Mini review

THE UBIQUITIN-PROTEASOME SYSTEM: A NOVEL TARGET FOR ANTICANCER AND ANTI-INFLAMMATORY DRUG RESEARCH [#]

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Abstract: The ubiquitin-proteasome system is responsible for the degradation of most intracellular proteins, including those that control cell cycle progression, apoptosis, signal transduction and the NF-κB transcriptional pathway. Aberrations in the ubiquitin-proteasome system underlie the pathogenesis of many human diseases, so both the ubiquitin-conjugating system and the 20S proteasome are important targets for drug discovery. This article presents a few

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Abbreviations used: AML – acute myeloid leukemia; ARF – acute renal failure; BMSCs – bone marrow stromal cells; βTrPC – β-transducin repeat containing protein; CDK – cyclin-dependent kinase; ChT-L – chymotrypsin-like; C-L – caspase-like; CLL – chronic lymphocytic leukemia; DHT – dihydroxytestosterone; DOCA – deoxycortycosterone; DUB – deubiquitinating enzyme; E1 – ubiquitin-activating enzyme; E2 – ubiquitin-conjugating enzyme; E3 – ubiquitin-protein ligase; ET-1 – endothelin-1; Hdm2 – human counterpart of Mdm2; HIF-1 – hypoxia inducible factor; IKK – IκB kinase; IL-1 – interleukin; INF-γ – interferon gamma; LMP – low-molecular-mass polypeptide; Mdm2 – murine double minute 2; Met-AP-2 – metionine aminopeptidase-2; MHC – major histocompatibility complex; MM – multiple myeloma; NF-κB – nuclear factor-kappaB; pIκBα – phosphorylated inhibitor-κB; Protacs – proteolytic targeting chimeric molecules; pVHL – phosphorylated von Hippel-Lindau tumor suppressor; RITA – reactivation of p53 and induction of tumor cell apoptosis; SCF – complex formed by Skp1, cullin and F-box protein; siRNA – small interfering RNA; SKP2 – S-phase kinase associated protein 2; SMPI – small molecule proteolysis inducers; T-L – trypsin-like; TNF – tumor necrosis factor; UPS – ubiquitin-proteasome system; VCAM – various leukocyte adhesion molecules; VEGF – vascular endothelial growth factor

of the most important examples of the small molecule inhibitors and modulators targeting the ubiquitin-proteasome system, their mode of action, and their potential therapeutic relevance in the treatment of cancer and inflammatory-related diseases.

INTRODUCTION

The ubiquitin-proteasome system (UPS) is of key importance in the targeted degradation of the bulk proteins (80-90%) in the cell [1]. Such proteins include misfolded or mutated proteins, viral proteins and many of the short-lived proteins that control cell division (e.g. cyclins, cyclin-dependent kinase inhibitors), apoptosis (e.g. p53, Bax, caspases), and signal transduction and gene expression (e.g. NF-κBp105, IκB, HIF-1, c-fos, c-jun). For the discovery of the ubiquitin-mediated protein degradation pathway, Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the 2004 Nobel Prize in Chemistry. Their pioneering work led to the discovery that abnormal activation or failure of ubiquitin-mediated proteolysis underlies the pathogenesis of various debilitating diseases (i.e. cancer, inflammation, cardiovascular diseases, neurodegenerative disorders) [1-4], indicating that the ubiquitin-proteasome system is an attractive target for pharmacological intervention [2, 5-7].
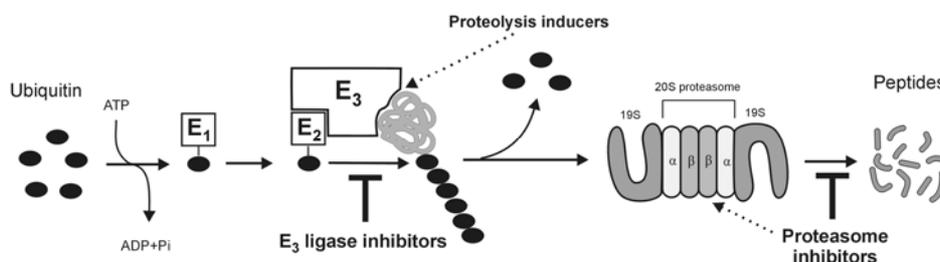

Fig. 1. The UPS as a target for pharmacological intervention [1, 2, 5, 6, 13]. One common ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent fashion. Then, the ubiquitin is transferred from the active-site cysteine in E1 to the catalytic cysteine in the active site of several different ubiquitin-conjugating enzymes (E2s). Several hundreds of different ubiquitin-protein ligases (E3s) select the target protein and transfer activated ubiquitin to generate the polyubiquitin chain. The polyubiquitinated protein is then recognized by the 19S regulator and directed to the 20S proteasome for destruction, following the removal of the ubiquitin chain by deubiquitinating enzymes (DUBs). E3 ligase inhibitors prevent protein ubiquitination and degradation. Small molecule proteolysis inducers direct disease-promoting proteins for ubiquitination and degradation. Proteasome inhibitors block the degradation of the ubiquitinated proteins by binding to the proteolytically active subunits located in two of the inner β-rings of the 20S core particle.

In general, protein degradation via the ubiquitin-proteasome pathway involves several successive steps employing different classes of enzyme, namely ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzymes (E2s), the ubiquitin-protein ligases (E3s), the 20S proteasome, and the deubiquitinating enzymes (DUBs). These can potentially be targeted for inhibition in the context of cancer and other diseases (Fig. 1). Indeed, a number of small-molecule inhibitors and modulators directed against the UPS components have been described in the biomedical literature as potential anticancer or anti-inflammatory drugs [5-9]. However, many of them have limited therapeutic usefulness due to their lack of selectivity and high levels of cytotoxicity against normal cells. Those with great therapeutic value include the small-molecule inhibitors of the E3 ubiquitin ligases specific for tumor suppressors (i.e. p53, p27) and IκB [7], the highly selective inhibitors of the 20S proteasome [8, 10-12], and the small molecule proteolysis inducers triggering tumor-promoting proteins for ubiquitination and degradation [13]. They are presented below.

TARGETING SPECIFIC E3 UBIQUITIN LIGASES IN CANCER

The most extensively studied E3 ligase as a drug target in cancer is the Mdm2 (murine double minute 2), which binds and ubiquitinates the tumor suppressor protein p53 [14]. p53 functions as a "guardian of the genome", inducing the expression of many genes regulating cell-cycle arrest, DNA repair and apoptosis after DNA damage, thus preventing mutagenesis and carcinogenesis. A reduced level of p53 and overexpression of the Mdm2 or Hdm2 (human counterpart of Mdm2) have been found for many tumors carrying wild-type p53, such as neuroblastoma, acute lymphoblastic leukemia (ALL), melanomas, and colorectal, lung and breast carcinomas [2, 7], and projects involving Mdm2 inhibition with either antisense oligonucleotides or small-molecule inhibitors are currently underway [5, 7, 15-17]. For example, a crystal structure analysis of the p53-binding pocket in Mdm2 and a screening of the National Cancer Institute chemical library led to the identification of two structurally different compounds, named Nutlins and RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis), which blocked Mdm2-p53 interaction and prevented p53 ubiquitination and degradation in many cancer cell lines [15-17]. Nutlins (cis-imidazoline analogs) bind directly to the p53-binding pocket in Mdm2 [15], whereas RITA (2,5-bis(5-hydroxymethyl-2-thienyl)furan) binds directly to p53 [16]. Both compounds have been shown to induce cell cycle arrest and apoptosis in cell-based studies, and to inhibit tumor growth in nude mouse tumor xenografts [15-17]. More studies are required to find out whether Nutlins and RITA target p53-related proteins (i.e. p63 and p73) and whether the *in vivo* activity of these compounds is exclusively limited to tumor tissues.

The SCF^{SKP2} ligase (a member of the Skp1-Cullin-F-box protein complex) recognizes and ubiquitinates several negative cell-cycle regulators, including the cyclin-dependent kinase (CDK) inhibitor p27 [1]. The high level of the

Skp2 component (S-phase kinase associated protein 2) and the low level of its substrate, p27, have been shown to correlate with a poor prognosis in many human cancer types, such as gliomas, lymphomas, and cancer of the prostate, colorectum, lung and breast [2, 18]. Several studies have demonstrated that inhibiting Skp2 using small interfering RNA (siRNA) or anti-Skp2 antibodies increases the level of p27, resulting in apoptosis induction and cell growth arrest *in vitro* and in animal models [5, 7]. Attempts to design small molecules that could disrupt the interaction of Skp2 and p27 are now in progress.

Another potential target for anticancer drug research is the SCF$^{\beta TRCP}$ ligase that ubiquitinates IκB, an inhibitor of the nuclear factor-κB (NF-κB) [19, 20]. In normal cells, NF-κB resides in the cytoplasm in an inactive form, bound to IκB. Upon cell exposure to various extracellular signals (e.g. proinflammatory cytokines, phorbol esters, growth factors, certain chemotherapeutic substances, and radiation), IκB is rapidly phosphorylated on Ser 32 and 36 by the multimeric IκB kinases, ubiquitinated by the SCF$^{\beta TRCP}$, and subsequently degraded by the 26S proteasome (Fig. 2). As a result, free NF-κB translocates into the nucleus, where it promotes the induction of many specific genes, the products of which
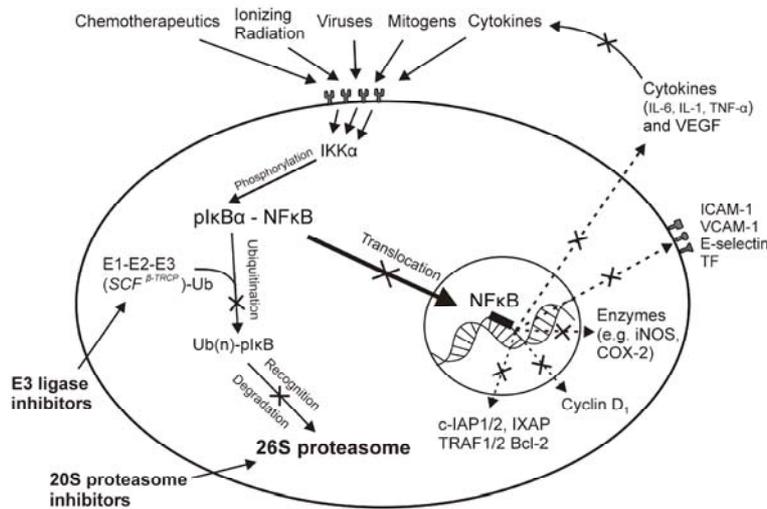

Fig. 2. The ubiquitin-proteasome inhibitors targeting the NF-kB activation pathway [7, 21, 24, 25]. Upon exposure of the cells to various stimuli, the IκB kinases (IKKs) rapidly phosphorylate the IκB, which is then ubiquitinated by the SCF$^{\beta TRCP}$ ligase and subsequently degraded by the 26 proteasome. The free NF-κB translocates into the nucleus, where it activates a number of genes involved in cancer progression and inflammation. The SCF$^{\beta TRCP}$ inhibitors prevent pIκB ubiquitination resulting in the inhibition of its degradation by the 26S proteasome. The inhibitors of the 20S proteasome block the degradation of ubiquitinated IκB, resulting in the blockage of NF-κB nuclear translocation and gene activation.

suppress apoptosis, induce cell proliferation, promote angiogenesis and metastasis, and potentate inflammation [19, 20]. Therefore, small-molecule inhibitors that target the NF-κB activation pathway, including proteasome inhibitors (see below) have great therapeutic potential in the treatment of many human cancers, particularly cancers resistant to conventional therapy, and in the treatment of inflammatory-related disorders [21]. It has been shown by many authors that silencing of the βTrPC component (β-transducin repeat-containing proteins) by siRNA inhibits NF-κB activation and reduces chemoresistance in cancer cell lines [5, 7, 21]. Moreover, identifying the putative recognition motif (pIκBα) in the βTrCP allowed the synthesis of a short IκB phosphopeptide antagonist that, after microinjection, inhibited IκBα degradation in TNF-stimulated HeLa cells [22]. It should be mentioned that βTrCP recognizes the same double-phosphorylated destruction motif in numerous other proteins, including the transcription factor β-catenin, which is known to be involved in cellular transformation [5, 7]. Furthermore, the novel, highly selective inhibitor Ro106-9920 blocks IκB ubiquitination and prevents NF-κB activation by targeting another, as-yet unidentified, IκB E3 ligase [23].

PHARMACOLOGICAL INHIBITORS OF THE 20S/26S PROTEASOME

The 20S proteasome, a catalytic core particle of the 26S proteasome, exhibits three main catalytic activities: chymotrypsin-like (ChT-L), trypsin-like (T-L) and caspase-like (C-L) [26]. These are respectively associated with three distinct subunits: β5, β2 and β1 (Fig. 1) [27]. Specific forms of the 20S proteasomes, the so-called immunoproteasomes, contain three novel interferon gamma-inducible protein subunits termed β5i (LMP7), β2i (MELC1, LMP10) and β1i (LMP2), instead of the standard subunits (β5, β2 and β1) [28]. The standard 20S proteasomes are present in most mammalian cells, while the immunoproteasomes are expressed constitutively in cells of lymphoid origin, where they play a major role in the generation of peptide antigens presented on MHC class I molecules [28].

A number of structurally different inhibitors of the 20S proteasome/ immunoproteasome were discovered and tested in experimental settings [8, 24, 25]. These include synthetic peptide-based inhibitors (i.e. peptide aldehydes, boronates, sulfonates), and compounds isolated from biological extracts on the grounds of their initial anticancer and anti-inflammatory activities (i.e. lactacystin, epoxomicin, eponemycin) [8]. The so-called "classical proteasome inhibitors" bind covalently to the catalytic active N-terminal threonine (Thr1Oγ), and predominantly inhibit its chymotrypsin-like activity, which is rate-limiting in intracellular protein degradation by the proteasomes [8, 27]. In preclinical studies, a dipeptide boronate PS-341 (bortezomib) fulfilled all the criteria for the treatment of cancer [24, 29-31]:

1. It selectively and reversibly blocked ChT-like activity;
2. It showed cytotoxicity against 60 cancer cell lines derived from multiple human tumors in the National Cancer Institute *in vitro* screen, and exhibited relatively few toxic effects on normal cells;
3. It induced apoptosis in tumor cells resistant to chemotherapy or radiation;
4. It down-regulated cytokine-induced expression of IL-6, TNF-α, VCAM-1 and VEGF; and
5. It showed effectiveness in human tumor xenograft models of a wide range of hematological malignancies and solid tumors, both as a single agent and in combination with standard chemotherapeutics.

On this basis, bortezomib (Velcade™) was approved by the US Food and Drug Administration (FDA) and by the European Medicines Agency (EMEA) for the treatment of patients with relapsed and refractory multiple myeloma (MM). The major molecular mechanisms through which it mediates anti-MM activity involve the induction of p53-dependent and p53-independent apoptotic pathways in MM cells resistant to conventional therapy, and down-regulation of the expression of several tumor-promoting proteins in bone marrow stromal cells (BMSCs) through the inhibition of the NF-κB activation pathway [12, 32, 33]. Moreover, gene expression profiling and proteomic analysis of the MM cells have demonstrated that bortezomib down-regulates the expression of several proteins involved in the cellular response to genotoxic stress [34]. Currently, bortezomib is in use in clinical trails for the treatment of other hematological neoplasias and solid tumors, including refractory indolent and aggressive B-cell lymphoma, mantle cell lymphoma, non-Hodgkin's lymphoma, relapsed leukemia, malignant metastatic melanoma, non-small lung cancer, and breast cancer [12, 35]. Detailed information concerning the pharmacology, pharmacokinetics and practical application of bortezomib in MM patients and other cancer patients can be found at the National Cancer Institute Web site (www.cancer.gov). In general, bortezomib appears to be well tolerated with mild/moderate and manageable side effects. In MM patients, it produced a 35% overall response rate and 10% complete responses.

A new generation of proteasome inhibitors that are now being explored in preclinical studies include noncovalent reversible inhibitors (e.g. indanone-substituted peptides, cyclic tripeptide TMC-95, 2-aminobenzyl-satatine derivative) [9, 27], and covalently bound inhibitors that block all three activities (i.e. ChT-L, T-L and C-L) or that specifically block immunoproteasomes [9-12, 36-38]. Two of them, namely NPI-0052 (salinosporamide A) and PR-171 (carfilzomib) have been found to induce apoptosis in multiple myeloma cells resistant to bortezomib and other chemotherapeutics [10, 37, 39]. NPI-0052 is an irreversible, lactacystin-related inhibitor that blocks all three proteasomal activities [36], and unlike bortezomib, it induces apoptosis predominantly through caspase-8 activation [39]. It also exerts strong antiproliferative and proapoptotic effects on lymphocytes from patients with chronic lymphocytic leukemia (CLL) [40]. The second inhibitor, PR-171, is a derivative of

epoxomicin that irreversibly inhibits the chymotrypsin-like activity of both the standard proteasomes and immunoproteasomes, and is a more effective inducer of apoptosis than bortezomib in multiple myeloma cells [37] and primary human acute myeloid leukemia cells (AML) [41]. Since immunoproteasomes are highly expressed in some cells of hematopoietic origin [28], it is suggested that small-molecule inhibitors targeting LMP-immunosubunits may have the ability to induce apoptosis only in hematological malignancies while sparing other tissues [12]. Both inhibitors (NPI-0052 and PR-171) showed effectiveness in preclinical models of multiple myeloma, and they are currently undergoing early clinical trials [10].

Due to the critical role of the ubiquitin-proteasome pathway in the activation of NF-κB, proteasome inhibitors have been also extensively studied in various animal models of inflammatory-related diseases [8, 25, 42]. For example, MLN-519 (a synthetic analogue of the *clasto*-lactacystin/β-lactone) exerted significant anti-inflammatory activity, and hence, limited the ischemic tissue damage in animal models of focal and middle cerebral ischemia [43-47], and myocardial reperfusion injury [48-50]. Currently, MLN-519 is in phase one clinical trials for safety in acute dosing regimens [42, 51]. Importantly, a structurally different proteasome inhibitor, CVT-634, has been also shown to block the NF-κB activation pathway and to reduce infarct volume in a focal model of cerebral ischemia [52]. Moreover, lactacystin/β-lactone and the peptide aldehyde PSI (Z-Ile-Glu (Ot-Bu)-Ala-Leucinal) have been shown to inhibit acute renal failure (ARF) in rats through the suppression of endothelin-1 (ET-1) production in the aorta and the kidney via NF-κB inhibition [53, 54], as well as to prevent the development of hypertension and vascular hypertrophy in an experimental model of deoxycortycosterone-salt-induced hypertension [53]. More recently, we demonstrated that PSI reduces thrombus formation in an experimental model of arterial thrombosis in renovascular hypertensive rats [55]. The exact mechanism through which PSI exerts antithrombotic activity is not yet known, but there exists evidence that the inhibition of 26S proteasome-mediated NF-κB activation is sufficient to block the expression of the tissue factor (TF) in monocytes during extracorporeal circulation [56], as well as in TNF- or angiotensin II-activated endothelial cells [57]. However, the major disadvantages of PSI and lactacystin/β-lactone as drug candidates include their poor stability and lack of specificity within cells. PSI inhibits calpains and cathepsin B [8], while lactacystin/β-lactone inhibits lysosomal cathepsin A [58] and cytosolic tripeptidyl peptidase II [59].

THE THERAPEUTIC POTENTIAL OF SMALL-MOLECULE PROTEOLYSIS INDUCERS IN CANCER

A new avenue of pharmacological intervention into the ubiquitin-proteasome system is to target cancer-promoting proteins for ubiquitination and degradation by the 26S proteasomes (Fig. 1) [13]. Several protein-based chimeric molecules

have been designated to destroy β-catenin, Rb protein, cyclin A, cdk 2; however, this class of compounds requires a special delivery system (viral or liposomal vehicle), which makes them poor drug candidates [13].

Two types of small molecule proteolysis inducers, named Protacs (proteolytic targeting chimeric molecules) [60] and SMPI (small molecule proteolysis inducers) [61] appear to be promising therapeutics for the future. They consist of an $SCF^{\beta-TRCP}$-binding phosphopeptide derived from IκBα (10-amino acid IκBαphosphopeptide domain) (Protacs) or a pVHL (phosphorylated von Hippel-Lindau tumor suppressor) ligase E3-binding octapeptide (SMPIs) linked covalently to the following ligands: 1. An anti-angiogenic inhibitor (ovalicin or fumagillol) that recognizes the metionine aminopeptidase-2 (Met-AP-2); 2. Estradiol, which noncovalently binds to the estrogen receptor implicated in the progression of breast cancer; or 3. Dihydroxytestosterone (DHT), which recognizes the androgen receptor, a known promotor of prostate cancer growth. Thus, following binding, the target protein is ubiquitinated by the $SCF^{\beta-TRCP}$ ligase or pVHL E3 ligase, and then degraded by the 26S proteasome. Both small-molecule proteolysis inducers have been successfully used in cell-free systems and in living cancer cells [60-62].

CONCLUSION

Targeting the ubiquitin-proteasome pathway is a new concept in therapy for many human diseases, particularly cancer and inflammatory-related diseases. Two different proteasome inhibitors entered clinical trails for the treatment of cancer (bortezomib) and stroke patients (MLN-519); the first is already on the market. Moreover, rapid progress in combinatorial chemistry, structure-based design and the screening of pharmaceutical companies' small-molecule libraries have accelerated developments in the identification of new classes of proteasome/immunoproteasome inhibitors. Researchers are cautiously optimistic that these may become anticancer drugs with better potency and lower toxicities. This is of particular relevance in the light of the findings that the UPS function is of paramount importance for the maintenance of cellular homeostasis, and its complete blockage is lethal for the individual cell and the organism as a whole. Because of this, much attention is now being paid to the development of small-molecule compounds that could block the degradation of cancer-preventing proteins at the level of their ubiquitination (i.e. E3 ligase inhibitors) or could target cancer-promoting proteins for ubiquitination and degradation (i.e. small molecule proteolysis inducers). It is now considered that such modulators may offer greater therapeutic promise in certain malignancies, and probably in other diseases, than the classical inhibitors of the 20S proteasome.

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