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Proteostasis and Aggresomes – as possible target for cancer therapy

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ABSTRACT

Efficient protein metabolism is essential to maintain intracellular homeostasis. Especially when the concentration of misfolded proteins increases. Cells evolve complex mechanisms to regulate protein synthesis, refolding, and degradation. There are two main mechanisms of protein degradation: the ubiquitin-proteasome system (UPS) is responsible for the degradation of small soluble proteins, and autophagy associated with hydrolytic lysosomal enzymes is responsible for the degradation of larger insoluble structures. In the process of carcinogenesis, genomic instability increases, and control mechanisms become insufficient. As a result, these two degradation pathways become insufficient, and the concentration of abnormal proteins increases. This favors the formation of aggresomes – the pericentriolar aggregates containing mostly insoluble proteins. Many studies confirm the cytoprotective nature of these structures.

Understanding the basis of molecular disorders in the cancer cell is crucial for developing novel therapies. Drugs affecting protein turnover induce endoplasmic reticulum stress. They also act preferentially to the cancer cells due to increased protein production. Proteasome inhibitors (PIs) – bortezomib, carfilzomib, and ixazomib – have been used to treat multiple myeloma and mantle cell lymphoma. PIs also act on numerous signaling pathways. In addition, immune-selective PIs are under evaluation in treating auto-immune diseases. Panobinostat – an inhibitor of histone deacetylases, including HDAC6, the regulator of transport to the aggresome, is also used in treating multiple myeloma. More research on drugs affecting proteostasis is needed to gain new therapeutic possibilities. Newly synthesized drugs enable more options in cancer therapy and the treatment of other diseases.

INTRODUCTION

Cellular metabolism and architecture complexity require constantly flowing newly synthesized proteins through the cell lifespan. An eukaryotic cell contains from 100 million to 100 billion molecules of proteins (Harper, 2016). For management of such huge number of components, cells develop a precise system to regulate protein synthesis and degradation. Similarly, the newly synthesized linear amino acid polymer becomes folded to the correct three-dimensional structure and is transported to a defined subcellular space to fulfill its function. If any stage of this mechanism fails, misfolded and incorrectly localized polypeptides disrupt other cellular processes (Chiti, 2017). Protein folding failure may occur due to changes in amino acid sequence resulting from DNA mutations, errors in the transcription and translation, or imbalance in protein synthesis (Bonifacino, 1989).

On the other hand, environmental stressors such as temperature, osmotic pressure, oxidative stress, or viral infections can also disturb protein folding (Johnston, 1998). Moreover, by exposure of hydrophobic domains, misfolded or denatured proteins may aggregate into toxic polymers (Garcia-Mata, 1999). Therefore, evolution equips cells with mechanisms that help them to manage unfolded or damaged proteins.

The system that is responsible for maintaining of protein homeostasis is called the proteostasis network. That system may move proteins with nonnative conformations to the pathways of refolding, degradation, or formation of aggregates. Indeed, years of research on cell biology revealed that these three mechanisms are closely related, complement each other, and many enzymes participate in two or all patterns (Johnston, 2021). Proteotoxicity leads to compensatory mechanisms of cellular response by reducing global protein transcription, producing proteostasis network components, and recruiting present ones. Misfolded proteins, primarily by recognition of presenting hydrophobic domains, are bonded by protein folding enzymes – molecular chaperones. Among many molecular chaperones, stress-induced heat-shock proteins 70 and 90 (HSP70, HSP90) are essential in response to proteotoxic stress. They rearrange damaged proteins and assist in the folding of *de novo* translated polypeptides, which are the most vulnerable to stress-induced misfolding (Jayaraj, 2020). Conversely, failure of protein refolding directs them to the degradation pathway. Also, an overload of chaperones' capacity and an insufficient supply of amino acids push proteins into the clearance system.

We can distinguish two main clearance pathways: the ubiquitin-proteasome system (UPS) and system associated with autophagy and involving hydrolytic lysosomal enzymes. UPS mainly degrades relatively small soluble proteins, while larger insoluble macromolecules and organelles are surrounded and degraded

by the autophagosome. These two pathways exist pararelly and complement each other. During endoplasmic reticulum (ER) stress proteins are retrotranslocated from the ER into the cytosol and exposed to degradation via UPS. When the amount of misfolded protein in ER overwhelms cellular transport capacity to cytosol, autophagy is induced to handle protein overload (Almanza, 2019).

USP – ON GUARD OF PROTEOSTASIS

The USP remains the most exploited cellular clearance pathway since it is ubiquitous and present in every eukaryotic cell in both cytosol and in nucleoplasm. UPS degrades over 80% of all cytoplasmic proteins (Glickman, 2002). Despite the clearance function, degradation via USP participates in various biological processes such as cell cycle regulation, signal transduction, and gene transcription (Wojcik, 2002).

The crucial mediator of UPS degradation and an essential label in protein trafficking is small globular polypeptide, ubiquitin (Ub) – it contains 76-amino acids and weights 7 kDa. For degradation by UPS at least four Ub has to be attached to the protein substrate. This highly conservative polypeptide is covalently attached by C-terminal glycine to lysine residues in protein structure in the ubiquitination process (Haglund, 2005). Although, recent reports confirm that, Ub could be linked to threonine, serine, and cysteine too (cytowanie). It is worth emphasizing that lysine – substrate bond in ubiquitin is the most intensively studied in eukaryotic cells (Ciechanover, 2014). Ub in its structure has seven lysine residues to be linked to other Ub-forming polyubiquitin chains. We can distinguish three types of ubiquitin attachment to a given protein depending on the number of ubiquitin molecules. Mono-ubiquitination: when a single Ub is linked to the substrate, resulting in transfer to endosomes, nuclear export, signal for DNA repair, and regulation of DNA transcription via chromatin remodeling. Multi-ubiquitination: when several single ubiquitin molecules are attached to several residues along the substrate, resulting in endo-cytosis. Lastly, polyubiquitination: the formation of polyubiquitin chains attached to the protein by the first ubiquitin (Pickart, 2004).

To add some more complexity to Ub-dependent protein trafficking, Ub in polyubiquitin chains can be linked to each other by one of seven lysines present in the Ub molecule, which determines the destination of protein with the attached Ub chain. For example, linking through 63 lysine (K63) leads proteins to aggre-gate with p62/SQSTM1 and degradation via autophagy (Wooten, 2008). Furthermore, the K63 chain also influences DNA repair and kinase activation (Acconcia, 2009). Finally, a chain consisting of at least four Ub monomers linked via K48 or K48/K11 is considered the most effective signal for degradation in proteasomes (Yau, 2017).

Polyubiquitination is controlled by three enzymes responsible for ubiquitin attachment. At the start, Ub is activated by forming an ATP-dependent thioester bond with the ubiquitin-activating enzyme (E1). Then activated Ub passes into the ubiquitin-conjugating enzyme (E2). Next, Ub linked with E2 is transferred to the target protein by ubiquitin-ligase enzyme (E3). Those actions repeat several times by bonding the next Ub to the previous one, creating a polyubiquitin chain (Hershko, 1998). In another pattern, when molecular chaperones fail to refold damaged proteins, another ubiquitin-ligase enzyme – cochaperone ubiquitin ligase carboxyl terminal of Hsp70/Hsp90 interacting protein (CHIP), performs ubiquitination and escorts proteins to the proteasome (Wojcik, 2002).

An ATP-dependent multi-catalytic protease (2,5 MDa) makes up the heart of the UPS – it is named 26S proteasome and is composed of 20S proteasome as tunnel-shaped core built up by 4 rings, each consisting of 7 globular particles: two peripheral alpha rings and two central beta rings; and two 19S regulatory subunits performing ubiquitin reception, deubiquitylating and unfolding amino acid chain, localized at the sides of the 20S proteasome. Three subunits in each beta ring determine the proteolytic activity of the 26S proteasome: beta1 with caspase-like activity, beta2 with trypsin-like activity, and beta5 with chymotrypsin-like activity (Manasanch, 2017). Oligopeptides leaving the 26S proteasome are further degraded by endo- and exopeptidases into amino acids (Saric, 2004).

UPS AND IMMUNITY

The UPS is involved in antigen turnover, especially endocellular antigen presentation, including viral antigen presentation. The IFN gamma facilitates both proteasome and antigen presentation for adequate clearance of viral infection (Seifert, 2010).

In somatic cells stimulated by interferon (INF)-gamma immunosubunits beta1i, beta2i, and beta5i are incorporated into proteasome structure in the places of beta1, beta2, and beta5 subunits. This newly rearranged structure is called an immunoproteasome (Basler, 2021). Changes in the structure of immunoproteasome result in altered proteolytic activity: Beta1-dependent caspase-like activity is reduced. Meanwhile, Beta5-dependent chymotrypsin-like activity is enormously increased. Resulting of these changes in proteolytic patterns, oligopeptides leaving proteasome mostly have hydrophobic amino acids on the c-terminal and are more efficiently presented by histocompatibility complex class I (MHC class I) (Groettrup, 2010). Through these changes, immunoproteasome increases peptide supply and provides more efficient antigen processing. Moreover, immunoproteasome helps to maintain proteostasis in the condition of increased oxidative stress promoted by INFs (Seifert, 2010). The altered proteasome is also involved in many processes inside the immunologic system or pathophysiology of neurodegenerative and inflammatory diseases (Basler, 2021).

Immunoproteasome manages with many different proteins, including DRiPS, which are particularly significant considering immune response (Seifert, 2010). Around 30% of newly synthesized proteins with an extremely short half-life (under 10 minutes) are called defective ribosomal products (DRiPs). They are defined as products of all mistakes in transcribing DNA information into protein (Qian, 2006; Anton, 2014). Even then, degradation via proteasome generates large quantities of antigens presented by MHC class I. Up to 70% of all antigens presented on cell surface come from DRiPs of cellular, viral, and neoplastic origin (Dolan, 2011). Moreover, they perform a crucial role in anti-viral response by rapidly presenting DRiPs-originated viral antigens enabling cytotoxic CD8+ T lymphocytes to recognize and kill infected cells before releasing new virions (Anton, 2014).

AUTOPHAGY AND UPS – ONE SYSTEM WITH TWO FACES

Cells under stress conditions, especially retrieval of nutrients, may activate autophagocytosis to obtain substrates for metabolism. While only specifically marked, small soluble proteins can be degraded by the proteasome, autophagy degrades protein polymers and all subcellular structures. Lysosomal hydrolases can digest different macromolecules: lipids, carbohydrates, proteins, intracellular aggregates, and even entire organelles inside endosomes fused with lysosomes. In the diversity of pathways leading to lysosome-mediated degradation, we can distinguish processes targeting monomeric molecules such as microautophagy and chaperone-mediated autophagy (Tekirdag, 2018). It is worth to add that macroautophagy is the essential type of autophagy in protein degradation. First, molecules are encircled by double-membraned structures called the autophagosome, then the material inside and the inner membrane are designated into degradation. Autophagy is considered the primary cellular response to acute nutrient depletion and is also involved in the degradation of protein aggregates, impaired organelles, and intracellular pathogens (Oh, 2018). Proteasomes can also be targeted by Ub and trafficked into autophagosomes in the process called proteophagy, probably as a mechanism of proteasome deactivation (Cohen-Kaplan, 2016). In amino acid deprivation, degradation via UPS is the first pathway to maintaining an adequate nutrient supply. Progressive amino acid deficiency blocks transcription and activates autophagy via the mTOR pathway (Suraweera, 2012).

Activation of ULK1 kinase in the pathway of the undefined enzymatic cascade induces the formation of the autophagosome and anchoring many members of the ATG8 protein family (a.o. LC3) to the phospholipids of growing membranes. Autophagic receptors (a.o. p62/SQSTM1), which direct particles to autophagosomes, have unique domains – LC3-interacting regions (Pohl, 2019). At the final stage of autophagy on SNARE-mediated fusion, autophagosomal and lysosomal membranes fuse. Then released lysosomal hydrolase in an acidic environment digests the inner autophagosomal membrane and phagosome contents into individual nutrients, which are transported to the cytosol (Yu, 2018). It is worth mentioning that p62 and other receptors equipped with Ubiquitin binding domains (UBDs) are situated at the crossroads of ubiquitination and autophagy (Rogov, 2014).

Interestingly, up to 50% of all selective autophagic particles are marked by Ub (Khaminets, 2016). p62 also acts in the UPS via its proteasome binding domains (PBD) by escorting ubiquitinylated molecules to the proteasome. According to recent studies, the shift between proteasomal and lysosomal degradation depends on the opposite polymerization and dimerization of p62 (Wurzer, 2015). Increased concentration of free Ub under prolonged stress or proteasome insufficiency probably block dimerization and promotes polymerization of p62, enhancing autophagy-dependent lysosomal degradation (Peng, 2017). Research

revealed complex correlations between UPS and autophagy, which preserve proteostasis. The pathways of these two systems overlap and complement each other. Nevertheless, in terms of insufficient degradation and formation of aggregates, a third cytoprotective mechanism is included – the formation of aggresomes.

AGGRESOME – SUBTLE RUBBISH BIN

Aggresomes are aggregates containing insoluble proteins, usually misfolded. They may also contain other molecules like cytoskeletal elements. Aggresomes are generally localized in the perinuclear region (Garcia-Mata, 1999; Johnston, 1998).

The massive juxtanuclear aggregates, later named aggresomes, had been described for the first time by Wójcik et al. in HeLa cells under proteasome inhibition. Researchers detected proteins designated for degradation, ubiquitin, and proteasome in these large insoluble aggregates and hypothesized lysosomal degradation of these structures under stress-free conditions (Wojcik, 1996). After the name "Aggresome" was proposed (Johnston, 1998), researchers revealed that the aggresomes are formed by the dynein-dependent retrograde transport of proteins and protein aggregates to the microtubule organizing center (MTOC) (Garcia-Mata, 1999). As a result, large unregular-shaped deposits are formed in the pericentriolar region near the Golgi apparatus. Interestingly, the accumulation of misfolded proteins around the centrosome does not interrupt ER-Golgi transport. Despite the total disorganization of MTOC, distal microtubules seem to perform their function unchanged (Garcia-Mata, 1999; Johnston, 1998). The formation of aggresomes interacts with the cytoskeleton in more ways. The intermediate filament (IF) cage established after the total reorganization of IFs surrounds the aggresome (Johnston, 1998). Aggresomes also contain damaged proteins, unbound ubiquitin, K48/K11, and K63 linked to the proteins, proteasome, chaperones Hsp40/Hsp70/Hsp90, ubiquitin ligases, histone deacetylase HDAC6, autophagy receptors (a.o. p62), tubulin and actin (Johnston, 2021). Aggresomes formation is crucial to cellular response when degradation via proteasome is overwhelmed by impaired proteins, both in proteasome inhibition, heat-shock response, and other stress conditions resulting in the accumulation of misfolded proteins. The cytoprotective character of aggresomes was confirmed by Nawrocki et al. in the condition of proteasome inhibition and combined disruption of aggresome formation (inhibition of HDAC6) survivability of cancerous cells was smaller than in cells affected only by proteasome inhibitor (Nawrocki, 2006; Mitsiades, 2004).

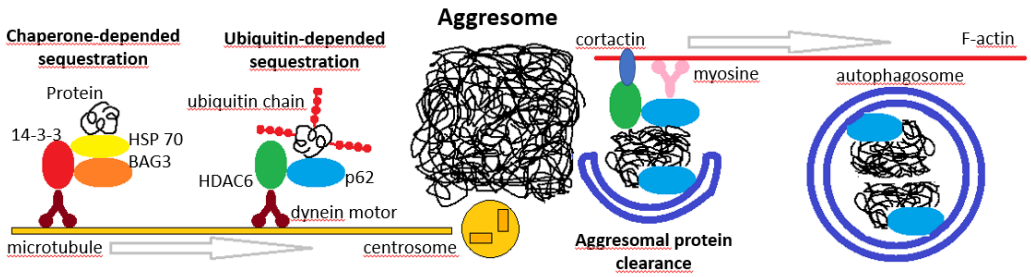

Figure 1. Formation and disruption of the aggresome. Two parallel processes can lead to aggresome formation. The first one: **Chaperone-dependent sequestration**: Involves BAG3 (co-chaperone) – that links misfolded protein tagged by HSP 70 to 14-3-3 protein. The latter 14-3-3 protein links the protein complex to dynein and traffic it to the centrosome. The second process: **Ubiquitin-dependent sequestration**: Involves poly-ubiquitin protein substrate that is linked to p62 protein and then is attached to microtubules via HDAC6 (Histone deacetylase). Both processes lead to formation of big protein aggregates in the cell center. The same two proteins, HDAC6 and p62 can promote: **Aggresomal protein clearance:** by association of the aggresome to cortactin (cortical actin binding protein) and motor it along actin filaments, depending on myosin, to the cell periphery where the aggresome can be cleared in autophagosome

HDAC6, despite its name, does not remodel chromatin but is involved in cytoplasmic pathways. This enzyme participates in heat-shock response as a co-chaperone regulating Hsp90 by deacetylation (Kovacs, 2005). More importantly, HDAC6 can deacetylate microtubules, thereby regulating dynein-dependent aggregates transport, or even function as a selective aggresome adaptor (Berdeja, 2021). Direct interactions between HDAC6 and p62 remain unknown, but their cooperation traffics ubiquitinated protein(K63)/p62 aggregates into aggresome rather than autophagy. Nevertheless, induced autophagy can also disrupt the formation of aggresomes. Phosphorylation of p62 blocks its LC3-interacting regions, thereby enabling autophagy and promoting protein sequestration into aggresomes, so inhibition of p62 phosphorylation

enhances autophagy and disrupts aggresomes formation (Zhang, 2022). On the other hand, p62, alongside HDAC6, promotes aggrephagy (autophagy of aggresome) by deacetylation of cortactin and myosin-dependent transport of misfolded protein aggregates from MOTC to peripheral cytosol, where aggregates are exposed for autophagosome (Yan, 2013).

Misfolded proteins may also be transferred to the aggresome in the Ub-independent pathway involving chaperones and BAG co-chaperone 3. BAG3 detects and directs the Protein-Hsp70 complex to the aggresome, using 14-3-3 protein. The whole transport complex is then linked to dynein, enabling retrograde transport along microtubules (Jia, 2014). This creates an additional chance to sequester into aggresome proteins which avoids ubiquitination. As well as p62-mediated transport, BAG3 can lead protein into selective autophagy (Gamerdinger, 2011).

Aggresome formation and disruption mechanisms remain primarily unexplored. Nevertheless, described interactions show how efficiently aggresome formation complements proteasomal degradation. Future research is needed to reveal the nature of these mechanisms, considering that aggresomes are involved in the pathogenesis of many diseases.

AGGRESOMES – AT THE CENTER OF CONFORMATIONAL DISEASES

Disturbed proteostasis underlies the pathogenesis of many severe and lethal diseases. Accumulation of misfolded proteins and their aggregation into insoluble deposits may impair cell functions and promote cell death. The presence of dysfunctional proteins promotes their aggregation into stable beta-pleated sheets of amyloid fibrils, plaques, or tangles, both endoplasmic and extracellular (Chiti, 2017; Babu, 2011). Amyloid formations present their characteristic morphological structure, even though their monomers originate from proteins with various native conformations. Aggregates lay at the basis of many chronic diseases, including multiple types of amyloidosis – a broad group of systemic and localized diseases with similar morphological appearances centered around amyloid deposits (Hazenberg, 2013). For example, deposits of the protein named islet amyloid polypeptide (IAPP) are connected to type 2 diabetes progression by damaging the islets of Langerhans (Shahnawaz, 2017). Misfolded proteins can also aggre-gate into soluble oligomers, which seem more cytotoxic than amyloid structures (Walsh, 2004; Caughey, 2003). This argument may support the hypothesis that aggregation is a cytoprotective mechanism that becomes impaired in conformational diseases.

Among other pathologies related to aggregate accumulation, neurodegenerative diseases have the most significant social impact. Besides genetic predispositions and environmental conditions, it was revealed that aging impact proteostasis by the expansion of misfolded proteins and limited proteolysis (Olzmann, 2008). This molecular mechanism justifies calling these diseases age-related. Due to their sophisticated struc-ture, neurons are especially vulnerable to the destructive effects of pathogenic inclusions. The aggregate formation is the crucial pathogenesis mechanism of Alzheimer's disease – beta-amyloid deposits and tau neurofibrils, Parkinson's disease – Lewy bodies, Huntington's disease – PolyQ bodies, and many other neurodegenerative disorders (Dugger, 2017).

Interestingly, these inclusions are biochemically and morphologically similar to aggresomes. Supposably their formation depends on the same pathways. Furthermore, it was shown that all-important particles of the aggresome: proteasome, chaperones, gamma-tubulin, HDAC6, and p62 are incorporated into the structure of Lewy bodies inside glial cells (Chiba, 2012). Parallelly, researchers revealed that aggresome adaptors – HDAC6, p62, and 14-3-3, play crucial roles in neurodegenerative disease pathogenesis, and more importantly, their pathways may have therapeutic significance (Jia, 2014; Ma, 2019).

PROTEASOME INHIBITORS – THE CLINICAL IMPLICATION OF PROTEOSTASIS

Progressing carcinogenesis is strictly correlated with the accumulation of mutations, genetic instability, and loss of control mechanisms. Rapid growth and short cell cycle of cancer cells require an increased flow of newly synthesized proteins (Hanahan, 2011). However, it makes neoplastic cells more vulnerable to disturbances in the UPS pathway, which is overcome by the supply of proteins. The primary mechanism of action of proteasome inhibitors (PI) in cancer therapy is the blockage of the UPS cytoprotective pathway. PIs increase the concentration of damaged proteins and induce unfolded protein response (UPR), which results in cell cycle arrest and apoptosis of neoplastic cells (Nunes, 2017). This pathway also explains why cancer cells that produce large quantities of protein are more vulnerable to PI-mediated proteostasis

disruption than healthy cells (Mlynarczuk-Bialy, 2014; Mlynarczuk-Bialy, 2006). This difference is especially useful in treating malignancies originating from plasma cells that produce large amounts of immunoglobins.

PIs also promote cell death affecting many other pathways. The best-described cytotoxicity mechanism of PIs, besides UPR inducement, is inhibiting the pro-survival anti-apoptotic NF-κB pathway, which is also involved in angiogenesis, metastasis, and invasion of cancer cells (Nunes, 2006). After receiving an activating stimuli, an inhibitor of this pathway – IκBα is degraded via UPS, which releases transcription factor NF-κB and transfers it into the nucleus (Palombella, 1994). Proteasome inhibition prevents the release of NF-κB, therefore, arresting the NF-κB pathway, effectively turning off pro-survival stimulation. In other patterns, PIs may induce apoptosis directly via activation c-Jun NH2-terminal kinase (JNK) pathway resulting in activation of caspase 8 and caspase 3 (Dai, 2003), overexpression of p53 regulating protein (An, 2006), or accumulation of pro-apoptotic Bim, Bik, and Bid proteins (Nunes, 2017; Breitschopf, 2000).

Proteasome inhibitors were initially developed as agents that prevent cancer-related cachexia by a decrease of UPS-mediated degradation and protein turnover. Subsequently, preclinical research revealed their pro-apoptotic activity *in vitro* and *in vivo* murine cancer models, which suggested potential chemotherapeutic utility (Manasanch, 2017). Two decades of development of PIs resulted in clinical validation and regulatory approval in the usage of three compounds for the treatment of multiple myeloma and mantle-cell lymphoma: bortezomib (PS-341, VELCADE) – a first-generation agent, carfilzomib (KYPROLIS) and ixazomib (NINLARO) – second-generation agents (Richardson, 2003; Alsina, 102; Philippe Moreau, 2015). Each presents inhibitory activity towards beta5 and beta5i subunits (in higher concentrations, these compounds inhibit beta1 and beta2 subunits as well), inhibiting both constitutive 26S proteasome and immunoproteasome (Wang, 2021). Interestingly, *in vitro* usage of selective constitutive proteasome or immunoproteasome inhibitors does not induce apoptosis in myeloma cells line (Eleftheriadis, 2017), which may confirm the involvement of immunoproteasome in the maintenance of proteostasis in hemato-poietic cells lines.

Table 1. Properties of proteasome inhibitors approved and in clinical trials

Proteasome inhibitor	Class	Targets	Administration	Approved in
Bortezomib	Boronate/reversible	Constitutive and immunoproteasome	IV and SC	multiple myeloma, mantle-cell lymphoma
Carfilzomib	Epoxyketone/irreversible	Constitutive and immunoproteasome	IV	multiple myeloma
Ixazomib	Boronate/reversible	Constitutive and immunoproteasome	IV and Oral	multiple myeloma
Marizomib	cyclic beta-lactone gamma-lactam/ irreversible	Constitutive and immunoproteasome	IV	-----
KZR-616	Epoxyketone/irreversible	immunoproteasome	SC	-----

PROTEASOME-TARGETED THERAPIES

Due to the impressive results of bortezomib treatment of relapsed/refractory multiple myeloma in phase II clinical trial (Richardson, 2003), FDA approved bortezomib on the expedited procedure as salvage therapy in 2003. Next, full regulatory approval of the first PI in multiple myeloma appeared in 2005, after an extended phase III trial (Richardson, 2005). Soon after, bortezomib was approved for treating mantle cell lymphoma (Fisher, 2006). Bortezomib consists of a dipeptide backbone connected to a boronate group, which interacts with catalytic threonine residue in proteasome subunits, reversibly inhibiting them (Schrader, 2016). Bortezomib is administrated as a mannitol ester by intravenous (IV) or subcutaneous (SC) route. Both administration routes present the same anti-tumor activity. Then, the drug quickly leaves the vascular compartment and, in therapeutic concentration, may result in maximum inhibition of proteasome up to 74% (Orlowski, 2002). Bortezomib is metabolized intrahepatic through oxidative deboronation performed by CYP450 enzymes and is excreted in bile and urine. Combination treatment with other chemotherapeutics has been referred to as more effective than bortezomib monotherapy. A widely used therapy option in treating multiple myeloma is a combination of bortezomib with dexa-methasone and lenalidomide- leading to prolonged remissions and improving overall survival compared to

dexamethasone-lenalidomide treatment (Durie, 2020). Bortezomib can also be combined with doxorubicin and dexamethasone as maintenance therapy in newly diagnosed multiple myeloma (Sonneveld, 2012). A combination of bortezomib and dexamethasone with daratumumab – an anti-CD38 monoclonal antibody, obtained stunning results in treating multiple myeloma (Palumbo, 2016). However, in this paper, we would like to distinguish the approved by FDA therapy for patients with multiple myeloma who have received at least two prior lines of treatment, a combination of dexamethasone, bortezomib, and panobinostat – HDAC inhibitor, which disturbs aggresome formation (San-Miguel, 2016; Dimopoulos, 2022). A severe problem for bortezomib-treated patients and a dose-limiting factor is the development of peripheral neuropathy. Even 80% of patients treated with bortezomib develop this condition (Richardson, 2010). Thrombocytopenia, nausea, diarrhea, fatigue, and neutropenia are the other side effects. Combination treatment with bortezomib used in another type of lymphoma, Waldenstrom's macroglobulinemia, is effective and is used in clinical practice (Ghobrial, 2010). Interestingly, bortezomib's potential to induce proteotoxicity helps treat light-chain amyloidosis, a disease caused by the deposition of light chains secreted by malignancy plasma cells into beta-pleated sheets of amyloid structures (Sanchorawala, 2015). Bortezomib did not show results in treating other hematological malignancies and solid tumors. The possible cause of these failures is relatively high toxicity, hence administration below the effective therapeutic dose. The solution to these problems may be to use second-generation PIs.

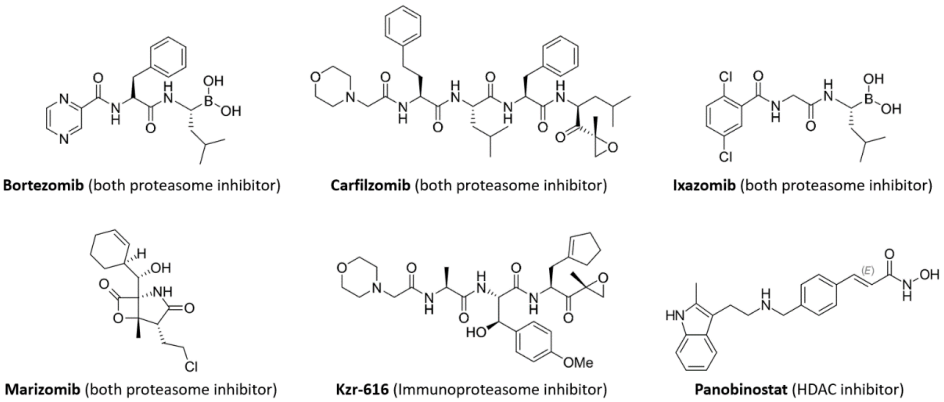

Figure 2. Drugs affecting proteostasis – proteasome inhibitors and Panobinostat

Carfilzomib consists of a tetrapeptide backbone and epoxyketone group as a warhead, which irreversible and specifically binds with threonine residue in catalytic proteasome subunits, unlike bortezomib. The drug is administered via the IV route, then rapidly leaves the vascular compartment. The T1/2 value for carfilzomib is under 30 min, which suggests extrahepatic metabolism. First, elimination includes epoxide hydrolyzation performed by microsomal epoxide hydrolases and peptidase cleavage. Next, carfilzomib is excreted in bile and urine (Alsina, 2012; Perel, 2016). Second-generation PI Carfilzomib was approved by FDA in 2012 as monotherapy, based on results of clinical application (Alsina, 2012), but nowadays is more often used in combination therapies. As bortezomib, carfilzomib combined with lenalidomide and dexamethasone is used to treat patients with multiple myeloma (Lendvai, 2014), and usage with panobinostat is under evaluation (Berdeja, 2015; Berdeja, 2021). The second widely used combination therapy that includes carfilzomib is treatment with melphalan and prednisone – drugs also used with bortezomib. Comparative phase III clinical trials do not indicate significant differences in anti-tumor activity in patients with multiple myeloma between these two combination therapies (Facon, 2019). Differences in the clinical application are mainly based on different toxicity profiles of bortezomib and carfilzomib, the latter not causing peripheral neuropathy but a dose-limiting deep vein thrombosis and febrile neutropenia. Other side effects are hypo and hypertension, fever, anemia, fatigue, cardiac failure, and shortness of breath (Manasanch, 2017).

Ixazomib, bortezomib analog bioavailable in oral administration, is the third PI approved by FDA in 2015 in combination therapy with dexamethasone and lenalidomide in patients with relapsed refractory multiple myeloma (Philippe Moreau, 2015). The mechanism of reversible proteasome inhibition resembles bortezomib action (Schrader, 2016). Ixazomib can be administrated in both IV and oral route as Ixazomib citrate, which rapidly converts into active form after exposure to plasma (Kupperman, 2010). The drug

absorbs quickly after oral administration (bioavailability of 58%) and is slowly removed from plasma with t1/2 of 9,5 days (Assouline, 2014). Metabolism of ixazomib is also like bortezomib once. In terms of toxicity, patients treated with ixazomib present peripheral neuropathy, thrombocytopenia, and gastro-intestinal disorders (Chen, 2021).

Marizomib (salinosporamide A) is another inhibitor of constitutive proteasome and immunoproteasome, but in contrast to others, PIs irreversibly inhibit all three catalytic subunits of the proteasome. It also belongs to another chemical group – cyclic beta-lactone gamma-lactam and is produced naturally by the marine bacteria *Salinispora Tropica* (Potts, 2011). The drug does not present significant toxicity. Side effects are limited to nausea, diarrhea, and fatigue. Marizomib is currently under evaluation for treating multiple myeloma and glioblastoma (Kisselev, 2021). In addition, due to its more hydrophobic structure, marizomib, unlike other PIs, can cross the blood-brain barrier, making it a potential option for treating brain tumors (Di, 2016).

Research on tool PI ONX 0914 found that selective immunoproteasome inhibition limits inflammatory activity without the cytotoxic effect of dual proteasome inhibition in preclinical models (Muchamuel, 2009). Soon after, researchers discovered the ability of the KZR-616 compound to inhibit immunoproteasomes selectively with good pharmaceutical properties. The KZR-616 is a tri-peptide analog of carfilzomib with an epoxyketone warhead, with binds irreversibly to beta5i (inhibits nearly 100% of chymotrypsin-like activity) and beta1i (inhibits almost 40% of caspase-like activity) (Johnson, 2018). The drug is administrated SC, with a bioavailability of nearly 100%. Like carfilzomib, absorption, and clearance of KZR-616 are rapid, but human metabolism is performed only by microsomal epoxide hydrolases (Fang, 2021). KZR-616 is being evaluated in patients with autoimmune disorders – lupus nephritis, polymyositis, and dermatomyositis in phase II clinical trials (Johnson, 2018). Patients also received standard immuno-suppressing drugs and corticosteroids without signs of drug-drug interactions. Inhibition of immunopro-teasome results in reduced cytokine expression, decreased Th1 and Th17 cell differentiation, reduced amounts of class-switched plasma cells, and a significant decrease in autoimmune antibody concentration (Basler, 2021; Kirk, 2021). Also, in the murine polymyositis model, KZR-616 significantly improves muscle function (Del Rio Oliva, 2022). No serious adverse events were reported in patients treated with KZR-616, in contrast to dual PIs. Side effects are limited to erythema in the place of injection (Kirk, 2021).

HDAC6 AND AGGRESOME FORMATION PATHWAY – WAY TO OVERCOME RESISTANCE TO PIS

Conversely, resistance to PIs in tumor cells limits their therapeutic applications (Merin, 2014). Mecha-nisms of resistance, despite the knowledge of some pathways, remain mostly undiscovered. Nevertheless, each newly discovered pathway brings us closer to understanding the effects of PIs treatment on cancer cells, which may help develop novel therapies. As one of the discovered mechanisms, some neoplastic cells resistant to PIs present a mutation in gene-coding beta5 catalytic subunits, preventing drugs from binding (Lu, 2009). Also, overexpression of proteasome subunits and chaperones may support proteostasis under stress induced by proteasome inhibition (Fuchs, 2008). An overload with toxic misfolded proteins during the insufficiency of UPS has to be either degraded by other enzymes, including lysosomal hydrolases in autophagy, or be sequestrated into aggresomes. Unsurprisingly, aggresomes were initially observed in cancer cell lines under proteasome inhibition (Wojcik, 1996). The cytoprotective character of aggresomes has a significant impact on tumor cells' survivability under proteotoxic stress. Thus, disruption of aggresome formation results in increased vulnerability to misfolded protein stress. The selective aggres-ome adaptor – HDAC6 that traffics misfolded proteins and smaller aggregates into aggresomes may be inhibited, which results in foreclosing of the aggresomes formation pathway from the proteostasis network (Rodriguez-Gonzalez, 2008). The importance of aggresome formation in cells resistant to PIs was revealed in many preclinical studies, and HDAC6 inhibition was proven to be an effective way of reducing the survivability of cancer cells in both *in vitro* and *in vivo* models (Nawrocki, 2006; Mitsiades, 2004). Moreover, HDAC6 inhibition proved to be effective in clinical applications.

Panobinostat (FARYDAC) – the first HDACs inhibitor that affects all enzymes of this class, did not present a therapeutic effect in monotherapy (Wolf, 2012). However, in phase III clinical trials combination treatment of bortezomib and dexamethasone proved to be effective in patients with refractory/relapsed multiple myeloma (San-Miguel, 2016), which resulted in FDA approval in 2015 of this therapy in patients who have been treated with at least two lines of treatment including bortezomib and an immunomodula-ting drug. Panobinostat inhibits all four classes of HDACs, mostly inactivating HDAC1-3 and HDAC6 (Berdeja,

2021). Inhibition of HDAC1-3 leads to epigenetic changes, transcription of suppressor genes, and enhanced synthesis of proteins. On the other hand, HDAC6 inhibition results in the inhibition of HSP90 and disruption of aggresome formation (Kovacs, 2005; Berdeja, 2021). In contrast to other HDACs inhibitors approved in clinical usage: vorinostat, belinostat, and romidepsin (which seem not to disturb proteostasis), panobinostat combination therapy mainly relies on dysregulation of protein metabolism. Panobinostat combination therapies with second-generation PIs – carfilzomib (Berdeja, 2015; Berdeja, 2021) and ixazomib plus dexamethasone (NCT02057640) are currently under evaluation. Panobinostat is a hydroxamic acid-based compound administered IV or orally (bioavailability of 21%), then rapidly absorbed. The T1/2 value of panobinostat is 31 hours (Srinivas, 2017). The drug is metabo-lized mainly by CYP450 enzymes in the liver and is excreted in bile and urine. The toxicity profile of panobinostat does not include serious side effects. The most common side effects include thrombocyto-penia, neutropenia, and gastrointestinal disorders (Berdeja, 2021).

DISCUSSION

Understanding the cellular mechanism and their pathology in cancer cells is crucial for developing new therapies. This also applies to the network of proteostasis and related therapies. The discovery of the proteasome and the UPS pathway enables the development of PIs and their application to the treatment of hematological malignancies (Richardson, 2007; Alsina, 2012; Philippe Moreau, 2015). Furthermore, there is still a place for research on the new PIs. Newly synthesized molecules may be more cytotoxic to cancer cells and have a better toxicity profile. As a result, they may be applied to the treatment of a broad spectrum of malignancies. Currently approved PIs can't be used in treating solid tumors, but the development of newly synthesized PIs may overcome this. Moreover, in the era of a personalized medicine board range of possible drugs, may help to suit the best therapeutic option for the patient.

Panobinostat shows that proteostasis mechanisms beyond the proteasome are also possible targets in cancer treatment (San-Miguel, 2016). Disturbance in aggresome formation by inhibition of HDAC6 acts parallelly with bortezomib. This approach makes the aggresome formation pathway a vital target in the therapy of relapsed refractory multiple myeloma. Furthermore, other drugs affecting the proteostasis network are under evaluation. Pevonedistat – an inhibitor of the ubiquitin-activating enzyme (E1) NEDD-8, and RG7112 – an inhibitor of the ubiquitin-ligase enzyme (E3) MDM2, have shown activity against acute myeloid leukemia (Manasanch, 2017). More research is needed to evaluate these drugs in a clinical approach.

The research on proteostasis revealed the main patterns in cellular protein turnover and degradation pathways. Nevertheless, details in these pathways remain unclear. May the discovery of detailed interaction in the proteostasis network will enable the development of new drugs in therapeutic options in cancer therapy.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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