolysis of ATP that is mediated by its AAA+ ATPase domain. Notably, the ATP-bound state of LonP1 (simulated by using AMP-PNP, the non-hydrolysable ATP analog) shows a shuttered entry pore to its proteolytic chamber that is gated by the axial pore loops (RTYVG; residues 563–567) [52]. In contrast, the ADP-bound state of LonP1 shows an unobstructed entry pore, suggesting the requirement of ATP hydrolysis in driving the entry of substrates into LonP1's proteolytic chamber [52]. Furthermore, the binding of ADP induces significant molecular rearrangements in LonP1, causing it to adopt a split-ring structure in the head region (Fig. 8.1c, left; the ring-splitting indicated by red dotted circles) that is not observed in the ATP-bound state (Fig. 8.1c, right). Nevertheless, it remains unclear if splitting the head of LonP1 has any functional significance with respect to its proteolytic cycle. Importantly, the N-terminal domain of LonP1 is shown to be essential for oligomerization and proteolysis, as deletion of the first 270 residues covering its MTS and part of the N-terminal domain prohibits the closing of the hexameric ring and abolishes its proteolytic activity *in vitro* [52].

8.2.2 Cellular Function of LonP1

The primary function of LonP1 is to maintain the mitochondrial proteome, mainly via the clearance of misfolded mitochondrial proteins that arise from oxidative damage [10, 13, 74]. The targeted degradation of oxidized mitochondrial aconitase (ACO2) is a well-characterized example of this process [10]. Notably, the clearance of misfolded proteins by LonP1 is an important step in the ATF5-mediated mitochondrial unfolded protein response (UPR^{mt}) during cellular stress induced by the formation of toxic protein aggregates in the mitochondria [8, 29].

In addition to clearing misfolded proteins from the mitochondria, LonP1 targets specific subunits of respiratory complexes for degradation as part of the cell's adaptation mechanism to stress or changes in respiratory condition. For example, LonP1 has been shown to directly interact and degrade specific peripheral arm subunits of Complex I (NDUFA9, NDUFS1, NDUFV1 and NDUFV2) to prevent production of reactive oxygen species (ROS) upon mitochondrial depolarization [77]. Similarly, LonP1 facilitates the turnover of SDHFA2, the flavination factor for subunit A of Complex II that is essential for its respiratory activity [9]. Furthermore, LonP1 has been shown to rapidly degrade isoform 1 of the cytochrome c oxidase subunit 4 (COX4-1) and is important for the cell's adaptation to hypoxia [30]. Outside of the respiratory chain, LonP1 has been shown to specifically mediate the rapid turnover of pyruvate dehydrogenase kinase 4 (PDK4), an inhibitory protein of the pyruvate dehydrogenase (PDH) complex, in response to changes in metabolic state of the mitochondria [22].

LonP1 has additional regulatory functions in multiple metabolic pathways. In renal glutamine catabolism, downregulation of the kidney isoform of mitochondrial glutaminase (glutaminase C) in the presence of diphenylarsinic acid (DPAA) occurs via its degradation by LonP1 [55]. In steroid biosynthesis, LonP1 mediates the turnover of the steroidogenic acute regulatory protein (StAR) [38], thereby suppressing the transfer of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) [38]. In heme biogenesis, LonP1 selectively targets the mature form of the nonspecific 5′-aminolevulinic acid synthase (ALAS1) for degradation as part of a heme-induced negative feedback regulatory mechanism for heme biosynthesis [92]. LonP1 also participates in the oxygen-sensitive degradation of specific heme-carrying proteins, such as cystathionine β-synthase (CBS) that catalyzes hydrogen sulfide formation, and heme oxygenase 1 (HO-1) that converts heme into biliverdin during heme catabolism [90]. With respect

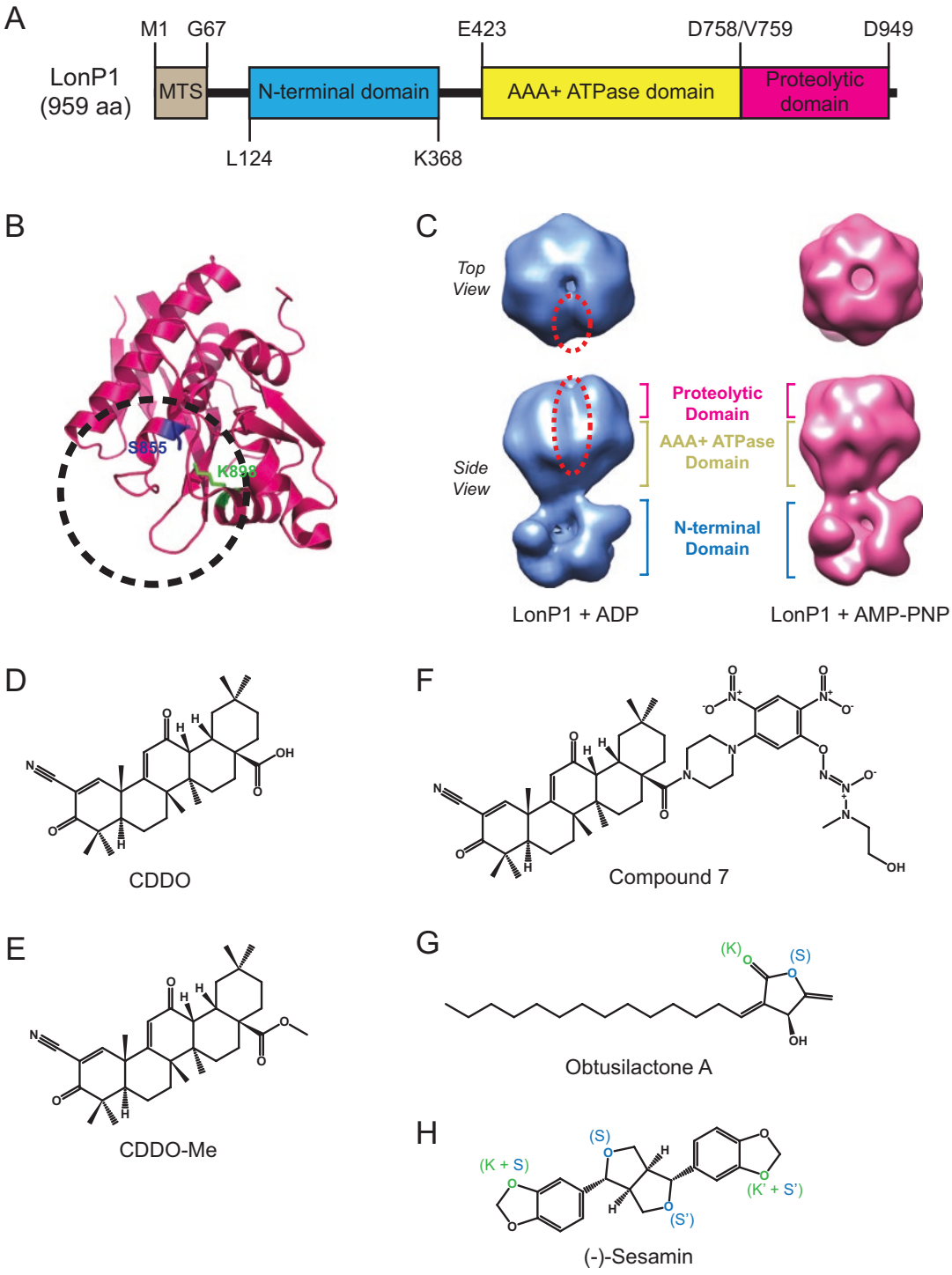

Fig. 8.1 Structure of mitochondrial LonP1 protease and its inhibitors. (**a**) Domain architecture of LonP1 illustrated as a bar diagram. Domain boundaries were determined from curated information obtained from UniProt (www.uniprot.org) or other public bioinformatics databases. *MTS* mitochondrial targeting sequence, *AAA* ATPase associated with diverse cellular activities. (**b**) Atomic structure of LonP1 (PDB: 2X36) [31]. Residues

to mitochondrial DNA (mtDNA) transcription, LonP1 mediates the downregulation of mitochondrial transcription factor A (TFAM) by selectively degrading its phosphorylated form that has less affinity for mtDNA, [69]. This in turn promotes mtDNA transcriptional activities that are necessary for mitochondrial homeostasis.

8.2.3 Role of LonP1 in Cancer

LonP1 is expressed at high levels in various types of solid cancers of the skin, lung, colorectal, bladder, cervix and the central nervous system [15, 26, 68, 71, 79], and in non-solid cancers such as B-cell lymphoma and acute myeloid leukemia [7, 37]. Suppressing LonP1 expression halts the proliferation of cancer cells and sensitizes them to chemotherapeutic reagents or sub-optimal growth conditions. For example, siRNA knockdown (KD) of LonP1 suppresses the proliferation of human bladder cancer cells ScaBER and UM-UC3 and increases the sensitivity of UM-UC3 cells to doxorubicin leading to caspase-dependent apoptosis [68]. Similarly, siRNA KD of LonP1 decreases the viability of human malignant glioma cells D-54 and U-251 and compromises their ability to survive under hypoxic conditions [26]. In non-small-cell lung cancer (NSCLC) H1299, shRNA KD of LonP1 suppresses cell proliferation and increases the occurrence of caspase-dependent apoptosis [98].

Conversely, the overexpression of a functional LonP1 promotes cancer cell proliferation. In hypopharyngeal squamous cell carcinoma (HSCC) FADU cells and oral cavity squamous cell carcinoma (OCSCC) OEC-M1 cells, overexpressing LonP1 significantly increases cellular proliferation [15]. Furthermore, LonP1-overexpressing cells exhibit enhanced colony formation compared to control cells [15]. Importantly, overexpression of LonP1 increases cellular resistance to apoptosis-inducing reagents. For example, overexpressing LonP1 in colon carcinoma cells RKO confers cellular resistance to the triterpenoid 2-cyano-3, 12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its C-28 methyl ester derivative (CDDO-Me) [33]. Both CDDO and CDDO-Me (Fig. 8.1d, e) have been characterized in detail with regard to their anticancer activity and will be discussed in Sect. 8.2.4.1.

A notable cellular effect resulting from high LonP1 expression is the altered expression and remodelling of specific respiratory complexes. In FADU and OEC-M1 cells, high LonP1 level leads to increased expression of Complex I subunits NDUSF3 and NDUFS8 [15]. This in turn raises intracellular ROS levels and promotes cell proliferation via Ras-ERK signalling, and cell migration and metastatic activity via both ERK and p38 MAPK signalling pathways [15]. Conversely, KD of LonP1 decreases ROS production and suppresses cell proliferation through downregulation of JNK phosphorylation and subsequent inhibition of MAPK signalling downstream [68]. In murine melanoma cells B16F10, high LonP1 levels increase the expression of specific subunits of Complex I and Complex V while decreasing the expression of other subunits, leading to significant remodelling of both complexes [79]. Consequently, respiratory oxidative phosphoryla-

Fig. 8.1 (continued) of the catalytic dyad are shown as sticks and coloured with Ser855 in blue and Lys898 in green. The active site of LonP1 in indicated with the black, dotted circle as shown. (**c**) Cryo-EM structures of LonP1 in complex with ADP (left; in blue) or AMP-PNP (right; in magenta). Both top views and side views of the structures are shown. Regions corresponding to the three functional domains are indicated. The red, dotted circles on LonP1 + ADP indicate the site of ring-splitting in LonP1's catalytic chamber as it adopts the split-ring conformation (see Sect. 8.2.1 for details). (**d**) Chemical structure of CDDO. (E) Chemical structure of CDDO-Me. (**f**) Chemical structure of Compound 7. (**g**) Chemical structure of Obtusilactone A. The oxygen atoms forming potential interactions with LonP1's catalytic dyad are coloured, with the oxygen interacting with Ser855 in blue and denoted with (S) and the oxygen interacting with Lys898 in green and denoted with (**k**). (**h**) Chemical structure of (−)-Sesamin. As in G, the oxygen atoms forming potential interactions with LonP1's catalytic dyad residues are coloured and denoted as before, with the exception that the piperonyl oxygen (shown in green) can likely interact with both Ser855 and Lys898, denoted by (K + S). The alternative LonP1-binding interface resulting from molecular symmetry is indicated by the coloured oxygen atoms denoted with (S′) or (K′ + S′).

tion (OXPHOS) is compromised, prompting the upregulation of the glycolytic pathway [79]. An increase in expression of the subunits of the spliceosome, the chaperone-containing TCP-1 (CCT) complex and the proteasome is also observed [79]. Collectively, these changes confer additional cellular protection against oncogene-induced senescence [79].

8.2.4 Chemical Inhibitors of LonP1 and Their Anti-cancer Properties

Given the central role of LonP1 in facilitating cancer cell proliferation and contribution to chemotherapeutic resistance, its inhibition using specific chemical inhibitors has been explored as a potential therapeutic strategy. Several classes of compounds have been identified to both inhibit the proteolytic activity of LonP1 and induce cytotoxicity in various types of cancer.

8.2.4.1 The Triterpenoid CDDO and Its Derivatives

Triterpenoids are structurally diverse compounds with a wide range of biological effects [105]. One notable example is the synthetic oleanane 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) (Fig. 8.1d), also known as Bardoxolone (RTA-401) that specifically inhibits the proteolytic activity of purified LonP1 [7]. Inhibition of LonP1 by CDDO occurs via direct molecular interactions, although the exact binding site has not been determined. In vivo, CDDO induces caspase-dependent apoptosis in various human cancer cells with high LonP1 expression, including Granta MCL (B-cell lymphoma), RKO (colon carcinoma), HepG2 (liver hepatocellular carcinoma) and MCF7 (breast ductal carcinoma) [7, 33] (Table 8.1). Furthermore, CDDO-induced cytotoxicity is increased when LonP1 is overexpressed, thus providing further confirmation on the specific targeting of LonP1 by the drug in vivo [33]. Importantly, CDDO exerts no cytotoxic effects on normal primary human fibroblasts [33], which supports its potential use as an anti-cancer agent against cancer cells that are CDDO-sensitive due to high LonP1 levels. Phase I clinical trials have been conducted on CDDO in treating acute myeloid leukemia [95] and advanced solid tumors [86], but so far they have fell short of confirming the drug's anti-cancer effects in patients.

Derivatives of CDDO have been developed and the biochemical and physiological effects characterized. For example, CDDO-Me, also known as Bardoxolone methyl (RTA-402) (Fig. 8.1e), is the C-28 methyl ester of CDDO, and it inhibits the proteolytic activity of LonP1 in vitro with greater efficacy than CDDO [7]. In vivo, CDDO-Me induces greater cytotoxicity than CDDO in RKO, HepG2, MCF7 and the taxol-resistant A549 (A549/Taxol) cells, but not in normal primary human fibroblasts [33, 47] (Table 8.1). Furthermore, the cytotoxicity of CDDO-Me is reduced when LonP1 is overexpressed [33], thus providing additional proof for the specific targeting of LonP1 by CDDO-Me.

Nevertheless, CDDO-Me exhibits potential off-target effects, given a recent report on the interaction and inhibitory effect of CDDO-Me on Hsp90 in vivo [78]. Similarly, CDDO-Me has been shown to interact with Keap1, a central regulator of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) mediated stress responses against electrophilic and oxidative conditions [18]. The interaction inhibits Keap1 and in turn activates Nrf2-mediated cytoprotective responses [102].

With regard to its clinical application, a Phase I clinical trial conducted with CDDO-Me for treating advanced solid tumors and lymphomas indicates that the drug exhibits objective anti-cancer activity with minimal toxicity on the patients [44], prompting further development of CDDO derivatives, such as Omaveloxolone (RTA-408), which has also undergone a Phase I trial for assessment against advanced solid tumors [21]. Aside from its anti-cancer properties, a Phase III trial has also been conducted in evaluating CDDO-Me as a treatment for stage 4

Table 8.1 Biological activities of small molecule compounds targeting different mitochondrial AAA+ proteases

Compound	Protease targeted	Molecular mechanism	Cell line	Disease model	Comments and references
CDDO	LonP1	Inhibition	Granta MCL	B-cell lymphoma	IC_{50} = 1.3 μM [7]
					Apoptosis induced at 2.5 μM [7]
			LS174T	Colon carcinoma	No effect on cell viability [7]
			RKO	Colon carcinoma	Inhibits cell proliferation at ≥2.5 μM [33]
					30–40% of cells in early apoptosis after 16-h exposure at 2.5 μM [33]
			HepG2	Liver hepatocellular carcinoma	Inhibits cell proliferation at ≥5 μM [33]
					20–30% of cells in late apoptosis after 16-h exposure at 5 μM [33]
			MCF7	Breast ductal carcinoma	Inhibits cell proliferation at ≥5 μM [33]
					30–40% of cells in late apoptosis after 24-h exposure at 5 μM [33]
			Human primary fibroblasts	Normal/ non-cancerous cells	No effect on cell proliferation [33]
CDDO-Me	LonP1	Inhibition	RKO	Colon carcinoma	Inhibits cell proliferation at ≥1 μM [33]
					50–60% of cells in early apoptosis after 16-h exposure at 2.5 μM [33]
			HepG2	Liver hepatocellular carcinoma	Inhibits cell proliferation at ≥2.5 μM [33]
					40–50% of cells in late apoptosis after 16-h exposure at 2.5 μM [33]
			MCF7	Breast ductal carcinoma	Inhibits cell proliferation at ≥2.5 μM [33]
					Apoptosis induced in 60–70% of cells in late apoptosis after 24-h exposure at 2.5 μM [33]
			Human primary fibroblasts	Normal/ non-cancerous cells	20–30% of cells in early apoptosis after 16-h exposure at 2.5 μM [33]
			A549/Taxol	Drug-resistant lung carcinoma	~10% of cells in apoptosis after 24-h exposure at 4.8 μM [47]
Compound 7 (CDDO derivative)	LonP1	Inhibition	A549/Taxol	Drug-resistant lung carcinoma	IC_{50} = 0.35 μM [47]
					30–40% of cells in apoptosis after 24-h exposure at 4.8 μM [47]
			A549	Lung carcinoma	IC_{50} = 1.07 μM [47]

(continued)

Table 8.1 (continued)

Compound	Protease targeted	Molecular mechanism	Cell line	Disease model	Comments and references
Obtusilactone A	LonP1	Inhibition	A549	Lung carcinoma	IC_{50} = 26.50 μM [98]
			H1299	Lung carcinoma	IC_{50} = 33.96 μM [98]
					Causes extensive DNA double-strand breaks [98]
					Induces JNK-mediated apoptosis after 12-h exposure at 40 μM [98]
			MRC-5	Normal/ non-cancerous cells	IC_{50} = 49.43 μM [98]
(−)-Sesamin	LonP1	Inhibition	H1299	Lung carcinoma	Causes DNA double-strand breaks, but less extensive than Obtusilactone A [98]
					No additional data provided on cytotoxicity or apoptotic induction [98]
A2-32-01	ClpP	Inhibition	TEX	Acute myeloid leukemia	IC_{50} ~25 μM [19]
			OCI-AML2	Acute myeloid leukemia	IC_{50} ~25 μM [19]
					Impairs complex II activity and delays growth of cells xenografted in SCID mice; no observable liver, muscle or renal toxicity [19]
			K562	Chronic myeloid leukemia	IC_{50} ~25 μM [19]
			HL-60	Acute promyelocytic leukemia	No effect on cell viability [19]
			143B	Osteosarcoma	IC_{50} ~25 μM [19]
			143B Rho (0)	Mitochondria depletion control for 143B	No effect on cell viability [19]
			Human primary AML cells	Acute myeloid leukemia	IC_{50} ranges from ~25 to ~125 μM, with cells expressing high ClpP levels being the most sensitive (R^2 = 0.9264) [19]
					Impairs proliferation of cells engrafted in NOD-SCID mice; no host toxicity observed [19]
			Human primary normal hematopoietic cells	Normal/ non-cancerous cells	Minimal to no effect on cell viability [19]
ADEP-28	ClpP	Dysregulated activation	HEK293 T-REx	N/A	IC_{50} = 0.36 μM [100]
			HEK293 T-REx *CLPP*$^{-/-}$	ClpP-deletion control for HEK293 T-REx	No effect on cell viability [100]

(continued)

Table 8.1 (continued)

Compound	Protease targeted	Molecular mechanism	Cell line	Disease model	Comments and references
ADEP-41	ClpP	Dysregulated activation	HEK293 T-REx	N/A	IC_{50} = 0.49 μM [100]
					Mcl-1-mediated apoptosis induced in ~10% of cells after 72-h exposure at 10 μM [100]
			HEK293 T-REx *CLPP*$^{-/-}$	ClpP-deletion control for HEK293 T-REx	No effect on cell viability [100]
					No apoptosis observed [100]
			HeLa	Endocervical carcinoma	IC_{50} = 0.54 μM [100]
			HeLa T-REx	Endocervical carcinoma	IC_{50} = 0.48 μM [100]
			U2OS	Osteosarcoma	IC_{50} = 0.58 μM [100]
			SH-SY5Y	Neuroblastoma	IC_{50} = 0.86 μM [100]

chronic kidney disease and type 2 diabetes mellitus, although the study has revealed serious side effects of the drug such as heart failure and fluid retention in susceptible patient subgroups [17].

Chemical hybrids of CDDO with different apoptotic inducers have also been explored. For example, Compound 7 (Fig. 8.1f) is a hybrid of CDDO and an O^2-(2,4-dinitrophenyl) diazeniumdiolate moiety [47]. O^2-(2,4-dinitrophenyl) diazeniumdiolates (e.g. PABA/NO, JS-K) generate intracellular nitric oxide (NO) upon activation by glutathione S-transferase π (GSTπ) that is highly expressed in many cancers [94]. The increase in NO level in turn creates nitrosative stress leading to cytotoxicity [45, 93]. Thus, a CDDO-diazeniumdiolate hybrid can target and inhibit LonP1 while simultaneously inducing nitrosative stress upon activation by GSTπ. Indeed, the hybrid Compound 7 is cytotoxic and induces apoptosis in lung carcinoma cells A549 and the Taxol-resistant equivalent A549/Taxol (Table 8.1) with greater efficacy than CDDO-Me (Table 8.1) and JS-K [47]. Furthermore, Compound 7 generates the highest levels of intracellular NO and is the most cytotoxic to A549/Taxol cells expressing a much higher level of GSTπ compared to A549 and the non-cancerous MRC-5 cells [47]. This highlights the potential of CDDO-diazeniumdiolate hybrids in targeting cancer cells that have developed resistance to traditional chemotherapeutic agents such as Taxol.

8.2.4.2 Obtusilactone A

In addition to CDDO and its derivatives, new types of LonP1-inhibiting compounds have also been discovered and characterized. For example, the γ-lactone Obtusilactone A (OA) (Fig. 8.1g) is a natural product isolated from the aromatic evergreen tree *Cinnamomum kotoense* Kanehira and has shown potential antioxidant and anti-cancer properties [16]. Further investigation has determined that OA inhibits the proteolytic activity of LonP1 both in vitro and in vivo [98]. Simulated docking analysis suggests that OA may bind to the active site of LonP1, with each of the two carbonyl oxygen atoms in its γ-lactone ring forming hydrogen bonds with Ser855 and Lys898 (Fig. 8.1b) of LonP1's catalytic dyad, respectively (Fig. 8.1g; interacting residues for each oxygen atom denoted with S for Ser855 and K for Lys898). At the cellular level, OA induces cytotoxicity in lung carcinoma cells A549 and H1299, resulting in extensive DNA double-strand breaks and subsequent apoptotic induction that is mediated through the c-Jun N-terminal kinase (JNK) pathway (Table 8.1) [98]. Treating the non-cancerous MRC-5 cells with OA also induces cytotoxicity, albeit it is less severe compared to A549 and H1299 (Table 8.1), which suggests higher drug sensitivity of cancer cells that express higher levels of LonP1 [98].

8.2.4.3 (−)-Sesamin

(−)-Seasamin (Fig. 8.1h) is another natural product isolated from *C. kotoense* that shows potential antioxidant and anti-cancer properties [16]. Like OA, (−)-Sesamin also inhibits LonP1 both in vitro and in vivo [98]. Docking analysis suggests that (−)-Sesamin may bind to LonP1's active site, with the oxygen atoms of its tetracyclic ether ring (Fig. 8.1h; shown in blue) and piperonyl group (Fig. 8.1h; shown in green) forming hydrogen bonds with Ser855 and Lys898 (Fig. 8.1h; S denotes the tetracyclic ether oxygen atom interacting with Ser855 and K + S denotes the piperonyl oxygen atom interacting with both Ser855 and Lys898) [98]. Notably, the structural symmetry of (−)-Sesamin suggests that the molecule has two putative binding interfaces for LonP1 (Fig. 8.1H; the alternative binding interface denoted with S′ for the tetracyclic ether oxygen and K′ + S′ for the piperonyl oxygen). Similar to OA, (−)-Sesamin has been shown to induce DNA double-strand breaks in H1299 cells (Table 8.1) albeit at lesser severity than OA [98], but its cytotoxicity in cancer cells has not been reported.

8.3 Mitochondrial ClpXP Protease Complex

8.3.1 Molecular Characteristics of Mitochondrial ClpXP

The mitochondrial ClpXP complex is another major AAA+ protease found in the mitochondrial matrix. ClpXP is constituted of two proteins: the 277-residue long serine protease ClpP and the 633-residue long AAA+ ATPase ClpX (Fig. 8.2a). ClpP and ClpX are encoded by the nuclear *CLPP* and *CLPX* genes, respectively. ClpP consists almost entirely of the serine proteolytic domain, with the addition of a 56-residue long MTS at its N-terminus that directs its localization to the mitochondrial matrix, which is cleaved off upon protein maturation [23]. It also has a 26-residue long C-terminal tail that modulates the ClpP-ClpX interaction and the proteolytic activity of the complex in vitro [49] (Fig. 8.2a).

ClpX consists of two domains: the N-terminal zinc-binding domain (ZBD) that facilitates the recognition and binding of substrate proteins [6, 91], followed by the AAA+ ATPase domain that mediates the oligomerization, ATP hydrolysis and protein unfoldase activity of ClpX [70, 88]. Like ClpP, a 56-residue long MTS is present at the N-terminus of ClpX.

The atomic structure of human mitochondrial ClpP has been solved at 2.1 Å resolution by X-ray crystallography (PDB: 1TG6) (Fig. 8.2b, c) [48]. The active site of ClpP carries the Ser153-His178-Asp227 catalytic triad (Fig. 8.2b) [28]. Like its bacterial counterparts, the functional state of mitochondrial ClpP is a cylindrical tetradecamer of two identical heptameric rings (Fig. 8.2c) [48]. In this configuration, axial entry pores are formed at the top and bottom ends of the ClpP tetradecamer (Fig. 8.2c, top view) that allows entry of substrates for degradation, while the generated peptidyl fragments likely exit via transient side pores (Fig. 8.2c, side view) [67]. Importantly, the 14 active sites are shielded and are exposed only within ClpP's proteolytic chamber, thereby effectively prohibiting unspecific proteolysis.

The functional form of mitochondrial ClpX is a hexamer [49], which is the most common among AAA+ ATPases [79]. Notably, the hexamerization of ClpX leads to the formation of six complete ATP-binding pockets with all the necessary conserved functional motifs (i.e. Walker A, Walker B, Sensor I, Sensor II and the Arginine finger) for ATP hydrolysis. The energy derived from ATP hydrolysis drives the allosteric movements and structural rearrangements in ClpX that are necessary for its substrate-unfolding activity [79].

To form the complete, functional ClpXP complex, one ClpX hexamer docks on each end of the ClpP tetradecamer such that the axial pores of both proteins are aligned on the same axis [49]. Importantly, interaction between ClpX and ClpP is facilitated by the docking of the essential IGF loops of ClpX at the hydrophobic binding sites on ClpP apical surface that are formed between every two ClpP subunits at the ClpX-ClpP interface. Furthermore, the docking of IGF loops at these hydrophobic pockets is a highly dynamic

process that contributes significantly to stabilizing the ClpX-ClpP interaction [4].

Protein degradation by ClpXP occurs via multiple steps. The first step involves the recognition and binding of the substrate protein to ClpX. Subsequently, the bound substrate is then unfolded and threaded through the axial pore of ClpX by the coordinated power strokes of specialized pore loops, utilizing energy derived from ATP hydrolysis [70]. Notably, this is a highly processive process that translocates the unfolded substrate into ClpP's proteolytic chamber, where it is degraded into small peptidyl fragments [67]. Ultimately, these fragments are expelled from the ClpP lumen through side pores that are formed via the dynamic, allosteric rearrangements of ClpP subunits [53]. The degradation cycle can then be repeated upon the binding of a new substrate molecule.

8.3.2 Cellular Function of Mitochondrial ClpXP

ClpXP contributes to mitochondrial proteome maintenance by facilitating the turnover of specific substrate proteins. Loss of ClpXP function thus results in the accumulation of misfolded proteins, impairing various cellular processes. For example, ClpP KD has been shown to induce the accumulation of misfolded subunit B of Complex II (SDHB), leading to the loss of Complex II function, impaired OXPHOS and ATP production, and resulting in oxidative stress [83]. Furthermore, the loss of ClpP expression has been shown to hinder the expression of mitochondrial unfolded protein response (UPR^{mt}) marker proteins, increase mitochondrial fragmentation, and attenuate the normal activities of respiratory complexes in muscle cells [24]. Notably, ClpXP expression has been shown to increase in response to the accumulation of misfolded mitochondrial proteins during UPR^{mt} [104], mitochondrial frataxin deficiency [40], and respiratory deficiency induced by mitochondrial DNA (mtDNA) mutagenesis [20].

In addition to maintaining the mitochondrial proteome, ClpXP provides additional modulatory functions in multiple biological processes. For example, ClpXP degrades both ALAS1 and the erythroid-specific ALAS2 as part of a negative feedback mechanism that regulates heme biosynthesis [61, 101]. ClpXP is also involved in the turnover of phosphatase and tensin homologue-induced kinase 1 (PINK1), which in turn affects the recruitment of the E3 ubiquitin ligase Parkin to the mitochondrial surface, potentially modulating the dynamics of mitophagy [39]. Furthermore, ClpXP has been shown to facilitate the turnover of the dynamin-related protein 1 (Drp1) and to modulate mitochondrial fission-fusion dynamics [24]. ClpXP has also been shown to recognize and degrade the nitric oxide-associated protein 1 (NOA1) that regulates mitochondrial translation [2]. Notably, ClpXP alters OXPHOS in response to stress or other external stimuli via the turnover of specific respiratory proteins, such as the peripheral arm subunits NDUFV1 and NDUFV2 of Complex I, to quench ROS production in depolarized mitochondria [77].

Unlike bacterial ClpXP, human mitochondrial ClpX has been shown to perform dedicated biochemical functions that are independent of ClpP. For example, ClpX functions as a molecular chaperone in the maturation of ALAS2 during erythropoiesis [50]. ClpX may also stabilize the mitochondrial transcription factor A (TFAM) to maintain proper mtDNA segregation [51]. In addition, ClpX physically interacts with the mitochondrial E3 ubiquitin-protein ligase XIAP (X-linked inhibitor of apoptosis proteins) and may potentially promote apoptosis by antagonizing XIAP from caspase inhibition in a similar manner as other known IAP-binding proteins [96]. Furthermore, ClpX alone has been shown to induce the upregulation of the transcriptional regulator C/EBP homologous protein (CHOP) during myogenesis and potentially stimulates UPR^{mt} [1].

8.3.3 Role of Mitochondrial ClpXP in Cancer

Expression of mitochondrial ClpXP has been characterized in various types of cancer. Notably, ClpP is upregulated to various degrees in solid

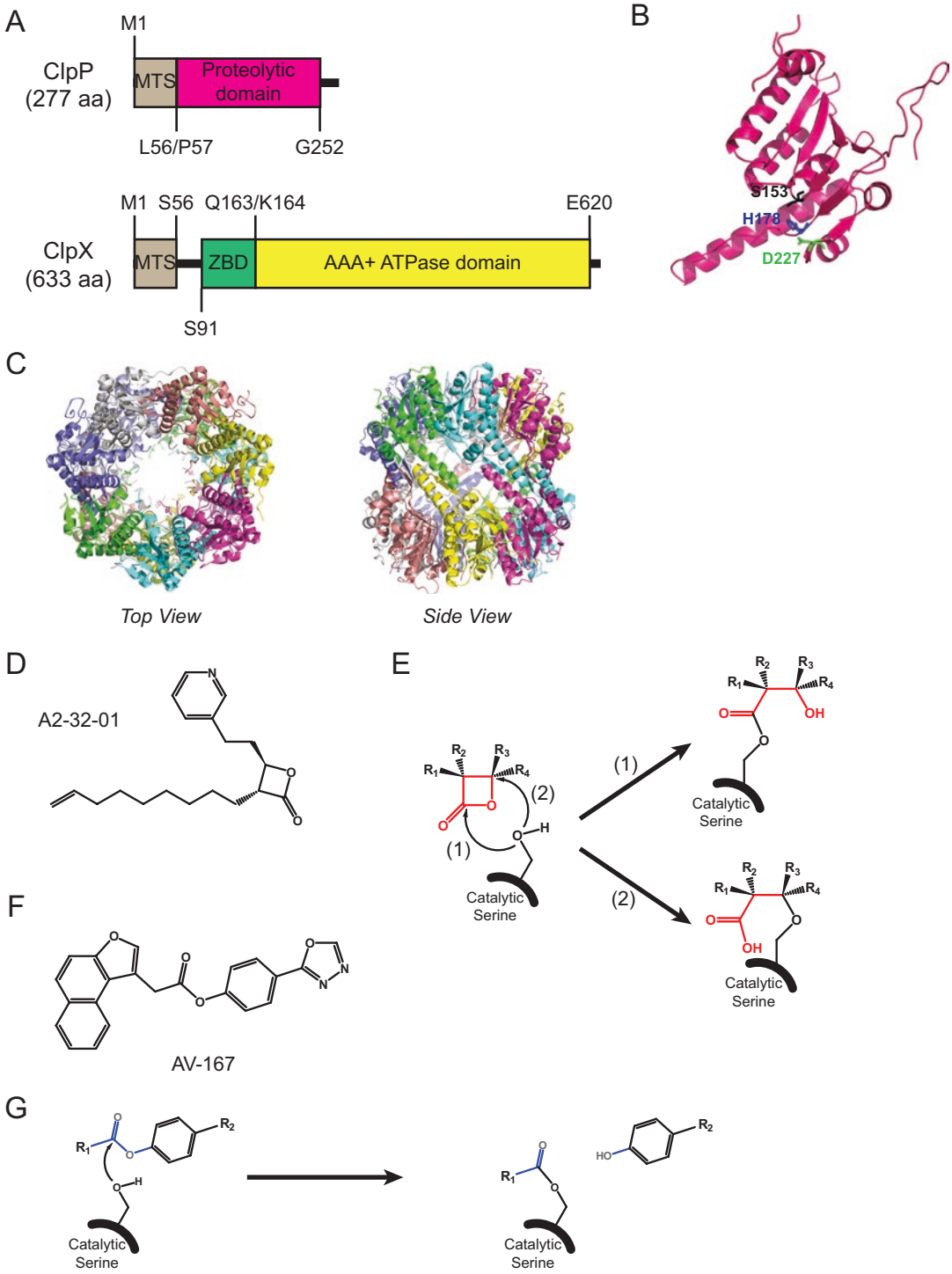

Fig. 8.2 Structure of mitochondrial ClpXP and its inhibitors. (**a**) Domain architecture of ClpP (top) and ClpX (bottom) illustrated as bar diagrams. Boundaries were determined from curated information in UniProt or other public bioinformatics databases. *ZBD* zinc-binding domain. (**b**) Atomic structure of the ClpP subunit in the apo tetradecamer (ZBD: 1TG6) [48]. The catalytic triad is shown as sticks and coloured with Ser153 in black,

cancers of almost all major organs or tissues, including breast, ovary, prostate, uterus, bladder, lung, stomach, liver, thyroid, prostate, testis and central nervous system [20, 72, 83], as well as in non-solid cancers such as acute myeloid leukemia [19].

Despite the fact that the functional roles of ClpXP in cancer pathology are not as well characterized as that of LonP1, the currently available evidence indicates the importance of ClpXP in the proliferation and metastasis of specific cancers. For example, depletion of ClpX or ClpP suppresses the proliferation and hinders colony formation of prostate cancer cells PC3 [83]. Furthermore, PC3 cells depleted of ClpX or ClpP are compromised in their ability to invade normal tissues and metastasize when xenografted into immunocompromised mice [83]. Nevertheless, the importance of ClpXP in cancer pathology depends heavily on cell type, as illustrated in the observation that KD of ClpX or ClpP has minimal effects on the proliferation of breast cancer cells MCF7 [83].

For non-solid cancer, ClpP depletion reduces the growth and viability of multiple leukemic cell lines expressing elevated levels of the protease [19]. The loss of ClpP expression also compromises the integrity and activity of Complex II, leading to reduction in OXPHOS and increase in ROS production [19]. Interestingly, while an increase in UPR^{mt} is consistently observed in leukemic cells with elevated ClpP expression, reduction in their viability as a result of ClpP KD has no observable effect on either the expression of UPR^{mt} markers or the mass and morphology of the mitochondria [19], which contradicts the findings in immortalized muscle cells [24] and fibroblasts [104], highlighting a potential variation in ClpP's role in UPR^{mt} among different cell types.

8.3.4 Chemical Inhibitors of Mitochondrial ClpXP as Potential Cancer Therapeutics

Relative to LonP1, the utilization of ClpXP as a potential drug target in cancer therapy has been explored to a lesser degree. Nevertheless, specific chemical inhibitors of ClpXP have been identified and their potential therapeutic effects against specific types of cancer have been characterized.

8.3.4.1 β-Lactones

β-Lactones have previously been developed as potential antibiotics to inhibit ClpP in *Staphylococcus aureus* and suppress its pathogenesis [60, 103]. Among the analogs developed, A2-32-01 (Fig. 8.2d) has been investigated further with regard to its inhibition of human mitochondrial ClpP and its therapeutic potential against acute myeloid leukemia [19]. Although the inhibitory mechanism of A2-32-01 on mitochondrial ClpP has not been investigated in detail, the drug might covalently modify ClpP's catalytic Ser residue via one of two possible reactions (Fig. 8.2e) that have been proposed for β-lactones in general [11].

In vivo, A2-32-01 induces cytotoxicity in OCI-AML2, TEX and the chronic myeloid leukemic K562 cells with IC_{50} at ~25 μM but has no effect on the acute promyelocytic leukemic HL-60 cells (Table 8.1) [19]. Notably, OCI-AML2, TEX and K562 cells all show highly elevated ClpP expression compared to HL-60 [19]. Furthermore, A2-32-01 induces cytotoxicity in osteosarcoma cells 143B (IC_{50} at ~25 μM) but not in the mitochondria-depleted 143B Rho (0) cells (Table 8.1) [19]. The combined data suggests that cellular sensitivity to A2-32-01 originates from the mitochondria.

Fig. 8.2 (continued) His178 in blue and Asp227 in green. (**c**) Top view (left) and side view (right) of the human apo ClpP tetradecamer [100]. Individual ClpP subunits are distinguished by different colours. (**d**) Chemical structure of A2-32-01. (**e**) Proposed general chemical mechanism in the covalent modification of ClpP's catalytic Ser residue by a β-lactone. The essential β-lactone ring is highlighted in red. R_1, R_2, R_3 and R_4 represent structural elements that are unique to individual compounds and do not participate in the reaction. (**f**) Chemical structure of AV-167. (**g**) Proposed general chemical mechanism in the covalent modification of ClpP's catalytic Ser residue by a phenyl ester. The essential ester group is highlighted in blue. R_1 and R_2 represent structural elements that are unique to individual compounds and that do not participate in the reaction

To further establish the therapeutic potential of A2-32-01, treating human primary leukemic cells expressing high levels of ClpP with the drug induces cytotoxicity, with IC_{50} ranging from ~25 to ~125 μM (Table 8.1) [19]. The specific targeting of A2-32-01 against leukemic cells is confirmed as the drug has minimal to no effect on the viability of normal primary hematopoietic cells. Furthermore, A2-32-01 has been shown to suppress the proliferation of OCI-AML2 and primary human leukemic xenografts in immunodeficient mice with no observable toxic effects on the animals (Table 8.1).

8.3.4.2 Phenyl Esters

Phenyl esters are a new class of ClpP inhibitors that are more potent and chemically stable than β-lactones [42]. They were originally developed as potential antibiotics against *S. aureus*, but, in a counter screen, AV-167 (Fig. 8.2f) was found to inhibit human ClpP from degrading fluorogenic peptidyl substrates [42]. Subsequent mass spectrometric analysis on the covalent modification of the catalytic Ser residue of SaClpP confirmed that AV-167 shares a common reaction mechanism with other active phenyl ester analogs, in which the catalytic serine's hydroxyl oxygen performs a nucleophilic attack on the carboxyl carbon of the drug and displaces the phenyl moiety that gets converted to an equivalent phenol moiety in the process (Fig. 8.2g) [42]. Data on the cytotoxicity of AV-167 on human cells is currently unavailable.

8.3.5 Activators of ClpP

The architecture of the ClpXP complex that segregates its ATP-dependent protein unfolding activity to ClpX and its proteolytic activity to ClpP provides a unique window for chemical interference via disabling the regulatory function of ClpX. Indeed, small molecules such as acyldepsipeptides (ADEPs) [54], activators of ClpP protease (ACPs) [64] and related compounds [14] have been identified that activate and dysregulate the protease activity of ClpP via physical displacement of ClpX, while keeping ClpP in its activated form. Importantly, dysregulation of ClpP by ADEP has been shown to be a promising venue for antibiotics development, with these molecules showing potent bactericidal activities against various pathogenic bacteria, including *S. aureus*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Bacillus subtilis*, *Enterococcus faecalis* and *Listeria innocua* [35, 36].

Recently, ADEP analogs that can specifically target the human mitochondrial ClpP have been shown to induce apoptotic cell death in immortalized human cancer cell lines with high potency [100], thus paving the way to the exploration of using ADEPs as novel anti-cancer drugs.

8.3.5.1 General Molecular Mechanism of ADEP-Based ClpP Dysregulation

The molecular mechanism for ClpP's activation by ADEP has been characterized in detail. Binding of ADEP occurs at the same hydrophobic pocket between ClpP subunits that normally accommodate the docking of ClpX's IGF loops [62]. The higher affinity of ADEP allows it to effectively out-compete the IGF loops for ClpP-binding [4]. Importantly, the docking and occupancy of ClpP's hydrophobic pockets by ADEP disrupts the dynamic docking of IGF loops that is essential in maintaining ClpX-ClpP interaction, resulting in the rapid dissociation of ClpX from ClpP [4]. Furthermore, ADEP-binding simulates the structural effects of ClpX-ClpP interaction, which forces ClpP to remain in its active state that is characterized by a widened axial pore [62] and structuring of its pore loops [66]. This in turn enables the unregulated access of peptides, molten globules and even folded proteins into ClpP's proteolytic chamber, where they are degraded in an unspecific manner [82].

8.3.5.2 Dysregulation of ClpP by ADEP Causes Cell Death via Intrinsic Apoptosis

ADEP analogs with high specificity for human mitochondrial ClpP have been identified and characterized. Among them, ADEP-28 (Fig. 8.3a) and ADEP-41 (Fig. 8.3b) were found to be potent activators of human ClpP's peptidase and protease activities against fluorogenic

model substrates [100]. The presence of ADEP at low concentrations is sufficient in dissociating ClpX from ClpP. Notably, both ADEP-28 and ADEP-41 induce cytotoxicity in ClpP-expressing HEK293 T-REx cells with IC_{50} measured at 0.36 μM and 0.49 μM, respectively (Table 8.1) [100]. Importantly, ADEP-induced cytotoxicity is dependent on ClpP expression, as the drug has minimal to no observable effect on HEK293 T-REx *CLPP*$^{-/-}$ cells even with high doses of the drug (Table 8.1) [100]. Furthermore, the severity of ADEP-induced cytotoxicity increases with respect to an increase in intracellular ClpP level.

In support of ADEP's anti-cancer potential, ADEP-41 was shown to induce cytotoxicity in various cancer-derived cell lines, including HeLa (endocervical carcinoma), U2OS (osteosarcoma) and undifferentiated SH-SY5Y (neuroblastoma), with IC_{50} ranging from 0.48 to 0.86 μM (Table 8.1) [100]. Analysis of the underlying cell death mechanism revealed that treatment of cells with ADEP activates the intrinsic, caspase-dependent apoptotic pathway, with the ADEP-treated cells showing characteristic phenotypes such as increase in DNA strand breaks, mitochondrial fragmentation, loss of OXPHOS and the proteolytic activation of caspase-9 and caspase-3 [100].

8.3.5.3 Structural Evidence for a Highly Dynamic ADEP-ClpP Complex

To further understand the molecular mechanism underlying ClpP's activation by ADEP, atomic structure of the mitochondrial ClpP in complex with ADEP-28 was solved at 2.8 Å resolution [100]. ADEP-28 binds to a highly complementary hydrophobic pocket that forms between two adjacent ClpP subunits (Fig. 8.3c) in the same manner as observed in other ADEP-bound ClpP. Notably, binding of ADEP-28 induces the formation of structured axial loops at the entrance pore (Fig. 8.3c). Furthermore, as with other ADEP-ClpP interaction, the binding of ADEP-28 to human ClpP widens the axial pore (Fig. 8.3d) so to enable the unregulated access of protein substrates, resulting in unspecific proteolysis [100].

Surprisingly, the ADEP-28-bound human ClpP oligomer adopts a compact conformation (Fig. 8.3e) that can be visualized in the rotation of one of its heptameric rings relative to the apposing one (Fig. 8.3e; indicated with tilt in the purple dotted line), resulting in the compaction of ClpP as indicated by the distance between the two rings of catalytic Ser residues being reduced by ~7.8 Å (Fig. 8.3e; upper and lower Ser rings coloured in red and blue, respectively). This provides the first structural evidence that ADEP-bound ClpP is in fact a dynamic structure that adopts multiple conformations over its proteolytic cycle, contrary to a previously proposed model in which ADEP locks ClpP into its active state with the extended conformation [32].

8.4 i-AAA and m-AAA Metalloproteases

The compartmentalization of mitochondria and the differential localization of mitochondrial proteins to these compartments give rise to the need for additional specialized AAA+ proteases for the clearance of proteins that are misfolded locally and that are not accessible by the matrix-localized LonP1 and ClpXP. In this regard, the membrane-bound i-AAA and m-AAA metalloproteases and paraplegin are responsible of maintaining the protein homeostasis of mitochondrial membranes and the inter-membrane space (IMS) in addition to the mitochondrial matrix. Other important functions of these proteases have also been identified.

8.4.1 i-AAA Metalloprotease

8.4.1.1 Molecular Characteristics of i-AAA

i-AAA is a 733-residue long integral membrane metalloprotease encoded by the nuclear *YME1L1* gene and is found in the inner membrane of mitochondria (IMM) [87]. It consists of a N-terminal domain carrying a cleavable MTS for localization to the IMM, followed by a single trans-

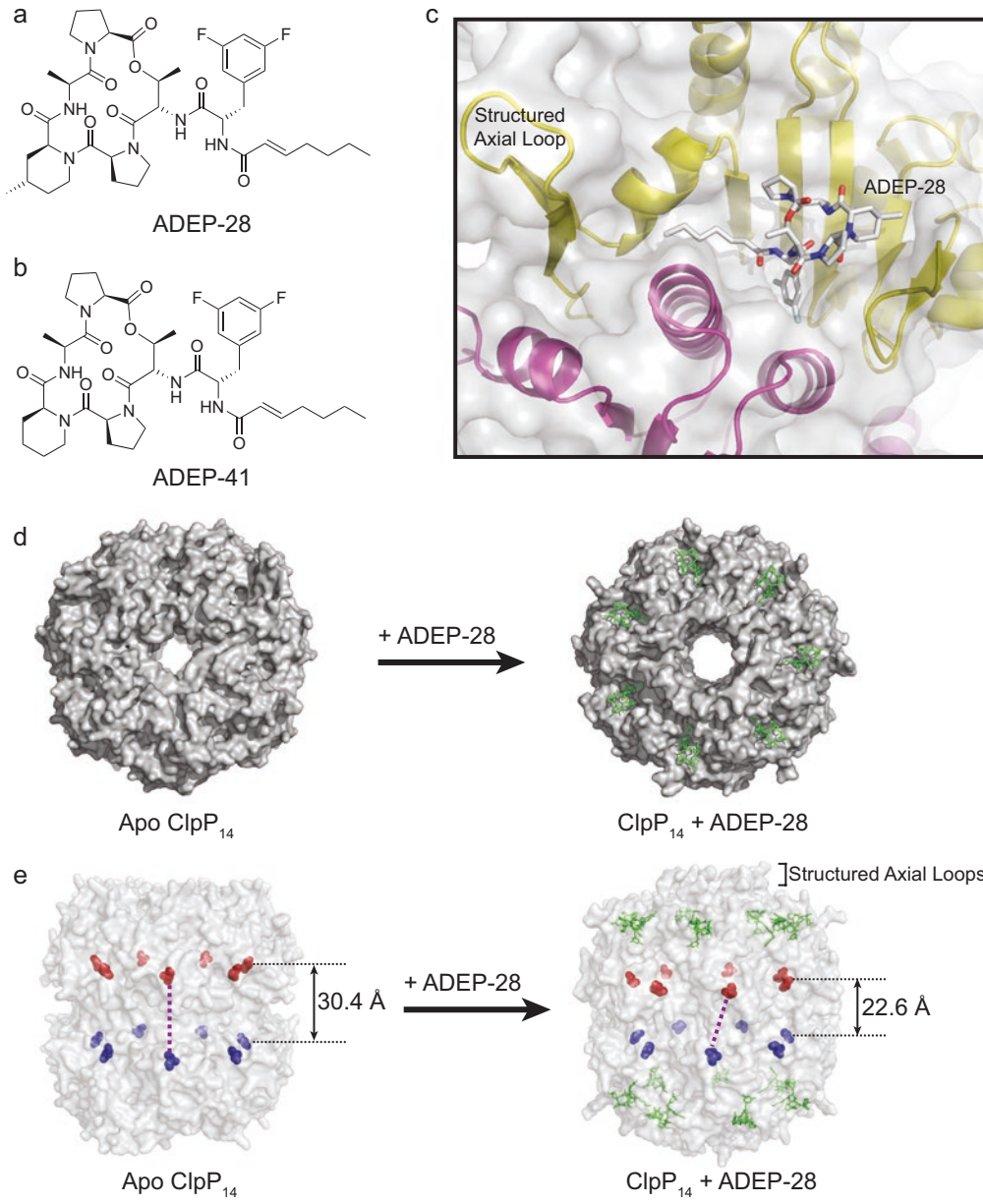

Fig. 8.3 Structural information of the activation of mitochondrial ClpP by ADEP**.** (**a**) Chemical structure of ADEP-28. (**b**) Chemical structure of ADEP-41. (**c**) ADEP-binding pocket in the atomic structure of human mitochondrial ClpP in complex with ADEP-28 (PDB: 6BBA) [100]. The bound ADEP-28 is shown as sticks and coloured by elements. The two ClpP subunits forming the ADEP-binding pocket are illustrated as ribbons with the structured axial loop of one subunit denoted. The molecular surface of the ClpP oligomer is used to highlight the contours of the ADEP-binding pocket. (**d**) Atomic structures of apo tetradecameric ClpP and ADEP-28-bound tetradecameric ClpP, showing the enlargement of ClpP's axial pore caused by ADEP-28-binding. ADEP-28 is shown as sticks and coloured in green. (**e**) Compaction of tetradecameric ClpP upon ADEP-28 binding is illustrated by the distance between two catalytic Ser residues from apposing rings (shown as spheres; top ring coloured in red and bottom ring coloured in blue). Rotation of the top ring relative to the bottom ring is illustrated as the tilting of the purple, dotted line linking the two foremost Ser residues. ADEP-28 is shown as sticks and coloured in green. Structured axial pores of ClpP that are present in ADEP-28-bound form are as indicated

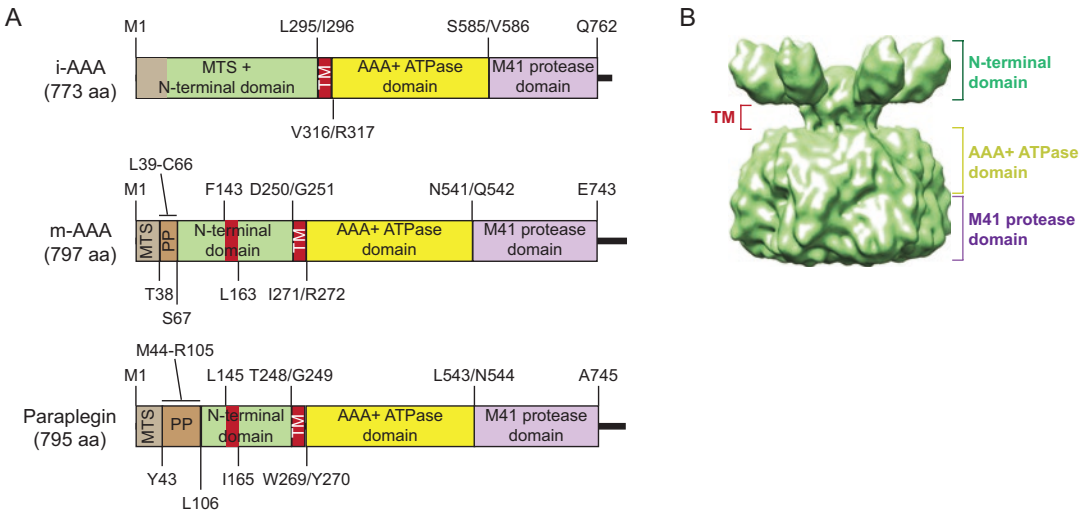

Fig. 8.4 Domain arrangement and structures of i-AAA, m-AAA and paraplegin. (**a**) Domain arrangement of i-AAA (top), m-AAA (middle) and paraplegin (bottom) are illustrated as bar diagrams. All domain boundaries were determined using curated information from UniProt or other public bioinformatics databases. For i-AAA, the boundary between the MTS and N-terminal domain is not well defined and thus is not explicitly shown. For m-AAA and paraplegin, the trans-membrane domain located within the N-terminal domain is indicated in the same red colour as the trans-membrane domain preceding the AAA+ ATPase domain. *PP* propeptide, *TM* trans-membrane domain. (**b**) Cryo-EM structure of the yeast Yta10/12 hetero-oligomer. The location of each functional domain is as indicated

membrane (TM) domain, an AAA+ ATPase domain, and a C-terminal proteolytic domain (Fig. 8.4a), which bears resemblance to LonP1's domain architecture (Fig. 8.1a). The N-terminal domain of i-AAA faces the mitochondrial matrix; its sequence is poorly conserved but is likely to mediate protein-protein interactions [65]. The singular TM domain forms the amphiphilic α-helical barrel upon oligomerization of i-AAA and anchor the protease to the IMM [65]. The AAA+ ATPase domain facilitates the hexamerization of i-AAA and provides the sites of ATP binding and hydrolysis [65]. The proteolytic domain of i-AAA belongs to the M41 metalloprotease family and has an active site with the sequence TAYHESGHAI (residues 596–615; essential residues are underlined) that coordinately binds a single divalent cation (usually Zn^{2+}), unlike LonP1 and ClpP that are serine proteases. Notably, HEXXH is a highly conserved sequence motif among M41 metalloproteases, in which the two His residues bind Zn^{2+} and the Glu residue is the proteolytic residue [65]. Both the AAA+ ATPase and proteolytic domains of i-AAA face the IMS [65].

8.4.1.2 Cellular Function of i-AAA

i-AAA generally functions in the proteolytic clearance of specific proteins that are misfolded in the IMM and IMS. Recognition by i-AAA is mediated by N-terminal or C-terminal degrons on the substrate that become exposed as the substrate protein misfolds [84]. This in turn allows i-AAA to engage in the active unfolding and degradation of these substrates in a processive manner [84]. Notably, in the degradation of integral membrane substrates, the energy required for extracting their TM domains is believed to be supplied by ATP hydrolysis, although the exact mechanism involved remains unclear [34].

In addition, i-AAA performs important regulatory functions via complete or partial proteolysis of specific protein targets in response to environmental stimuli. Notably, i-AAA participates in reciprocal proteolysis with the integral IMM metalloprotease OMA1 under stress

conditions causing mitochondrial depolarization. Specifically, i-AAA degrades OMA1 when the cellular ATP supply is ample, whereas OMA1 degrades i-AAA when the ATP pool is depleted [80]. Importantly, the mutual proteolytic regulation of i-AAA and OMA1 in turn modulates the differential processing of the integral IMM dynamin-like GTPase OPA1. The processing of OPA1 by i-AAA results in the GTPase's activation that promotes maintenance of normal mitochondrial morphology and protein homeostasis, while processing by OMA1 yields to an inactive form of OPA1 that promotes mitochondrial fragmentation instead [80].

Other cellular functions of i-AAA have also been identified. For example, i-AAA was shown to have a direct impact on the lamellar morphology of mitochondrial cristae and the turnover rates of the subunits of Complex I (NDUFB6 and ND1) and Complex IV (COX4) [87]. KD of i-AAA thus results in accumulation of these respiratory subunits that remain unassembled, loss of Complex I activity, increase in oxidative stress protein carbonylation and increased mitochondrial fragmentation, leading to reduced cellular proliferation and sensitization to apoptotic inducing reagents [87]. Furthermore, i-AAA modulates apoptosis by mediating the turnover of PRELID1 (protein of relevant evolutionary and lymphoid interest 1) [43], which facilitates the accumulation of cardiolipin in mitochondrial membranes and suppresses apoptotic onset [76].

8.4.1.3 i-AAA Expression Is Linked to Cancer Progression

Compared to LonP1 and ClpP, the link between i-AAA and cancer is not as well established. The limited available data nevertheless suggests that it may suppress cancer cell growth. For example, the expression of i-AAA has been shown to be downregulated by the proto-oncogene product c-Myc in rat pheochromocytoma cells PC12 [41]. On the other hand, i-AAA overexpression has been shown to suppress the growth of human hepatocellular carcinoma cells SMMC7721 [97]. Furthermore, *YME1L1* is among the genes showing consistent genetic alterations in primary human glioma cells that are associated with poor prognosis [12].

Presently, there are no published data on the development of chemical reagents that can specifically target and modulate i-AAA, or on utilizing i-AAA as a drug target in cancer therapeutics.

8.4.2 m-AAA Metalloprotease

8.4.2.1 Molecular Characteristics of m-AAA

m-AAA is a 797-residue long integral membrane metalloprotease encoded by the *AFG3L2* gene that preferentially localizes to the inner boundary membrane (versus the cristae membrane) of IMM [89]. It shares the same domain architecture as i-AAA (Fig. 8.4a), but with the addition of a propeptide (PP) located between the MTS and the N-terminal domain that is removed upon maturation of the protease, and a second TM region that is located within the N-terminal domain (Fig. 8.4a; TM represented as red bars). Notably, the presence of two TM segments contributes to m-AAA having the opposite topology compared to i-AAA, with the N-terminal domain of m-AAA facing the IMS and its AAA+ and proteolytic domains facing the mitochondrial matrix. As with i-AAA, the formation of m-AAA hexamers is mediated by its AAA+ ATPase domain, and its proteolytic activity is contributed by its M41 metalloprotease domain with the active site sequence VAYHEAGHAV (residues 571–580; essential residues are underlined) for Zn^{2+}-binding and proteolytic catalysis, as discussed previously in Sect. 8.4.1.1.

m-AAA shows normal proteolytic activity as a homo-oligomer or as a hetero-oligomer when in complex with the architecturally similar paraplegin (Fig. 8.4a), a homologue of m-AAA encoded by the nuclear *SPG7* gene that is co-localized to IMM and shares the same topology [25]. Notably, the interaction of m-AAA with paraplegin has been proposed to be important for modulating its substrate specificity [58]. This is further supported by the observation that in

hereditary spastic paraplegia (HSP7), paraplegin deficiency causes axonal degradation in neurons, despite m-AAA homo-oligomers being a functional substitute for m-AAA-paraplegin hetero-oligomers in enzymatic activities [58].

Structural analyses on full-length or individual domains of m-AAA and paraplegin homologues from various species have revealed collectively that these proteins share a highly similar structure, as represented by the yeast m-AAA hetero-oligomer Yta10/12 [63] (Fig. 8.4b). Notably, docking analysis using the atomic structures of domains of m-AAA and paraplegin homologues from human, yeast and *Thermatoga maritima* has shown highly complementary spatial fittings of these domains to Yta10/12 [34].

8.4.2.2 Cellular Function of m-AAA

Both m-AAA and paraplegin are important in maintaining normal cellular respiration by mediating the processing and maturation of various respiratory proteins. Depletion of these proteases leads to various respiratory defects and gives rise to deleterious cellular phenotypes. For example, loss of m-AAA and paraplegin has been shown to impair the proper assembly of Complex I and to increase cellular sensitivity to oxidative stress in human fibroblasts [5]. Similarly, expression of catalytically inactive mutants of m-AAA in respiratory-deficient yeast cells results in impaired OXPHOS due to the reduced activity and expression of cytochrome *c* oxidase subunits, highlighting the role of m-AAA in Complex IV maturation [25].

Notably, m-AAA performs vital functions that are indispensable in neuronal cells. For example, m-AAA is essential in maintaining normal mitochondrial structure and in facilitating proper mitochondrial ribosome assembly in Purkinje cells (large neurons found in the cortex of the cerebellum) [3]. Furthermore, m-AAA also suppresses the hyperphosphorylation of the tau protein and facilitates normal anterograde mitochondrial transport in mouse cortical neurons [56]. Importantly, m-AAA mediates the turnover of the essential subunit of the mitochondrial Ca^{2+} uniporter (MCU), EMRE [57]. This process counters the activity of the MAIP1 complex that promotes EMRE biogenesis and ensures the efficient assembly of MCU with gate-keeping subunits, thereby preventing mitochondrial Ca^{2+} overload and the accelerated opening of the mitochondrial permeability transition pore (MPTP), preserving neuronal cell viability [57].

While mutations in m-AAA and paraplegin that give rise to spinocerebellar ataxia (SCA28) and hereditary spastic paraplegia (HSP7), respectively, have been well-documented and the underlying pathological mechanisms have been characterized [25, 75], there are no current reports that link either m-AAA or paraplegin to cancer.

8.5 Concluding Remarks

The studies discussed here have provided new insights for deciphering the molecular mechanisms by which mitochondrial AAA+ proteases contribute to cancer pathology. These insights are invaluable for the continuous development of new cancer therapeutic strategies. Importantly, the structural characterization of AAA+ proteases in complex with their respective activity-modulating compounds has revealed key allosteric events to provide the essential structural basis for improving the potency and specificity of these compounds and for developing new compounds that will produce the same biomolecular effects in the AAA+ proteases via alternative mechanisms. Furthermore, the characterization of cellular events induced by these compounds leading up to apoptosis has revealed distinct pharmacological vulnerabilities in cancer cells that can be exploited via the synergistic use of multiple drugs, or by creating hybrid molecules that combine the pharmacological characteristics of the originals. Hence, continued efforts at understanding the structure and function of these AAA+ proteases are critical to the development of novel anticancer compounds.

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