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Powering Down the Mitochondrial LonP1 Protease: A Novel Strategy for Anticancer Therapeutics

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Abstract

Introduction—Mitochondrial LonP1 is an ATP-powered protease that also functions as an ATP-dependent chaperone. LonP1 plays a pivotal role in regulating mitochondrial proteostasis, metabolism and cell stress responses. Cancer cells exploit the functions of LonP1 to combat oncogenic stressors such as hypoxia, proteotoxicity and oxidative stress, and to reprogram energy metabolism enabling cancer cell proliferation, chemoresistance and metastasis.

Areas covered—LonP1 has emerged as a potential target for anti-cancer therapeutics. We review how cytoprotective functions of LonP1 can be leveraged by cancer cells to support oncogenic growth, proliferation, and survival. We also offer insights into small molecule inhibitors that target LonP1 by two distinct mechanisms: competitive inhibition of its protease activity and allosteric inhibition of its ATPase activity, both of which are crucial for its protease and chaperone functions.

Expert opinion—We highlight advantages of identifying specific, high-affinity allosteric inhibitors blocking the ATPase activity of LonP1. The future discovery of such inhibitors has potential application either alone or in conjunction with other anticancer agents, presenting an innovative approach and target for cancer therapeutics.

Keywords

Cancer; LonP1; Lon protease; ATP-powered protease; AAA^+ protease; mitochondria; CDDO-Me; allosteric inhibitor; drug development

1. Introduction

Oncogenic transformation of somatic cells is driven by a complex interplay of genetic and epigenetic alterations, which either activate oncogene-dependent pathways or deactivate

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tumor-suppressive mechanisms. Genotoxic stress often triggers replication stress, which is a critical initiator of tumorigenesis in its early stages. Furthermore, aneuploidy frequently arises early in tumorigenesis, disrupting the balance of proteins and promoting proteotoxicity caused by protein misfolding, misassembly and aggregation. In their quest to proliferate and survive, cancer cells must overcome other cellular stressors, which include hypoxia, oxidative and metabolic stress, amongst others. Cancer cells meet these challenges by adaptively reprogramming normal cytoprotective mechanisms, to sustain and increase their oncogenic potential.

Mitochondrial LonP1 has emerged as a potential target for anti-cancer therapeutics. It belongs to the superfamily of **A**TPases **A**ssociated with diverse cellular **A**ctivities (AAA$^+$), which govern various cellular processes including DNA replication, gene expression, membrane fusion, and protein degradation [1]. Within human mitochondria, there are four AAA$^+$ proteases- LonP1 and ClpXP are soluble complexes in the matrix, while m-AAA and i-AAA are transmembrane complexes with active sites in the matrix and intermembrane space, respectively [1]. LonP1 plays versatile roles in regulating cellular homeostasis and mediating adaptive responses to cell stress. Here, we review how the cytoprotective functions of LonP1 are likely exploited by cancer cells to promote their oncogenic growth, proliferation, and survival. We provide perspective on recently identified small molecule inhibitors of LonP1 that allosterically inhibit its ATPase activity necessary for protease and chaperone-like functions. We suggest that a novel and promising therapeutic approach lies in targeting the allosteric inhibition of ATP hydrolysis by LonP1 and other mitochondrial matrix AAA$^+$ proteases. Administering these inhibitors, either as standalone treatments or in combination to potentiate existing anti-cancer chemotherapeutics, may be an innovative avenue for effectively combating cancer.

2. LonP1 in cellular homeostasis and cell stress responses

Human LonP1 is highly conserved throughout evolution from archaea, eubacteria to eukaryotes. In mice, the homozygous deletion of LonP1 causes early embryonic lethality [2]. In eukaryotes, LonP1 is located in the mitochondrial matrix where it preserves cellular homeostasis and responds to cell stress [3] (Figure 1).

Multifaceted roles of LonP1 in the maintenance of mitochondrial homeostasis.

LonP1 plays diverse roles in regulating mitochondrial proteostasis, metabolism and cell stress responses. As a protein quality control protease, it degrades misfolded, misassembled, and oxidatively damaged proteins [3]. As a regulator of mitochondrial metabolism LonP1 also degrades key rate-limiting proteins involved, for example, in cholesterol metabolism, heme biosynthesis, and mitochondrial transcription. LonP1 degrades: 1) StAR, the steroidogenic acute regulatory protein, which mediates the rate-limiting transfer of cholesterol from the mitochondrial outer membrane to the inner membrane [4]; 2) ALAS-1, delta-aminolevulinate synthase 1, which catalyzes the rate-limiting step in heme biosynthesis [5]; and TFAM, transcription factor A of mitochondria, which is required for the activation of mitochondrial transcription and the maintenance of mitochondrial DNA (mtDNA) [6]. LonP1 also functions as an ATP-dependent chaperone, independent

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of its proteolytic activity, which can promote protein folding and assembly, and prevent aggregation. LonP1 is required for the proper folding of mitochondrial Hsp70 and its cognate co-chaperone DNAJA3/Tid1 [7]. LonP1 helps maintain the solubility of newly synthesized polypeptides translocated into the mitochondrial matrix, thereby preventing aggregation and supporting proper folding through its ATPase (not protease) activity [8]. The chaperone activity of LonP1 has been implicated in facilitating the assembly and regulation of oxidative phosphorylation (OXPHOS) complexes [9].

As a regulator of cellular metabolism, LonP1 is critical for modulating pyruvate dehydrogenase (PDH) [10,11], the gatekeeper linking glycolysis with the TCA cycle. In isolated mouse heart mitochondria, LonP1 degrades PDH kinase 4 (PDK4) [10], preventing the phosphorylation of the PDH E1α subunit, which inhibits PDH. In human fibroblasts, LonP1 also degrades phosphoE1α, providing another mechanism by which it regulates PDH activity [11]. Together, these data suggest that LonP1 fine-tunes glucose and fatty acid oxidation by modulating the phosphorylation and activity of PDH.

Additionally, LonP1 has a DNA-binding property that is conserved from bacteria to humans [12–14]. In human cells and worms, LonP1 binds mtDNA within the non-coding region [13,15] where replication and transcription are initiated. It plays a critical role in maintaining the integrity and expression of mtDNA, which encode essential subunits of OXPHOS complexes, as well as tRNAs and rRNAs vital for mitochondrial translation [6,13]. Notably, the embryonic demise of homozygous LonP1 null mice coincides with a substantial decline in mtDNA copy number [2].

LonP1 is crucial during pre- and post- natal development (Figure 1). Homozygous, compound heterozygous, and de novo mutations in the *LONP1* gene cause a broad spectrum of rare developmental diseases, which can affect multiple organ systems as in CODAS syndrome, characterized by **c**erebral, **o**cular, **a**uricular, **d**ental, and **s**keletal anomalies [16,17]. Dysfunctional LonP1 in CODAS syndrome may explain the ocular and skeletal abnormalities, which are the key clinical hallmarks of this disease. LonP1 may be indispensable for protecting tissues during cartilage formation and bone ossification where ROS-dependent signaling is heightened, and in interior regions of the eye that are avascular and relatively hypoxic.

Roles of LonP1 in cell stress responses.

LonP1 mitigates cell stress in several ways (Figure 1). During hypoxia, low oxygen up-regulates LonP1, which facilitates the remodeling of the cytochrome c oxidase complex (COX) in the electron transport chain (ETC) [18]. Hypoxia-inducible factor-1 alpha (HIF-1α), activates the transcription of LonP1, and increased levels of LonP1 degrade the COX subunit 4–1 [18]. At the same time, HIF-1α activates the expression of an alternate subunit COX4–2, which is assembled into the COX complex. This subunit switching has been proposed to optimize the efficiency of the ETC to hypoxic conditions [18].

In response to oxidative stress, LonP1 degrades oxidatively proteins, but it also employs its protease and chaperone activities to abrogate deleterious processes that threaten cell survival. A study in neuroblastoma and HeLa cell lines showed that LonP1 functions

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in concert with ClpXP to extinguish ROS in mitochondria by degrading the domain of Complex I of the ETC, which generates ROS [19]. Another study using colon cancer cells and mouse embryonic fibroblasts showed that elevated ROS prompts p53 translocation from the cytosol into the mitochondrial matrix triggering necrosis [20]. Similarly, experiments using oral squamous carcinoma and HEK293(T) cell lines showed that oxidative stress led to p53 accumulation in the matrix, resulting in cytochrome c release and apoptosis [21]. However, overexpression of wild-type LonP1 almost completely abrogated cytochrome c release and apoptosis, whereas a chaperone mutant of LonP1 (K529R) failed to block apoptosis elicited by oxidative stress [21].

LonP1 participates in the integrated stress response (ISR) as well as the unfolded protein response in the endoplasmic reticulum (UPR^{ER}) and mitochondria (UPR^{mt}) [22,23]. UPR^{ER} and UPR^{mt} are characteristically induced by misfolded and misassembled proteins in these respective organelles, whereas the ISR is induced by amino acid deprivation, viral infection, heme deficiency and other cellular stressors including ER stress. Although the knockout or knockdown of yeast and mammalian *Lon* homologs lead to mitochondrial dysmorphology and the accumulation of electron dense inclusions within mitochondria, which are often large and extensive, organisms and cells continue to proliferate often comparably to corresponding controls and do not die prematurely [16,24]. What remains unanswered is whether these inclusions are proteinaceous or elemental in nature, and whether they represent a cytoprotective response by sequestering toxic forms of proteins and elements (e.g., calcium, iron, phosphate).

A role for LonP1 in regulating metabolic flexibility is demonstrated by its selective degradation of PDK4 [10], a component of the PDH complex bound to the E2 subunit [10]. Metabolic flexibility is the ability of cells to generate ATP by preferentially using glucose or lipids (e.g., fatty acids and ketone bodies). For instance, in a cardiac muscle cell line cultured with glucose, LonP1 rapidly degrades PDK4 [10]. However, when these cells are cultured with fatty acids, PDK4 degradation is suppressed leading to phosphorylation of PDH E1α and PDH inhibition [10]. Additionally, in fibroblasts cultured in glucose, LonP1 degrades phosphoE1α, thereby preventing PDH inhibition [11]. Further work is required to understand the coordinated roles of LonP1 in regulating metabolic flexibility.

3. Exploitation of LonP1 in Cancer: A Survival Strategy

Cancer cells face various oncogenic stressors like hypoxia, oxidative, proteotoxic, and metabolic stress, which align with processes influenced by LonP1 (Figure 1, red shading). In leukemia cell lines, upregulated LonP1 degrades COX4–1 and enhances COX4–2 adapting OXPHOS efficiency to hypoxia [18,25]. The same study also showed increased levels of PDH kinase 1 (PDK1) during hypoxia [25], raising the possibility that modulation of PDK1 degradation by LonP1 may be involved. Additionally, in prostate adenocarcinoma cells, LonP1 and ClpP (the proteolytic component of ClpXP) mitigate oxidative and proteotoxic stress as the knockdown of both AAA^+ proteases increased production of mitochondrial ROS, accumulation of aggregated and misfolded proteins, and induced cell death [26]. A key hallmark of cancer cells is the reprogramming of metabolism from OXPHOS to aerobic glycolysis (i.e., Warburg effect). One mechanism promoting this metabolic switch is via

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PDH inhibition. LonP1 may be involved in mediating this shift by increased degradation of PDH phosphatases (PDPs) and decreased degradation of PDKs and phosphoE1α; studies are required to test this possibility. Alternatively, a study in a prostate cancer cell line subjected to hypoxia, showed that LonP1 safeguards OXPHOS. LonP1 was phosphorylated by Akt (aka protein kinase B), leading to its enhanced protein quality control of OXPHOS complexes, and dampened ROS generation, resulting in increased tumor cell migration and invasion [27].

4. LonP1 Expression in Cancer Cells

Normal tissues- proteomics and transcriptomics.

According to Human Protein Atlas (HPA) proteomics data, LonP1 is constitutively expressed throughout most tissues of the body. The HPA uses an immunohistochemistry approach to annotate protein expression *in situ* using 44 normal tissue types from 144 individuals [28]. High protein expression levels are seen in the adrenal glands, gastrointestinal tract (appendix, colon, duodenum, rectum), gallbladder, and kidneys. The highest RNA expression is seen in adrenal gland and liver tissues based on the HPA and the Genotype-Tissue Expression (GTEx) RNA-Seq datasets [28,29].

Cancer cell lines and tissues- proteomics and transcriptomics.

HPA cancer proteomics data (216 cancer patients, 20 different cancer types) show most malignant tissues displayed immunoreactivity to anti-LonP1 antibodies, with the highest expression in colon/rectum, prostate, head/neck, and liver [28]. Transcriptomics data from The Cancer Genome Atlas (TCGA) (>20,000 primary cancer and matched normal samples across 33 cancer types) show LonP1 expression across all TCGA cancer types. In matched samples, increased LonP1 transcripts were seen in cervical squamous cell carcinoma, cholangiocarcinoma, colon adenocarcinoma, and esophageal carcinoma. Although higher levels of LonP1 are not prognostic, they are associated with lower survival in multiple cancers, including low-grade glioma and invasive breast carcinoma [30].

Mutation Rate.

Alterations in the *LONP1* gene are seen in 5.54% of cases profiled in the 2020 TCGA pan-cancer analysis of whole genomes, and only 2.64% were amplifications [30].

LonP1 Isoforms.

Recently in 2022, one study [31] investigated the differential expression of three alternatively spliced LonP1 isoforms (ISO −1, −2, and −3) across cancers based on the TGCA Splicing Variants Database (TSVdb). To our knowledge, this is the only study that has addressed the expression of these LonP1 isoforms. ISO1 (959 aa) contains the complete amino-terminal mitochondrial targeting sequence (MTS); ISO2 (859 aa), has a truncated MTS; and ISO3 (763 aa), lacks an MTS. As compared to normal tissues, transcripts of ISO1 were significantly increased in 4 of the 9 cancer types analyzed- lung, prostate, breast, and bladder; while ISO2 were significantly elevated in 8 of 9 cancer types- prostate, breast, colon, rectum, cervical, head/neck, bladder, and renal. By contrast, transcripts of ISO3 were undetected in normal tissues except for head/neck and bladder and showed increased

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expression in 6 of 9 cancer types- breast, rectum, cervical, head/neck, bladder, and renal. To determine their subcellular localization, GFP fusion proteins were expressed in colorectal cancer cells; ISO1 was exclusively in mitochondria, ISO2 in mitochondria and cytoplasm, and ISO3 exclusively in the cytosol. Further work is needed to determine whether ISO2 and ISO3 have ATPase, protease, and chaperone-like activities. Future studies examining LonP1 must be cognizant of these isoforms.

5. Small Molecule Inhibitors of LonP1

Only a handful of small molecules have been shown to inhibit LonP1; none are specific or of high affinity. Bortezomib and MG262 are dipeptide boronic acid inhibitors of the proteasome [32], which also inhibit LonP1 at substantially lower efficacy [33,34]. When comparing these inhibitors in parallel, bortezomib inhibited the peptidase activity of purified human LonP1 with an IC_{50} of 17 nM, which is ~7 orders of magnitude lower activity than that of the 20S proteasome with an IC_{50} of 2.3 nM [6] (Table 1). The cryoEM structure of bortezomib bound to human LonP1 shows that it binds at the proteolytic active site [35]. Bortezomib is used clinically to treat multiple myeloma and mantle cell lymphoma [32]. As the knockdown of LonP1 in mantle cell lymphoma cells leads to cell death [24], it is unknown whether the inhibition of LonP1 by bortezomib might be of therapeutic benefit in treating hematologic malignancies. One study showed that in multiple myeloma cells, increased LonP1 levels resulted in decreased efficacy of proteasome inhibitors, conferring partial resistance [36]. This suggests that the simultaneous inhibition of the proteasome and LonP1 by a single compound or by the combination of protease-specific compounds may be an effective approach for abrogating resistance to proteasome inhibition and effectively treating these hematologic cancers.

Non-peptide inhibitors of LonP1 have also been described. A boronic acid-based non-peptide inhibitor called compound 14 shows high affinity for LonP1 with an IC_{50} of 0.059 μM, with no cross-reactivity with the proteasome [37] (Table 1). Whether this compound inhibits LonP1-mediated degradation of protein substrates in mitochondria has not been solidly demonstrated. Rather, compound 14 was shown to increase the levels of certain proteins co-immunoprecipitated with LonP1 in compound-treated cells [37]. In addition, another study showed that non-peptide coumarinic derivatives compounds 4 to 8 inhibit recombinant LonP1 [38] (Table 1). The ability to inhibit mitochondrial LonP1 in cells was not examined.

Additionally, two classes of plant-derived LonP1 inhibitors, Obtusilactone A and Sesamin, have been shown to block proteolysis by recombinant LonP1 with IC_{50} values of 34.1 μM and 19.9 μM, respectively, and in cultured cells they stabilize the reported endogenous protein substrate mitochondrial aconitase [39]. Computer modeling using the crystal structure of the *E. coli* Lon protease domain suggest that these compounds interact with the proteolytic active site. These compounds were found to induce apoptosis in human lung cancer cells. However, further work is required to determine whether the inhibition of LonP1 by these compounds plays a role in promoting cancer cell death.

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More recent work demonstrates that the synthetic triterpenoids CDDO and its derivatives inhibit LonP1 via a novel allosteric mechanism, blocking ATPase activity in a non-competitive manner [34]. ATP hydrolysis is required for the degradation of folded and partially folded protein substrates and for the chaperone function of LonP1 [7,9,40]. By contrast, the cleavage of small peptides does not require ATP hydrolysis, however, peptidase activity is stimulated by binding of ATP or non-hydrolyzable ATP analogs [41]. CDDO derivatives inhibit the ATP-dependent protease activity of purified LonP1 in the micromolar range- CDDO (IC_{50} = 13 μM), CDDO-Me (methyl) (IC_{50} = 1.9 μM), and CDDO-Im (imidazole) (IC_{50} = 2 μM). Notably, CDDO-Me selectively inhibits the ATPase activity of purified LonP1 but not that of the purified 26S proteasome. The ability of CDDO derivatives to inhibit LonP1 within the mitochondrial matrix is supported by data showing the co-localization of biotinylated CDDO with the organelle stain Mitotracker Green [42] and, moreover, data showing that these compounds inhibit LonP1-dependent degradation of its endogenous protein substrates [6,34]. Importantly, it must be noted that CDDO derivatives have multiple cellular targets and are not LonP1-specific. Their direct binding sites have been identified for Keap1 [43], IκB kinase beta (IKK-β) [44], Jak1 and Stat3 [45]. Nevertheless, CDDO and its derivatives offer opportunities for identifying the specific allosteric binding pocket(s) within LonP1 and for the discovery of specific, high affinity compounds effectively inhibiting this key AAA^+ protease.

6. Expert opinion

Allosteric modulators provide several physicochemical advantages compared to compounds that bind to the active site of a target protein. These modulators are typically lipophilic, enabling them to establish stronger hydrophobic interactions with higher affinity and selectivity that can reduce off-target effects. Additionally, these compounds tend to exhibit a high degree of rigidity, which confers greater overall stability. The discovery of specific, high-affinity allosteric inhibitors of the LonP1 ATPase holds promise for disabling the protease and chaperone-like functions of this stress response protein, which is hijacked by cancer cells for their proliferative advantage.

The combinatorial inhibition of AAA^+ proteases such as the 26S proteasome, LonP1 and ClpXP is a potentially efficacious and novel approach for anticancer therapeutics. Simultaneously inhibiting LonP1 and the proteasome could potentially overcome the partial resistance to proteasome inhibitors observed in multiple myeloma [36]. Similarly, a combined strategy for inhibiting LonP1 and the mitochondrial ATP-dependent protease ClpXP may be efficacious in promoting cancer cell death as suggested by the finding that depleting the *LONP1* and *CLPP* genes in prostate cancer cells synergistically attenuated cell growth and induced cell death [26]. Small molecules inhibiting or hyperactivating ClpP have been identified, which selectively induce cancer cell death to a greater extent than normal cell death [46], further supporting a combinatorial therapeutic approach. In the future, a more detailed and comprehensive mechanistic understanding of cancer cell reliance on mitochondrial AAA+ proteases like LonP1, ClpXP and related proteases is essential. This knowledge will inform the development of small molecules capable of disabling these specific functions, thereby facilitating the discovery of innovative anticancer therapeutics and approaches.

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Abbreviations

AAA$^+$	ATPases Associated with diverse cellular Activities
Akt	Protein Kinase B
CDDO	2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid
CDDO-Im	CDDO-imidazole
CDDO-Me	CDDO-methyl
COX	Cytochrome c oxidase complex
ETC	Electron transport chain
GTEx	Genotype-Tissue Expression
HIF-1α	Hypoxia-inducible factor-1 alpha
HPA	Human Protein Atlas
ISR	Integrated Stress Response
mtDNA	Mitochondrial DNA
OXPHOS	Oxidative phosphorylation
PDH	Pyruvate dehydrogenase
PDK1 and PDK4	PDH kinases 1 and 4
PDP	PDH phosphatase
ROS	Reactive oxygen species
UPR^{ER}	Unfolded Protein Response of the Endoplasmic Reticulum
UPR^{mt}	Mitochondrial Unfolded Protein Response
TCGA	The Cancer Genome Atlas
TSVdb	TGCA Splicing Variants Database

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Article highlights

- LonP1 has multifaceted functions as an ATP-dependent protease that degrades abnormal proteins and rate-limiting protein in mitochondrial metabolism, as an ATP-dependent chaperone promoting protein folding and assembly, and preventing aggregation, and as an evolutionarily conserved DNA-binding protein.
- Upregulation of LonP1 in many cancer tissues and its involvement in cancer-associated pathways make it a promising new target for pharmacological intervention.
- Allosteric inhibitors of LonP1 that block its ATPase activity provide an opportunity for identifying the allosteric compound binding pocket(s) and the discovery of specific, high-affinity inhibitors.
- The combination of LonP1 inhibitors and other cytotoxic agents may be a novel approach to overcome drug resistance in some cancers.

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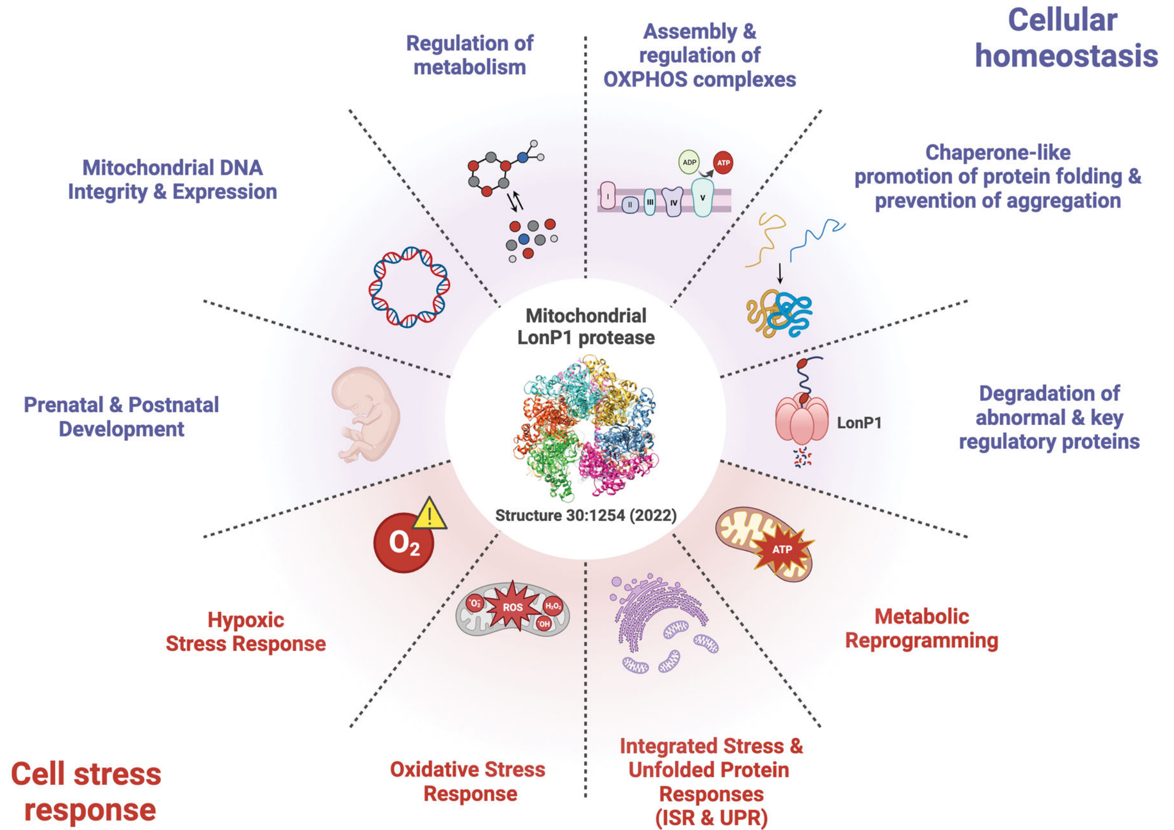

Figure 1.

Functions of LonP1 in cellular homeostasis and cell stress responses that can be exploited by cancer cells (created using BioRender.com).

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Table 1.

Small molecule inhibitors of LonP1.

Drug	IC50	Mechanism of Action	Reference
Bortezomib	17 nM	Proteolytic active site inhibitor	[6]
CDDO	13 μM	Allosteric ATPase inhibitor	[34]
CDDO-Me	1.9 μM	Allosteric ATPase inhibitor	[34]
CDDO-Im	2 μM	Allosteric ATPase inhibitor	[34]
Compound 14[1]	0.059 μM	Proteolytic active site inhibitor[2]	[37]
Coumarinic derivatives 4–8	nd	Proteolytic active site inhibitor	[38]
MG262	122 nM	Proteolytic active site inhibitor	[33]
Obtusilactone A	34.1 μM	Proteolytic active site inhibitor[2]	[39]
Sesamin	19.9 μM	Proteolytic active site inhibitor[2]	[39]

[1] Boronic acid-based non-peptide inhibitor

[2] Suggested by computational modelling

nd – Not determined