Molecular Pathways: Turning Proteasomal Protein Degradation into a Unique Treatment Approach

Sebastian Stintzing and Heinz-Josef Lenz

Abstract

Cancer treatment regimens have evolved from single cytotoxic substances affecting all proliferative tissues toward antibodies and kinase inhibitors targeting tumor-specific pathways. Treatment efficacy and cancer survival have improved overall, and side effects have become less frequent. The ubiquitin-proteasome system–mediated proteasomal protein degradation is the most critical pathway to regulate the quantity of signal proteins involved in carcinogenesis and tumor progression. These processes are, as well as protein recycling, highly regulated and offer targets for biomarker and drug development. Unspecific proteasome inhibitors such as bortezomib and carfilzomib have shown clinical efficacy and are approved for clinical use. Inhibitors of more substrate-specific enzymes of degradation processes are being developed and are now in early clinical trials. The novel compounds focus on the degradation of key regulatory proteins such as p53, p27kip1, and β-catenin, and inhibitors specific for growth factor receptor kinase turnover are in preclinical testing. Clin Cancer Res; 20(12); 3064–70. ©2014 AACR.

Background

Ubiquitin-proteasome system

The closely regulated ubiquitin-proteasome system (UPS) clears the cell plasma from damaged, misfolded, and aged proteins. More than 80% of intracellular proteins are processed by the UPS (1), and the remaining proteins are handled by the lysosome system. UPS is also involved in the inactivation of regulatory proteins by initiating the post-translational addition of multiple ubiquitin motifs, which sorts intracellular proteins for degradation.

Ubiquitin is a small and highly conserved protein of 76 amino acids. Polyubiquitination is facilitated by isopeptide bonds between the last amino acid of ubiquitin (glycine) and one lysine (K) of another ubiquitin that functions as the substrate. Ubiquitin has seven lysine positions (K6, K11, K27, K29, K33, K48, and K63) with K48 and K63 being the most common positions where polyubiquitination occurs. The position of polyubiquitination determines whether a protein will be degraded (K48-linked) or will be activated (K63-linked; ref. 2). Little is known about ubiquitination at the other lysine positions.

Ubiquitin-like proteins

More than 20 ubiquitin-like proteins, such as NEDD8 (neural precursor cell expressed, developmentally down-regulated 8), SUMO (small ubiquitin-related modifier), and ISG15 (IFN-1-induced-17-kDa protein), have been described that play important roles in posttranslational protein modification (3).

NEDD8 most importantly is modifying the ubiquitin-dependent degradation process by interacting with cullin-like E3 ligases (4). It activates cullin E3 ligases, leading to a higher rate of polyubiquitination, and therefore drives the degradation of proteins that are turned over by cullin E3 ligases (5).

SUMO, like ubiquitin, facilitates lysine amino acids within the substrate to bind to other proteins. SUMOylation therefore competes with ubiquitylation and can inhibit ubiquitin-dependent proteolysis (6). It has been described in neurodegenerative disorders such as Alzheimer and Parkinson disease (7). SUMO modification of multiple substrates supports their physical interaction (SUMO glue) and thereby stimulates complex formation. This complex formation plays an important role in DNA repair mechanisms, ribosomal biogenesis, and genome maintenance (8) and links SUMOylation to multiple diseases such as melanoma and renal cell carcinoma and to cell stemness, making it an interesting field for drug development (9).

ISG15 also modifies proteins by a lysine-glycin isopeptide bond and is involved in the inflammatory response to IFN-1. Its role in alternating lethality to virus infection has been investigated to a wide extent (10). As virus replication is for the most part not affected and function differs between different viruses and host species, many questions remain unaddressed. It is known that ISG15 targets newly translated virus and host proteins under the influence of IFN-1 (11) and therefore that it is involved in the modulation of immune response to viral infections.
Ubiquitin activation (E1)

The UPS can be separated into four different processes: (i) ubiquitin activation by E1 enzymes, (ii) ubiquitin conjugation by E2 enzymes, (iii) ubiquitin ligation by E3 enzymes, and (iv) the proteolysis of the substrate in a 26S-proteasome (12). Deubiquitinases (DUB) can reverse this process by dissociating ubiquitin from the substrate and enable protein recycling. Ubiquitin activation is ATP dependent and is achieved by one of the two known E1 human enzymes. UBE1, the principal ubiquitin activating protein in eukaryotes, and the recently described UBE1L2, add an energy-rich thioester bond to the C-terminal end of ubiquitin. Inhibitors of E1 enzymes are designed to interfere with this thioester bond.

Ubiquitin-conjugating enzymes (E2)

Ubiquitin-conjugating enzymes (E2) are capable of transferring the activated ubiquitin onto a ubiquitin ligase (E3) enzyme–substrate complex. About 50 E2 enzymes have been identified. The central functional motif is a ubiquitin-conjugating catalytic (UBC) fold. The UBC exhibits a catalytic cysteine residue that, together with the thioester bond of the activated ubiquitin, forms a high-energetic conjugate. E2 enzymes define the position of ubiquitination (e.g., K48 vs. K63) and consequently determine the further destiny of the protein substrate (12). Characterized by the extensions to the UBC, four different classes of E2 enzymes have been defined: class I, no extension; class II, N-terminal extension; class III, C-terminal extension; and class IV, extension on both ends (13).

Ubiquitin ligases (E3)

E3 ligase enzymes are highly substrate specific with more than a thousand enzymes estimated (14). The principal function of E3 ligases is to recruit specific proteins (substrates) and to interact with E2 enzymes to catalyze the covalent binding of ubiquitin. Three major classes of E3 enzymes have been defined according to the structure of the catalytic domain (15): (i) HECT (homologous to the E6AP carboxyl terminus), (ii) U-Box, and (iii) RING (really interesting new gene) E3s. An important subgroup of RING E3s is cullin RING ligases (CRL), which ubiquitinate proteins with key roles in cell-cycle progression and signal transduction. The functional motif of many CRL is the SCF (Skp-Cullin-F-box containing) complex, making all three components interesting targets for drug development. F-box is a structural motif of about 50 amino acids that mediates protein–protein interactions (16). Skp (seventeen-kDa protein) is a trimeric periplasmic chaperone that assists outer membrane proteins in their folding and insertion into membranes. Cullins are a family of proteins scaffolding the E3 ligase activity that are regulated by neddylation (4). The addition of NEDD8 to CRLs drives the turnover of multiple regulatory proteins toward degradation such as growth factor receptor proteins (5).

26S proteasome

Degradation within the UPS is processed by the 26S proteasome, which consists of a 20S core particle and two regulatory 19S regulatory caps (17). Polyubiquitinated proteins are broken down by proteolysis. In cancer cell lines, augmented proteasome activity is a common phenomenon, including degradation of proteins involved in tumor progression, apoptosis, and cell-cycle regulation.

Clinical–Translational Advances

Novel inhibitors of the UPS have been developed targeting key proteins of the major circuits of carcinogenesis, as defined by Hanahan and Weinberg (ref. 18; Fig. 1). Most of the compounds (Table 1) are still in early clinical development (phase I) and therefore are under examination for toxicity and tolerability. As single substance efficacy is not anticipated, studies testing drugs in further development (phase Ib/II) use combinations of standard-of-care chemotherapeutic substances such as antimetabolites (e.g., cytarabine, 5-fluorouracil) or mitotic inhibitors (e.g., paclitaxel) depending on the underlying disease.

Cell-cycle regulation

Accelerated degradation of regulatory cell-cycle proteins causing lower intracellular expression of tumor suppressors such as p53 has been demonstrated in a variety of neoplasias. E3 ligases reducing intracellular p53 levels are associated with carcinogenesis and prognosis. Examples are E6-AP ubiquitin-protein ligase (E6-AP), which is activated by human papillomavirus (HPV; ref. 13) and leads to HPV-associated carcinomas, and RING finger and CHY zinc finger domain-containing protein 1 (aka Pirh2), which is overexpressed in hepatocellular carcinoma (HCC), head and neck cancers, lung cancer, and prostate cancer and correlates with poor overall survival in HCC (19).

Given the central role of p53 in DNA damage repair and cell-cycle regulation, the turnover of p53 has been extensively studied and several potential regulatory proteins of p53 degradation have been identified. Higher levels of Hdm2, which is the most important E3 ligase for p53, were identified in leukemias, lymphomas, and solid tumors (12), making it a valuable target for drug development. Substances interacting with the Hdm2-p53–binding site have been shown to increase p53 levels in p53 nonmutant cell lines and consequently led to cell-cycle arrest and apoptosis (20). Among those, nutlins were the first class discovered and two members (RO5045337 and RO5503781) made it to phase I trials, but results have not been reported. Recently, clinical data on serدميتسن (JN-26854165), a tryptamine compound belonging to the second class of Hdm2 antagonists, have been reported (21). Serدميتسن has been shown to increase p53 levels radio-sensitizing tumors in xenograft models (22). It was well tolerated in a phase I trial. Spiro-oxindoles are the third class of small inhibiting molecules interacting with Hdm2 (23), with MI-773 (SAR405838) currently being tested in two clinical trials (NCT01636479 and NCT01985191). Other compounds interacting with Hdm2 are being developed and are in early
clinical testing (see Table 1). Their specific mechanisms of action have not been reported.

Targeting DUBs play an important role in the regulation of p53 levels. Both the DUB HAUSP (herpesvirus-associated ubiquitin-specific protease) and USP10 (ubiquitin-specific protease 10), targeting polyubiquitinated p53, have been shown to restore p53 levels even when Hdm2 is overexpressed (24, 25).

It has also been shown that inhibition USP7 and USP2a, which are deubiquitases of Hdm2, increases Hdm2 proteolysis and stabilizes p53 levels in multiple myeloma cells (26, 27), enabling the initiation of apoptosis. The inhibition of USP7 with HBX 41,108, a DUB to Hdm2, has been shown to induce p53-dependent apoptosis with an IC50 in submicromolar concentrations. The same principle was demonstrated with other compounds such as P5091 (26) and P22077 (28) in preclinical models; however, no deubiquitinase inhibitor has entered clinical development.

Other potential targets in cell-cycle regulation, such as cyclin E, C-Myc, and p27Kip1, are known to be degraded by F-box containing SCF E3 ligases.

Mutations leading to a loss of function in the F-box FBXW7 have been identified in solid and hematologic malignancies (29), leading to increased proliferation. The
| Table 1. Modulators of the UPS in clinical development for cancer treatment |
|---------------------------------|-----------------|--------------------------------------------------|
| **Target structure name**       | **Clinical stage** | **Remarks**                                      |
| **Proteasome inhibitors**       |                  |                                                  |
| Bortezomib                      | Approved for MM and MCL | Multiple clinical trials testing bortezomib in combination with chemotherapeutic substances in MM and MCL. |
| Carfilzomib                     | Approved for MM after two prior therapies | Tested in a phase I study of advanced malignancies (including mCRC) with hepatic impairment (NCT01949545) and in combination with irinotecan in a phase I/II study of irinotecan-sensitive advanced malignancies (including mCRC; NCT01941316) |
| Oprozomib ONX 0912              | Phase I          | Oral proteasome inhibitor under evaluation for the treatment of MM and lymphomas and in a phase I study of advanced malignancies (including mCRC; NCT0129349) |
| Marizomib NPI-0052              | Phase I/II       | Tested in a phase I study for advanced solid malignancies (including mCRC; NCT00629473) |
| Delanzomib CEP-18770            | Phase I          | Tested in a phase I study for advanced solid malignancies (including mCRC) or non-Hodgkin lymphoma (NCT00572637) |
| Ixazomib MLN9708                | Phase I/II       | Tested in a phase I study for advanced solid malignancies (including mCRC; NCT00830869) |
| **Nedd8 activating enzyme**    |                  |                                                  |
| NAE MLN4924                     | Phase I          | Currently tested in AML (NCT01814826), large B-cell lymphoma (NCT01415765) and advanced malignancies (NCT00677170) in combination with chemotherapeutic drugs |
| **E3 ligases interacting Hdm2-p53** |                  |                                                  |
| RO5045337                       | Phase I          | Nutlin derivate tested in multiple phase I trials (NCT00559533), results not reported yet |
| RO5503781                       | Phase I          | Nutlin derivate currently being tested in AML (NCT01773408), advanced malignancies except leukemia (NCT01462175) |
| DS-3032b                        | Phase I          | Tested in a phase I study for advanced solid malignancies or lymphomas (NCT01877382) |
| SAR405838                       | Phase I          | Spiro-oxindole tested in phase I studies for advanced malignancies (NCT01636479) and in combination with pimasertib (NCT01985191) |
| JNJ-26854165                    | Phase I          | Successful phase I study with good tolerability and modest efficacy (21) |
| MK-8242                         | Phase I          | Tested in phase I studies for advanced solid malignancies (NCT01463696) and in combination with cytarabin in AML (NCT01451437) |
| CGM097                          | Phase I          | Tested in a phase I study for advanced solid malignancies (NCT01760525) |
| p28                             | Phase I          | Tested in pediatric patients with recurrent or progressive central nervous system tumors (NCT01975116) |
| **E3 ligases interacting with apoptotic proteins** |                  |                                                  |
| Birinapant TL32711              | Phase II         | Testing the efficacy for ovarian, primary peritoneal or fallopian tube cancer (NCT01681368) |
| AEG35156                        | Phase I/II       | Preclinical studies showed activity but data of clinical phase I/II studies were disappointing |
| LCL161                          | Phase I/II       | Tested in solid tumors (NCT01098838), in combination with paclitaxel in solid tumors (NCT01240655), and in breast cancer (NCT01617668) |
| AT-406 (Debio 1143)             | Phase I          | Encouraging data in mouse xenotransplant models of human ovarian cancer (56), tested in phase I design (NCT01078649) |
| GDC-0917                        | Phase I          | (NCT01226277) |
| GDC-0152                        | Phase I          | Tested in solid tumors (NCT00977067) |
| HSG1029                         | Phase I          | Tested in solid tumors (NCT00708006) and lymphoid malignancies (NCT01013818) |
| CUDC-427                        | Phase I          | Tested in solid tumors and lymphomas (NCT01908413) |

NOTE: The authors make no claim for completeness of clinical trials listed in this table.
Abbreviations: mCRC, metastatic colorectal cancer; MM, multiple myeloma; NCT, National clinical trial number.
reported frequency of FBXW7 loss of function mutations in T-cell acute lymphoblastic leukemia (T-ALL) is approximately 20% (30), but these mutations are also frequently found in cholangiocarcinoma (∼35%), colorectal (∼10%), ovarian, and endometrial cell lines (29, 30). Modulating FBXW7 degradation is promising for novel drug development in a variety of tumors (15).

Degradation of p27\(^{kip1}\) is mediated by a specific SCF Skp2, which plays a role in cellular senescence, cancer progression, and metastasis (31). No specific Skp2 inhibitor has been reported in clinical development.

Viability pathways

The downregulation of apoptosis and upregulation of survival pathways are characteristic for cancer development and progression. Inhibitors of apoptosis proteins (IAP) are E3 ligases regulating caspase activity that is required for apoptosis. In a number of hematologic diseases, including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, and lymphomas, IAPs are overexpressed (32), making them a promising target for drug development.

A detailed review of IAP inhibitors in cancer has been recently published (33) and multiple IAP inhibitors are currently being tested in phase I and II trials (Table 1). AEG 35156 is the only substance with clinical outcome data. Unfortunately, due to a lack of efficacy shown with this agent in two phase II trials (34, 35), further development was terminated.

NF-κB is a transcriptional factor involved in inflammatory processes, cell proliferation, and cell survival. Active NF-κB is released after degradation of inhibitor of κB (IκB). Canonical release through TNF-α signaling and noncanonical activation through NF-κB inducing kinase are both dependent on cellular IAPs (cIAP; ref. 33). Birinapant is an antagonist inhibiting cIAP1 and cIAP2 and was shown to restore TNF-α-dependent apoptosis in breast and melanoma cancer cells (36, 37). Birinapant is currently being tested in phase II trials in ovarian, fallopian tube, and primary peritoneal cancer.

Another target for novel drug development interacting with viability circuits is USP9x, which deubiquitinases the induced myeloid leukemia cell differentiation protein Mcl-1. USP9x is overexpressed in lymphomas, chronic myelogenous leukemia, and multiple myeloma, where higher Mcl-1 levels cause a block in apoptosis (38). In preclinical models, the USP9x inhibitor WP1130 has been shown to increase proapoptotic proteins and to decrease antiapoptotic proteins (39) and therefore increase tumor cell sensitivity to multiple chemotherapeutic agents (40).

Further clinical testing will depend on whether promising data are generated in preclinical models.

Regulation of cell motility

A key regulatory protein that is commonly associated with a large number of cancers and hematologic tumors is β-catenin. Depending on its intracellular localization, this protein has distinct functions in cell proliferation and cell motility. Usually bound to cytoskeleton proteins, cell plasma levels are critical for the function as a transcription factor. The F-Box E3 ligase SCF β-TrCP (41) and Siah-1–SIP-Skp1 (42) degrade cell plasma β-catenin, reducing wnt-β-catenin signaling. FAS-associated factor 1 (FAF1) has been shown to increase β-catenin degradation by activating the β-TrCP F-Box complex (43). K63 ubiquitination of β-catenin with the help of the E2 ligase RAD6 (ubiquitin-conjugating enzyme E2 B) increases cell plasma β-catenin. K63 polyubiquitinated β-catenin is not available for K48 ubiquitination (44) but is functionally active. RAD6 has been shown to be overexpressed in breast cancer (45), and inhibitors are in preclinical development (46).

Growth factor–dependent pathways

Growth factor pathway–dependent carcinogenesis, proliferation, and metastasis are activated through extracellular ligands, transmembrane receptors, or by activating mutations within the intracellular part of the pathways. The amount of receptors and ligands expressed on the cell surface is dependent on recycling and degradation processes.

Solid tumors are in need of growth factors to migrate, proliferate, and create their own vessel system through angiogenesis. These signaling pathways are often regulated through growth factor receptor kinases such as ErbB family members or members of the VEGF receptor family. After ligand binding, the growth factor receptor is internalized, the tyrosine activity is shut down, and the protein is degraded with the help of c-Cbl, an SCF (Skp1-Cullin-F-box) ligase that is activated by neddylation (47, 48). Only inhibitors of neddylation (ML4232) are in clinical development (4).

The von Hippel–Lindau (VHL) syndrome is caused by a mutation in the E3-ligase (VHL), reducing degradation of hypoxia-inducible factor-1α (HIF1α) and leading to increased signaling of proangiogenic cytokines (49), which further supports the key role of degradation in the regulation of growth factor signaling.

DNA repair mechanisms

There are no inhibitors of the UPS-associated DNA repair mechanisms available; however, alterations in the BRCA and FANC E3 ligases are a good example for the clinical significance of degradation for diagnosis, prognosis, prevention, and treatment strategies. Reduced DNA repair mechanisms lead to accumulation of DNA damage, promoting carcinogenesis. One of the best known hereditary cancer syndromes is caused by mutations within the BRCA1 gene, which is an E3 ligase involved in DNA repair, emphasizing the importance of alterations in the UPS in carcinogenesis and tumor progression. Loss-offunction mutations in FANC E3 ligases that are involved in the ubiquitination of the FANC-FANCD2, which is also associated with DNA repair, cause Fanconi anemia (50) and are associated with childhood T-ALL and testicular seminoma (51).
Inhibitors of neddylation and the 26S proteasome

Inhibition of the 26S proteasome. Inhibition of the 26S proteasome causes an accumulation of intracellular proteins that leads to an inhibition of NF-κB activity and angiogenesis, alters degradation of cell cycle and apoptotic proteins, and affects endoplasmatic reticulum stress (1, 52). The reversible inhibitor, bortezomib (53), and the irreversible inhibitor, carfilzomib (54), have been approved for treatment of multiple myeloma. Bortezomib is also approved for the clinical use of mantle cell lymphoma (MCL) after disease progression after one prior therapy and is currently being tested in clinical trials in combination with chemotherapeutic agents in AML and ALL. More potent and less toxic proteasome inhibitors (Table 1) are under evaluation in clinical trials for patients with multiple myeloma and advanced solid tumors.

Inhibiting ubiquitin and NEDD8-activating enzymes. Inhibitors of E1 enzymes have been identified using high-throughput screening for substances targeting p53 and p27. PYR-41 was the first ubiquitin-activating enzyme (UAE) inhibitor to be tested in preclinical models and was able to inhibit p53 degradation and downregulate cytokine-induced NF-κB signaling (12). In addition to p53 levels, the structurally related substance PYZD-4409 was able to stabilize cyclin D3 levels and to induce cell death by induction of endoplasmatic reticulum stress (55). The UAE inhibitors have shown activity in preclinical models, but no clinical trials are under way, so far. Many E3 ligases are cullin-RING-ubiquitin ligases, which are activated by neddylation. Neddylation accelerates K48-linked polyubiquitination of multiple regulatory proteins such as p53, p21, p27kip1, growth factor receptor tyrosine kinases, and apoptosis proteins. One inhibitor of NEDD8 activation (MLN4924) is in early clinical development.

Conclusions

Degradation plays a key regulatory role in all major cell circuits representing the hallmarks of cancer. The challenge to translate these novel compounds successfully into the clinic is to identify the tumor tissue-specific degradation processes to personalize therapy with specific inhibitors.

Targeting the UPS for cancer treatment is a unique approach that has been proved to be effective, as the proteasome inhibitors bortezomib and carfilzomib are established in the treatment of multiple myeloma and MCL. Inhibitors of specific UPS enzymes of key regulatory proteins of carcinogenesis and tumor progression such as apoptosis proteins and p53 are currently being tested in phase I and phase II trials in a variety of cancers, including leukemia and solid tumors.

Increased molecular understanding of the regulation of protein degradation in cancer and liquid tumors will be essential for development of more specific and more effective and less toxic compounds. Identification of tumor-specific UPS enzymes in cancers and liquid tumors will be critical for selection of patients who will benefit the most from specific UPS inhibitors.

Disclosure of Potential Conflicts of Interest

S. Stintzing reports receiving speakers bureau honoraria from Amgen, Merck KgaA, Roche/Genentech, and Sanofi and is a consultant/advisory board member for Bristol-Myers Squibb, Merck KgaA, and Roche/Genentech. H.-J. Lenz reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Bristol-Myers Squibb and Merck.

Authors’ Contributions

Conception and design: S. Stintzing, H.-J. Lenz
Development of methodology: S. Stintzing, H.-J. Lenz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-J. Lenz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Stintzing, H.-J. Lenz
Writing, review, and/or revision of the manuscript: S. Stintzing, H.-J. Lenz
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-J. Lenz
Study supervision: H.-J. Lenz

Grant Support

H.-J. Lenz is supported by the NIH (R30CA014089) and the Nancy Bernstein Research Fund. S. Stintzing is supported by a postdoctoral fellowship from the German Cancer Aid (Mildred-Scheel Foundation).

Received January 9, 2014; revised March 24, 2014; accepted April 2, 2014; published OnlineFirst April 22, 2014.
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doi:10.1158/1078-0432.CCR-13-3175

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