Role of Ubiquitin Ligases and the Proteasome in Oncogenesis: Novel Targets for Anticancer Therapies

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ABSTRACT

The ubiquitin proteasome system (UPS) regulates the ubiquitination, and thus degradation and turnover, of many proteins vital to cellular regulation and function. The UPS comprises a sequential series of enzymatic processes using four key enzyme families: E1 (ubiquitin-activating enzymes), E2 (ubiquitin-carrier proteins), E3 (ubiquitin-protein ligases), and E4 (ubiquitin chain assembly factors). Because the UPS is a crucial regulator of the cell cycle, and abnormal cell-cycle control can lead to oncogenesis, aberrancies within the UPS pathway can result in a malignant cellular phenotype and thus has become an attractive target for novel anticancer agents. This article will provide an overall review of the mechanics of the UPS, describe aberrancies leading to cancer, and give an overview of current drug therapies selectively targeting the UPS.

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INTRODUCTION

The 2004 Nobel Prize in Chemistry was awarded to Drs Ciechanover, Hershko, and Rose for the discovery of the ubiquitin proteasome system (UPS), the pathway responsible for the degradation and turnover of intracellular proteins within the eukaryotic cell.^{1,2} This pathway is critical for the regulation and turnover of important cellular proteins and maintains homeostasis within the cell by controlling the half-life of specific proteins based on cellular needs. The UPS targets a variety of proteins, including those that are misfolded, mutated, or otherwise damaged—the cellular version of quality control.³ This waste-disposal function was discovered in the 1940s, but not until the 1980s did it become apparent that the UPS also degrades normal, functional proteins involved in basic cellular processes, such as enzymes and cell-cycle and transcriptional regulators that are no longer needed.^{2,3} This flux of proteins being continuously synthesized and degraded results in a dynamic state; however, without appropriate protein homeostasis maintained by the UPS, healthy cells can undergo malignant transformation. This review will focus on the UPS and its potential as a novel anticancer target.

UPS

The UPS uses a two-step process to selectively target proteins for diverse physiologic roles, including subcellular localization, cellular signaling, and degrada-

tion. The first step involves the covalent attachment of a 76-amino acid polypeptide tag, ubiquitin, to lysine residues on the target protein. In the second step, the targeted protein becomes polyubiquitinated and, depending on the particular lysine residue involved and the length and characteristics of the ubiquitin chain, results in distinct physiologic functions. Ubiquitin linkages vary depending on specific lysine residue involvement and polyubiquitin versus multiple monoubiquitin chains, and these variations confer a distinct protein fate. Lys63 residue involvement preferentially involves the lysosomal pathway, whereas Lys11-, Lys29-, and Lys48-linked chains are generally targeted for degradation.^{4,5}

The covalent bonding of ubiquitin is achieved through the sequential action of four key enzyme families (Fig 1): E1 (ubiquitin-activating enzymes [UAEs]), E2 (ubiquitin-carrier proteins), E3 (ubiquitin-protein ligases), and E4 (ubiquitin chain assembly factor).^{3,6} Initially, ubiquitin is activated by E1 in an ATP-dependent reaction and is then transferred from E1 to E2. Next, E3 transfers and conjugates ubiquitin from the E2-ubiquitin complex to the substrate protein.⁷⁻⁹ E3 enzymes perform a critical role in this process through the selective binding of protein substrates. Approximately 1,000 different E3 enzymes comprise multisubunit families that allow for a high degree of substrate specificity.² E3 enzymes are divided into subclasses based on their structural and biochemical features: HECT (homologous to E6-AP carboxy terminus), RING (really

Fig 1. Protein ubiquitination and subsequent degradation is dependent on several integrated enzymatic cascades. The formation of the polyubiquitin chain is catalyzed by a cascade of enzymes. The E1 enzyme (ubiquitin-activating enzyme [UAE]) activates ubiquitin (Ub) and then transfers it to one of dozens of E2 enzymes (ubiquitin-carrier protein [UCP]). The E2-Ub complex then interacts with a specific E3 enzyme to catalyze the covalent formation of a polyubiquitin chain on a substrate protein specifically recruited by that E3. E3 ubiquitin ligases can be divided into three classes: HECT (homologous to E6-AP carboxy terminus), U-box, and RING (really interesting new gene). Within the RING family, there are two additional subclasses: cullin-containing RING-finger ligases (CRLs) and other RINGs. CRLs represent a unique subclass in that they must undergo neddylation to become activated and participate in the ubiquitin proteasome system (UPS). Neddylation is an enzymatic cascade homologous to ubiquitination involving E1 (NEDD8-activating enzyme [NAE]) and E2 (Ubc12) enzymes. Deubiquitinases (DUBs) function to deubiquitinate proteins, thus altering their fate within the UPS. Novel targeted inhibitors of enzymes within the UPS, including UAE, NAE, E3, DUBs, and proteasome inhibitors, are in preclinical and clinical development. UPL, ubiquitin-protein ligase.

interesting new gene) fingers, and U-box domains.⁸ The RING finger subclass is subdivided into two subfamilies: cullin-containing RING-finger ligases (CRLs) and those in which the RING-finger and substrate binding domains are contained on the same polypeptide.¹⁰ E3 substrates are recognized, polyubiquitinated, and degraded under a variety of conditions. Some are degraded based on physiologic conditions, and in some cases, the substrate must undergo post-translational modification, such as phosphorylation, oxidation, or a conformational change, to be recognized by the appropriate E3 enzyme.^{2,8} This specificity confers the discrimination used by the UPS to tightly regulate protein homeostasis.^{11,12} E3 ligases mediate the mono- or multiple-monoubiquitination of proteins, and recently, a new class of ubiquitin ligases—the E4 ligases—has been recognized as a mediator of ubiquitin chain elongation. The first E4 protein, Ufd2, was originally discovered in yeast, and subsequently, its human homolog, UBE4B, was identified.⁶ The E3 ubiquitin ligase MDM2 only mediates the mono- or multiple-monoubiquitination of p53 degradation.¹³⁻¹⁶ Wu et al¹⁷ demonstrated UBE4B to be an essential cofactor for MDM2-promoted p53 polyubiquitination and degradation. This group also found an association between UBE4B overexpression and decreased p53 levels in brain tumors. Members of this rare group of ubiquitin ligases continue to grow, with Hul5 being the most recent addition.¹⁸ The cooperative action of the ubiquitin ligase enzymes, repeated multiple times, results in target-protein polyubiquitination.

It is now appreciated that there are homologous ubiquitin-like proteins (UBLs) that are also conjugated in a similar enzyme cascade.¹⁹ The alternative UBL pathways serve to insert an additional layer of selectivity into protein degradation through activation of specific E3 enzymes in the UPS. For example, NEDD8 is a UBL that after conjugation to the CRL enzyme complex, activates the complex

and facilitates the transfer of ubiquitin to the protein substrate using this now-activated E3 enzyme.^{10,20} One function of the NEDD8 pathway is to regulate the rate of ubiquitination, and thus the degradation kinetics, of the subset of proteins, the ubiquitination of which is dependent on CRLs.²¹ Many proteins with key roles in cell-cycle progression and signal transduction are substrates for CRL-mediated polyubiquitination. Without NEDD8-activating enzyme (NAE)—assisted neddylation of the CRL, these proteins are not polyubiquitinated and accumulate within the cell.²¹ The ability to selectively affect a subset of proteins involved in these cellular processes renders NAE inhibition an appealing anticancer target. Examples of known substrates involved in cancer pathogenesis and targeted by CRLs for ubiquitination and destruction include p27, p-IkBa, c-MYC, mTOR (mammalian target of rapamycin), and HIF1 (hypoxia-inducible factor-1).²²⁻²⁷

Just as there is a complex system for ubiquitinating proteins and targeting them for modification and/or degradation, there also exists a process for deubiquitination, which is carried out by a large group of proteases known as deubiquitinases (DUBs) or ubiquitin-specific proteases.²⁸ The removal of ubiquitin chains can rescue proteins from degradative pathways or lead to reversion of ubiquitin signaling. Because the nature of the ubiquitin chain linkage defines the fate of the protein, modification of the ubiquitin chain by DUBs can ultimately enhance protein stability.^{29,30} The broad functional diversity of DUBs renders them powerful participants in many aspects of cell-cycle control, cell-signaling pathways, and DNA repair, and thus, they are relevant to oncogenesis. Accordingly, because some DUBs function in both tumor suppressor and oncogenic roles, appropriate targeting is critical.³¹ There are several recent publications discussing new therapeutic strategies involving DUB inhibition, but currently, this area is in

the early stages of development, and a broader understanding of all DUB substrates and regulatory functions is needed before the advancement of such agents into the clinic.^{28,32-35} The Appendix (online only) provides an extended review of DUBs and DUB inhibitors.

The UPS is a complex pathway regulating the levels and function of proteins with roles in both oncogenesis and tumor suppression. This is further complicated by the cellular environment and context of protein activity, making inhibition of this pathway challenging and not without risks, because the potential to promote cancer via stabilization of oncogenic proteins or inhibition of tumor suppressors exists. The development and success of bortezomib, a downstream inhibitor of protein degradation at the level of the proteasome, have illustrated that despite nonspecific targeting of the UPS, potent and clinically relevant anticancer effects can be achieved. Further development of drugs targeting this pathway need to be fully examined for procancer effects before human trials; however, our need for a broader understanding of the cellular processes resulting from inhibition of this pathway should not pose an obstacle to further development, because clinical success using these strategies has already been achieved and can likely be improved.

UBIQUITIN PROTEASOME PATHWAY ABERRATIONS

The UPS pathway is critical in the regulation of many intracellular processes, and thus, aberrations in this pathway result in clinically distinct phenotypes, including several malignancies.^{2,36,37} Because the emergence of a malignant cell is dependent on concurrent events, and the UPS is critical for the proper turnover of both oncoproteins and tumor suppressor proteins, when this balance is disrupted by either a loss or gain of function mutation in the UPS pathway, malignant transformation of a cell can result (Table 1).

MDM2 REGULATION OF P53 FUNCTION

The p53 tumor suppressor protein is a ubiquitous transcriptional activator that performs a critical role in the response of a cell to errors

induced by stress, such as DNA damage and oncogene activation. This pathway serves to eliminate potentially tumorigenic cells via cell-cycle arrest and apoptosis and thus, when dysfunctional, directly contributes to the malignant phenotype. Cellular p53 levels are tightly regulated by cellular localization and degradation mechanisms. Although it is now appreciated that degradation of p53 can be regulated by several E3 ubiquitin ligases, including COP1, Pirh2, and ARF-BP1, the major regulators of p53 turnover are MDM2 and its structural homolog, MDM4 (also known as MDMX).^{36,62-66} MDM2 regulates p53 activity in three ways: one, through direct binding to p53, thereby preventing transcriptional activity; two, as the major E3 ubiquitin ligase for p53, targeting it for degradation by the 26S proteasome; and three, by mediating its nuclear export.⁶⁷⁻⁷⁰ Accordingly, with the loss of p53 function, either through mutation or increased degradation, DNA damage goes unrepaired, or there is no induction of apoptosis, resulting in the potential for malignant transformation. The critical role of MDM2 in the regulation of p53 has been supported by the fact that although the deletion of the *MDM2* gene in mice results in embryonic lethality, the lethality is reversed by p53 deletion, suggesting that the major role of MDM2 is to control p53 activity.^{71,72}

MDM2 overexpression has been reported in 10% to 20% of malignancies, particularly in cancers of the bone and salivary gland and in Hodgkin lymphoma.⁷² Some data suggest that elevated MDM2 levels are associated with increased metastasis and accelerated cancer progression.^{44-47,73,74} Additionally, other data have shown that transformed cells are more sensitive to p53-induced apoptosis than their normal counterparts, thus possibly providing a method for tumor-specific cell killing.^{75,76}

UBLs, such as SUMO and NEDD8, also play a role in p53 regulation. The processes by which ubiquitin and NEDD8 are ligated to p53 are analogous and even use some of the same E3 enzymes.⁷⁷ Furthermore, shared ubiquitination, neddylation, and sumoylation lysine sites have been identified on p53. Because these processes share modification sites, it is unclear what dictates the process of ubiquitination versus neddylation or sumoylation.⁶² Furthermore, the effects of sumoylation are not entirely clear, with some evidence indicating that SUMO E3 ligase-mediated sumoylation enhances p53 transcriptional activity, whereas other data have shown increased cytoplasmic translocation.^{78,79} The balance between sumoylation and ubiquitination of p53 is not fully understood and is likely cell-context dependent. However, it is clearly important, because the outcomes of these two modifications are divergent with respect to their effects on cellular regulation.

FBW7 TUMOR SUPPRESSOR

FBW7, also known as FBXW7, is an E3 ubiquitin ligase with well-known substrates, including cyclin E, MYC, JUN, and Notch, all of which have central roles in cell division, growth, and differentiation. Many of these substrates function as dominant oncogenes, and thus, FBW7 functions as a tumor suppressor for these particular substrates. Conversely, p100, an inhibitor of the procancer signal nuclear factor- κ B (NF- κ B), has been identified as a substrate of FBW7.⁸⁰ In this context, FBW7 functions instead as an oncogene and further highlights the complexity and dual roles of many proteins functioning within the UPS. Mutations in FBW7 have been described in a number of malignancies, including T-cell

Table 1. UPS Mutations Associated With Malignancies

Malignancy	Mutation/Defect	Reference
Breast and ovarian cancers	<i>BRCA1</i> ; loss of tumor suppressor function	38-40
Lung cancer, clear cell carcinoma, VHL-disease cancers	<i>VHL</i> mutation; loss of tumor suppressor function	41, 42
Various malignancies	<i>MDM2</i> overexpression; loss of p53 tumor suppressor function	43, 44-47
Prostate cancer	<i>USP2a</i> overexpression; increased <i>cMYC</i> oncogene expression; <i>SPOP</i> mutation	48-50, 51
Leukemia, cholangiocarcinomas, GI and endometrial cancers	<i>FBW7</i> mutation; loss of tumor suppressor function; potential oncogene	52, 53
Colorectal, breast, and prostate cancers	<i>SKP2</i> overexpression; loss of tumor suppressor function of p27	54, 55-58
Cervical cancer	HPV-mediated degradation of p53	59-61

Abbreviations: HPV, human papilloma virus; UPS, ubiquitin proteasome system; VHL, von Hippel-Lindau.

acute lymphoblastic leukemia, cholangiocarcinomas, and GI and endometrial cancers. Mutations resulting in decreased tumor suppressor activity occur in both the gene loci as well as the FBW7 binding domains on target substrates.^{52,53}

SKP2 REGULATION OF P27 CYCLIN-DEPENDENT KINASE INHIBITORS

Cyclin-dependent kinases (CDKs) regulate progression through the cell cycle via the phosphorylation of specific substrates. In turn, CDKs are themselves regulated by CDK inhibitors, one of which is p27,^{81,82} which inhibits cell-cycle transitions and thus functions as a tumor suppressor.⁸³ By inhibiting cell-cycle transitions, p27 functions as a tumor suppressor via cell-cycle inhibition. Skp2, an E3 ligase in the cullin-RING family, regulates p27 via ubiquitination and 26S proteasome-mediated degradation.^{54,84,85} Biochemical and genetic experiments have shown that Skp2 is necessary for the degradation of p27 both in vivo and in vitro.^{54,84} Although heterozygous loss of one p27 allele is not uncommon in human malignancies, homozygous gene deletions are extremely rare.^{86,87} The current hypothesis is that the low levels of p27 found in several malignancies result from increased proteolysis, and not decreased production as a result of gene deletions, defects, or post-translational modifications. Elevated levels of Skp2 result in increased degradation of p27 and thus decreased tumor suppressor activity. Several studies have demonstrated elevated Skp2 levels and a correlation between p27 expression and clinical outcomes in colorectal, breast, and prostate cancers.⁵⁴⁻⁵⁸ The exact mechanism by which Skp2 contributes to oncogenesis is currently under investigation.^{2,88}

E6 VIRAL ONCOPROTEIN

Cervical cancer has a well-established causal link to infection by human papilloma virus (HPV), a small DNA virus that infects mucosal or cutaneous epithelia. In addition to cervical cancer, infection can also lead to anogenital tumors and squamous cell carcinoma of the head and neck. High-risk HPV strains 16, 18, and 31 can result in squamous intraepithelial lesions that can progress to invasive cancer of the cervix.² The HPV genome encodes for an oncoprotein—E6. When E6 binds to E6-associated protein (E6-AP), an E3 enzyme, p53, becomes the targeted protein for ubiquitination and degradation. Furthermore, the E6-AP enzyme only recognizes p53 when it is associated with E6. The E6-AP enzyme activity of HPV-infected cells ultimately results in the increased degradation of p53.^{59,60} Studies have demonstrated that p53 levels in high-risk HPV-infected cervical cells are substantially lower than in non-HPV-infected cells. Interestingly, the low-risk HPV strains 6 and 8 encode for an E6 that cannot interact with E6-AP and thus do not increase the degradation of p53, likely accounting for their inability to induce malignant transformation.^{60,61}

SPOP MUTATIONS IN PROSTATE CANCER

Recent studies using exome sequencing to identify recurrent mutations in prostate cancer specimens found that *SPOP*, a cullin 3-ubiquitin ligase complex, was mutated at a frequency of 6% to 15% in localized and metastatic prostate tumors, whereas no mutations

were found in benign tissues.⁴⁸ Most of these mutations were in the substrate binding domain, which recognizes members of the hedgehog and JNK signaling pathways, suggesting a biologic role in neoplasia.^{49,50} Although the functional significance of these mutations remains unclear, mutant protein expression resulted in increased invasion of prostate cancer cells with no change in cell growth. Of note, *SPOP*-mutant tissues lacked the *ETS* family gene rearrangements seen in 50% of prostate tumors, suggesting the *SPOP* mutants may represent a distinct molecular subtype of prostate cancer.⁴⁸

UPS AS A TARGET FOR NOVEL ANTICANCER THERAPEUTICS

UPS dysfunction, with demonstrated contribution to the development of cancer, renders it a feasible and rational target for novel therapies. The development of proteasome inhibitors was a historic milestone in the understanding and targeting of the UPS; many reviews of this topic can be found in the literature. This review serves to highlight advances in the development of new drug targets within the UPS (Table 2).

E1-ACTIVATING ENZYME INHIBITORS

MLN4924 (Millennium Pharmaceuticals, Cambridge, MA) is a small-molecule inhibitor of NAE. Inhibition of the pathway is facilitated through the generation of an adenosine sulfamate analog that binds to NAE and forms a covalent adduct with NEDD8. MLN4924 mimics the first intermediate in the E1 reaction cycle, but it cannot be used in the subsequent enzymatic reactions, thereby resulting in enzyme inhibition.^{36,89} NEDD8 conjugation with cullin proteins is required for the ubiquitin ligase activity of the CRLs, and thus blocking NAE results in the inhibition of CRL activity and the stabilization of CRL substrates, many of which are important in cell-cycle regulation. With the abnormal accumulation of these substrates, specifically Cdt-1, a critical factor required for licensing origins of DNA replication, rereplication is induced, leading to cell apoptosis.²¹ Preclinical studies have verified increased levels of the DNA damage marker, CHK1, and the

Table 2. UPS Inhibitors Currently in Clinical Development

Name	Developer	Inhibitory Target	Clinical Development Status
MLN4924	Millennium Pharmaceuticals	NEDD8-activating enzyme (E1)	Phase I/phase II
R7112	Roche	p53-MDM2 binding pocket	Phase I
Bortezomib	Millennium Pharmaceuticals	20S core subunit	FDA approved
Carfilzomib	Onyx Pharmaceuticals	20S core subunit	Phase IIb
CEP-18770	Cephalon	20S core subunit	Phase I
MLN9708	Millennium Pharmaceuticals	20S core subunit	Phase I
Salinosporamide A (NPI-0052)	Nereus Pharmaceuticals	20S core subunit	Phase I
ONX-0912	Onyx Pharmaceuticals	20S core subunit	Phase I

Abbreviations: FDA, US Food and Drug Administration; UPS, ubiquitin proteasome system.

apoptosis markers cleaved caspase 3 and cleaved PARP in treated HCT-116 cells. Furthermore, DNA profiles were analyzed, and a significant fraction of treated cells demonstrated greater than tetraploid DNA content. MLN4924 selectively inhibits NAE and stabilizes only proteins that are regulated by this E3, which may reduce toxicity and improve therapeutic index as compared, for example, with 26S proteasome inhibition.¹⁰ On the contrary, although many NAE substrates have been identified, there are undoubtedly substrates that have not yet been identified, and thus, MLN4924 has the potential to alter the degradation of proteins, the functional impact of which is not yet fully understood. Further evidence of the effectiveness of MLN4924 as an anticancer agent was demonstrated in vivo against colon cell-line xenograft tumors, where growth inhibition was observed at doses and schedules that were well tolerated.²¹ In acute myeloid leukemia models, MLN4924 treatment disrupted the ability of cells to grow and form colonies and led to the induction of apoptosis in vivo, whereas in diffuse large B-cell lymphoma, the agent demonstrated inhibition of the NF- κ B pathway, a pathway on which many hematologic malignancies are dependent.⁹⁰⁻⁹³ Milhollen et al⁹⁴ confirmed the role of MLN4924 in inhibition of the NF- κ B pathway via decreased polyubiquitination of I κ B proteins, which in turn bind NF- κ B transcription factors in the cytoplasm and prevent activation.⁹⁵ These data illustrate the critical but mechanistically distinct role NAE plays in cell-cycle regulation, making it an attractive anticancer target for a variety of malignancies.

MLN4924 is currently in phase I testing in advanced solid tumors, lymphoma, myeloma, melanoma, and acute myeloid leukemia. There have been limited toxicity and three complete responses to date; it is expected to advance to additional clinical testing within the next year.⁹⁶ Additional preclinical work aimed at identifying potential mechanisms of resistance to MLN4924 has identified a heterozygous mutation of *NAE β* as a mediator of resistance. These results provide a rationale for patient selection and the design of second-generation NAE inhibitors.⁹⁷

PYZD-4409 (Chembridge, San Diego, CA), a pyrazone-derived compound, has been proposed as a UAE inhibitor, with activity in vitro and in vivo against murine cancer models.⁹⁸ Another pyrazone derivative, PYR-41, blocks the formation of a ubiquitin thioester at the UAE E1 enzyme, resulting in inhibition of cytokine-induced NF- κ B and p53 stabilization. Previous work has suggested that p53 stabilization is a valid target in cells containing wild-type p53, ultimately resulting in the apoptosis of malignant cells.⁹⁹ PYR-41 has demonstrated effective UAE E1 inhibition as well as some off-target inhibition of other ubiquitin regulatory enzymes and signal-transducing proteins, suggesting it is a nonspecific inhibitor.¹⁰⁰ This nonspecificity may lead to undesirable off-target effects, but studies have shown anticancer activity in animals.⁹⁸ PYZD-4409 and PYR-41 are currently being investigated for their feasibility as therapeutic agents.

E3 UBIQUITIN LIGASE INHIBITORS

The identification of E3 ubiquitin ligase inhibitors has been limited, because researchers have focused primarily on disrupting the interaction between E3 ligases and their substrates, which is selective to particular E3 ligase-substrate pairs. In comparison to kinase inhibitors, finding compounds to disrupt the interface of two proteins can be intrinsically more difficult to achieve than searching for small

molecules that block catalytic activity. That being said, there has been some success in this area of drug development. p53 serves a vital role in the induction of apoptosis in cells containing damaged DNA. As described previously, upregulation of the E3 ligase MDM2 leads to increased p53 degradation, which has been associated with several malignancies. There are several new compounds in development with inherent MDM2 antagonistic effects, which in turn enhance p53 function and thus apoptosis of cells containing damaged DNA.

Cis-imidazolines are a recently discovered class of MDM2 antagonists. Also known as nutlins (Roche, Nutley, NJ), this well-characterized class of compounds binds MDM2 in the p53 binding pocket, dislodging p53 and enabling its activation, which leads to growth inhibition and the induction of p53-mediated apoptosis. Recently, it has been shown that nutlins also promote p73-mediated apoptosis via MDM2 inhibition and HIF1- α inactivation.^{101,102} Nutlin-3 has shown to be effective against a number of cancer cell lines, including gastric and squamous cell cancers, leukemia, and lymphoma.¹⁰³⁻¹⁰⁵ Additionally, in vivo experiments with nutlin-3 in mice bearing osteosarcoma xenografts demonstrated significant tumor growth inhibition and tumor shrinkage.¹⁰⁶ Nutlin-3 has demonstrated potent antitumor activity against human cancer cells with wild-type p53 and shows promise as a single agent and in combination with conventional or novel anticancer drugs. Currently, there are active phase I trials with R7112, a nutlin-3 analog, in patients with solid tumors or leukemia (NCT0062387, NCT00559533).

Spiro-oxindoles, another class of small-molecule inhibitors of the p53-MDM2 protein-protein interaction, contain a spiro-oxindole scaffold, and within that scaffold exists an oxindole ring that mimics a critical binding site for MDM2.¹⁰⁷ Recently, a new spiro-oxindole compound, known as MI-219 (Ascenta Therapeutics, Malvern, PA), with promising pharmacokinetic and preclinical data, has been reported. MI-219 inhibited cell growth in osteosarcoma and prostate cancer cell lines expressing wild-type p53 and stimulated p53 activation, with subsequent inhibition of cell proliferation, induction of apoptosis, and tumor growth inhibition in xenograft models. MI-219 was well tolerated in mice, without significant toxicity.¹⁰⁸ MI-43 (Ascenta Therapeutics) is another spiro-oxindole shown to induce apoptosis and cell death in colon cancer cells with wild-type p53. Interestingly, MI-43 induces cell-cycle arrest but does not induce apoptosis in normal cells.¹⁰⁹

The use of small molecules to disrupt protein-protein interactions extends beyond the MDM2-p53 complex. A recent report described the development of the first small-molecule inhibitor of another well-known E3 ligase with a known role in cancer development—the von Hippel-Lindau protein, along with its primary substrate, HIF1- α .¹¹⁰ Additionally, two recent publications identified novel specific inhibitors of SCF E3 ligase.^{111,112} Although these compounds are still in development, they are a sign of future development in this area.

PROTEASOME INHIBITORS

The 26S proteasome is a complex cytoplasmic organelle composed of one 20S core subunit and two 19S regulatory subunits responsible for the degradation of polyubiquitinated proteins into small peptides and amino acids. Proteasome inhibitors target one of the three active sites within the proteasome via covalent modification; with this inhibition,

both promoters and inhibitors of the cell cycle accumulate, which leads to conflicting signals within the cell, resulting in cell-cycle arrest and induction of apoptosis.¹¹³

Currently, bortezomib is US Food and Drug Administration approved for the treatment of patients with multiple myeloma or mantle-cell lymphoma, but somewhat surprisingly, it has not demonstrated significant activity against other solid malignancies.¹¹³ This conundrum has spurred the development of several next-generation proteasome inhibitors, including MLN9708 (Millennium Pharmaceuticals), carfilzomib (Onyx Pharmaceuticals, South San Francisco, CA), ONX0912 (Onyx Pharmaceuticals), salinosporamide A (formerly NPI-0052; Nereus Pharmaceuticals, San Diego, CA), and CEP-18770 (Cephalon, Frazer, PA).^{36,114} Although these compounds share the same target, the 20S core subunit, their targeted active site and enzyme kinetics vary, which may confer differences in activity depending on tumor type and location.¹¹⁵⁻¹¹⁷

Agents inhibiting 26S proteasome-mediated degradation lack specificity and ultimately result in the accumulation of a wide variety of proteins destined for degradation by the proteasome, thus likely contributing to clinical toxicity.¹¹⁸ The evolution of specific upstream inhibitors of the UPS, such as MLN4924, aims to improve specificity by limiting protein targets within the cell, ultimately resulting in fewer adverse effects with similar anticancer activity.⁹⁶

FUTURE DIRECTIONS

The degree to which the ubiquitin proteasome pathway is involved in the regulation of such a diverse set of biologic processes is substantial. The UPS is involved in a broad array of cellular functioning transmitted via protein signaling and interactions. Any system required to manage such a complex cellular process must itself be complex, and such is the case for the UPS. Perhaps the greatest challenge ahead for scientists attempting to manipulate the UPS in the malignant cell will be deciphering the effects of targeting proteins with dual roles as both tumor suppressors and oncogenes. The role of such proteins can be influenced by a multitude of factors, some of which are

undoubtedly still unknown. The context in which these proteins are assigned their function will need to be further understood before the full effects of inhibition can be predicted, and until that time, the risk of manipulating the UPS is still great. Additional research and a better understanding of the UPS continue to evolve and, with this evolution, will undeniably yield a host of new therapeutic targets. The precedent for UPS inhibition and its role in cancer treatment has been established by bortezomib; second-generation proteasome inhibitors as well as other UPS targets exist and continue to move forward in clinical development. As the cellular mechanisms responsible for malignant transformation and propagation are exposed, the role of selective therapies will only become more valuable, and the UPS clearly comprises a range of such selective targets.

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REFERENCES

- Wilkinson KD: Ubiquitin: A Nobel protein. *Cell* 119:741-745, 2004
- Reinstein E, Ciechanover A: Narrative review: Protein degradation and human diseases—The ubiquitin connection. *Ann Intern Med* 145:676-684, 2006
- Hershko A: The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. *Cell Death Differ* 12:1191-1197, 2005
- Dammer EB, Na CH, Xu P, et al: Polyubiquitin linkage profiles in three models of proteolytic stress suggest the etiology of Alzheimer disease. *J Biol Chem* 286:10457-10465, 2011
- Ikeda F, Dikic I: Atypical ubiquitin chains: New molecular signals. *EMBO Rep* 9:536-542, 2008
- Koegl M, Hoppe T, Schlenker S, et al: A novel ubiquitination factor, E4, is involved in multi-ubiquitin chain assembly. *Cell* 96:635-644, 1999
- Haas AL, Warms JV, Hershko A, et al: Ubiquitin-activating enzyme: Mechanism and role in protein-ubiquitin conjugation. *J Biol Chem* 257:2543-2548, 1982
- Glickman MH, Ciechanover A: The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. *Physiol Rev* 82:373-428, 2002
- Ardley HC, Robinson PA: E3 ubiquitin ligases. *Essays Biochem* 41:15-30, 2005
- Soucy TA, Smith PG, Rolfe M: Targeting NEDD8-activated cullin-RING ligases for the treatment of cancer. *Clin Cancer Res* 15:3912-3916, 2009
- Hershko A, Heller H, Eytan E, et al: The protein substrate binding site of the ubiquitin-protein ligase system. *J Biol Chem* 261:11992-11999, 1986
- Hershko A: Ubiquitin-mediated protein degradation. *J Biol Chem* 263:15237-15240, 1988
- Grossman SR, Deato ME, Brignone C, et al: Polyubiquitination of p53 by a ubiquitin ligase activity of p300. *Science* 300:342-344, 2003
- Lai Z, Ferry KV, Diamond MA, et al: Human mdm2 mediates multiple mono-ubiquitination of p53 by a mechanism requiring enzyme isomerization. *J Biol Chem* 276:31357-31367, 2001
- Li M, Brooks CL, Wu-Baer F, et al: Mono-versus polyubiquitination: Differential control of p53 fate by Mdm2. *Science* 302:1972-1975, 2003
- Rodriguez MS, Desterro JM, Lain S, et al: Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. *Mol Cell Biol* 20:8458-8467, 2000
- Wu H, Pomeroy SL, Ferreira M, et al: UBE4B promotes Hdm2-mediated degradation of the tumor suppressor p53. *Nat Med* 17:347-355, 2011
- Crosas B, Hanna J, Kirkpatrick DS, et al: Ubiquitin chains are remodeled at the proteasome by opposing ubiquitin ligase and deubiquitinating activities. *Cell* 127:1401-1413, 2006
- Schulman BA, Harper JW: Ubiquitin-like protein activation by E1 enzymes: The apex for downstream signalling pathways. *Nat Rev Mol Cell Biol* 10:319-331, 2009
- Kerscher O, Felberbaum R, Hochstrasser M: Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* 22:159-180, 2006
- Soucy TA, Smith PG, Millhollen MA, et al: An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature* 458:732-736, 2009
- Podust VN, Brownell JE, Gladysheva TB, et al: A Nedd8 conjugation pathway is essential for proteolytic targeting of p27Kip1 by ubiquitination. *Proc Natl Acad Sci U S A* 97:4579-4584, 2000

23. Read MA, Brownell JE, Gladysheva TB, et al: Ned8 modification of cul-1 activates SCF(betaTrCP)-dependent ubiquitination of IkappaBalpha. *Mol Cell Biol* 20:2326-2333, 2000
24. Yada M, Hatakeyama S, Kamura T, et al: Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. *EMBO J* 23:2116-2125, 2004
25. Mao JH, Kim IJ, Wu D, et al: FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. *Science* 321:1499-1502, 2008
26. Liakopoulos D, Büsgen T, Brychzy A, et al: Conjugation of the ubiquitin-like protein NEDD8 to cullin-2 is linked to von Hippel-Lindau tumor suppressor function. *Proc Natl Acad Sci U S A* 96:5510-5515, 1999
27. Jin J, Arias EE, Chen J, et al: A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Mol Cell* 23:709-721, 2006
28. Fraile JM, Quesada V, Rodríguez D, et al: Deubiquitinases in cancer: New functions and therapeutic options. *Oncogene* 31:2373-2388, 2012
29. Amerik AY, Hochstrasser M: Mechanism and function of deubiquitinating enzymes. *Biochim Biophys Acta* 1695:189-207, 2004
30. Pickart CM, Rose IA: Ubiquitin carboxyl-terminal hydrolase acts on ubiquitin carboxyl-terminal amides. *J Biol Chem* 260:7903-7910, 1985
31. Stevenson LF, Sparks A, Allende-Vega N, et al: The deubiquitinating enzyme USP2a regulates the p53 pathway by targeting Mdm2. *EMBO J* 26:976-986, 2007
32. Cohen P, Tcherpakov M: Will the ubiquitin system furnish as many drug targets as protein kinases? *Cell* 143:686-693, 2010
33. Colland F, Formstecher E, Jacq X, et al: Small-molecule inhibitor of USP7/HAUSP ubiquitin protease stabilizes and activates p53 in cells. *Mol Cancer Ther* 8:2286-2295, 2009
34. Liu J, Xia H, Kim M, et al: Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. *Cell* 147:223-234, 2011
35. Turk B: Targeting proteases: Successes, failures and future prospects. *Nat Rev Drug Discov* 5:785-799, 2006
36. Bedford L, Lowe J, Dick LR, et al: Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. *Nat Rev Drug Discov* 10:29-46, 2011
37. Rahimi N: The ubiquitin-proteasome system meets angiogenesis. *Mol Cancer Ther* 11:538-548, 2012
38. Ohta T, Fukada M: Ubiquitin and breast cancer. *Oncogene* 23:2079-2088, 2004
39. Brzovic PS, Keffe JR, Nishikawa H: Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex. *Proc Natl Acad Sci U S A* 100:5646-5651, 2003
40. Sato K, Sundaramoorthy E, Rajendra E, et al: A DNA-damage selective role for BRCA1 E3 ligase in claspin ubiquitylation, CHK1 activation, and DNA repair. *Curr Biol* 22:1659-1666, 2012
41. Zhang Q, Yang H: The roles of VHL-dependent ubiquitination in signaling and cancer. *Front Oncol* 2:35, 2012
42. Lipkowitz S, Weissman AM: RINGS of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. *Nat Rev Cancer* 11:629-643, 2011
43. Toledo F, Wahl GM: Regulating the p53 pathway: In vitro hypotheses, in vivo veritas. *Nat Rev Cancer* 6:909-923, 2006
44. Rayburn E, Zhang R, He J, et al: MDM2 and human malignancies: Expression, clinical pathology, prognostic markers, and implications for chemotherapy. *Curr Cancer Drug Targets* 5:27-41, 2005
45. Polsky D, Bastian BC, Hazan C, et al: HDM2 protein overexpression, but not gene amplification, is related to tumorigenesis of cutaneous melanoma. *Cancer Res* 61:7642-7646, 2001
46. Oliner JD, Kinzler KW, Meltzer PS, et al: Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* 358:80-83, 1992
47. Eymin B, Gazzeri S, Brambilla C, et al: Mdm2 overexpression and p14(ARF) inactivation are two mutually exclusive events in primary human lung tumors. *Oncogene* 21:2750-2761, 2002
48. Barbieri CE, Baca SC, Lawrence MS, et al: Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. *Nat Genet* 44:685-689, 2012
49. Liu J, Ghanim M, Xue L, et al: Analysis of Drosophila segmentation network identifies a JNK pathway factor overexpressed in kidney cancer. *Science* 323:1218-1222, 2009
50. Wang C, Pan Y, Wang B: Suppressor of fused and Spop regulate the stability, processing and function of Gli2 and Gli3 full-length activators but not their repressors. *Development* 137:2001-2009, 2010
51. Benassi B, Flavin R, Marchionni L, et al: MYC is activated by USP2a-mediated modulation of microRNAs in prostate cancer. *Cancer Discov* 2:236-245, 2012
52. Welcker M, Clurman BE: FBW7 ubiquitin ligase: A tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat Rev Cancer* 8:83-93, 2008
53. Cheng Y, Li G: Role of the ubiquitin ligase Fbw7 in cancer progression. *Cancer Metastasis Rev* 31:75-87, 2012
54. Carrano AC, Eytan E, Hershko A, et al: SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol* 1:193-199, 1999
55. Palmqvist R, Stenling R, Oberg A, et al: Prognostic significance of p27(Kip1) expression in colorectal cancer: A clinico-pathological characterization. *J Pathol* 188:18-23, 1999
56. Cote RJ, Shi Y, Groshen S, et al: Association of p27Kip1 levels with recurrence and survival in patients with stage C prostate carcinoma. *J Natl Cancer Inst* 90:916-920, 1998
57. Gstaiger M, Jordan R, Lim M, et al: Skp2 is oncogenic and overexpressed in human cancers. *Proc Natl Acad Sci U S A* 98:5043-5048, 2001
58. Tan P, Cady B, Wanner M, et al: The cell cycle inhibitor p27 is an independent prognostic marker in small (T1a,b) invasive breast carcinomas. *Cancer Res* 57:1259-1263, 1997
59. Scheffner M, Werness BA, Huibregtse JM, et al: The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 63:1129-1136, 1990
60. Scheffner M, Takahashi T, Huibregtse JM, et al: Interaction of the human papillomavirus type 16 E6 oncoprotein with wild-type and mutant human p53 proteins. *J Virol* 66:5100-5105, 1992
61. Werness BA, Levine AJ, Howley PM: Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* 248:76-79, 1990
62. Brooks CL, Gu W: p53 regulation by ubiquitin. *FEBS Lett* 585:2803-2809, 2011
63. Hakem A, Bohgaki M, Lemmers B, et al: Role of Pirh2 in mediating the regulation of p53 and c-Myc. *PLoS Genet* 7:e1002360, 2011
64. Le Cam L, Linares LK, Paul C, et al: E4F1 is an atypical ubiquitin ligase that modulates p53 effector functions independently of degradation. *Cell* 127:775-788, 2006
65. Lee JT, Gu W: The multiple levels of regulation by p53 ubiquitination. *Cell Death Differ* 17:86-92, 2010
66. Patel S, Player MR: Small-molecule inhibitors of the p53-HDM2 interaction for the treatment of cancer. *Expert Opin Investig Drugs* 17:1865-1882, 2008
67. Oliner JD, Pietsenpol JA, Thiagalingam S, et al: Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* 362:857-860, 1993
68. Haupt Y, Maya R, Kazanietz A, et al: Mdm2 promotes the rapid degradation of p53. *Nature* 387:296-299, 1997
69. Honda R, Tanaka H, Yasuda H: Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* 420:25-27, 1997
70. Kubbutat MH, Jones SN, Vousden KH: Regulation of p53 stability by Mdm2. *Nature* 387:299-303, 1997
71. Montes de Oca Luna R, Wagner DS, Lozano G: Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* 378:203-206, 1995
72. Jones SN, Roe AE, Donehower LA, et al: Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 378:206-208, 1995
73. Zheleva DI, Lane DP, Fischer PM: The p53-Mdm2 pathway: Targets for the development of new anticancer therapeutics. *Mini Rev Med Chem* 3:257-270, 2003
74. Clegg HV, Itahana K, Zhang Y: Unlocking the Mdm2-p53 loop: Ubiquitin is the key. *Cell Cycle* 7:287-292, 2008
75. Lu C, El-Deiry WS: Targeting p53 for enhanced radio- and chemo-sensitivity. *Apoptosis* 14:597-606, 2009
76. Vazquez A, Bond EE, Levine AJ, et al: The genetics of the p53 pathway, apoptosis and cancer therapy. *Nat Rev Drug Discov* 7:979-987, 2008
77. Xirodimas DP, Saville MK, Bourdon JC, et al: Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell* 118:83-97, 2004
78. Bischof O, Schwamborn K, Martin N, et al: The E3 SUMO ligase PIASy is a regulator of cellular senescence and apoptosis. *Mol Cell* 22:783-794, 2006
79. Carter S, Bischof O, Dejean A, et al: C-terminal modifications regulate MDM2 dissociation and nuclear export of p53. *Nat Cell Biol* 9:428-435, 2007
80. Busino L, Millman SE, Scotto L, et al: Fbxw7α and GSK3-mediated degradation of p100 is a pro-survival mechanism in multiple myeloma. *Nat Cell Biol* 14:375-385, 2012
81. Morgan DO: Principles of CDK regulation. *Nature* 374:131-134, 1995
82. Sherr CJ, Roberts JM: Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* 9:1149-1163, 1995
83. Bloom J, Pagano M: Deregulated degradation of the cdk inhibitor p27 and malignant transformation. *Semin Cancer Biol* 13:41-47, 2003
84. Tsvetkov LM, Yeh KH, Lee SJ, et al: P27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol* 9:661-664, 1999

85. Sutterlüty H, Chatelain E, Marti A, et al: P45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. *Nat Cell Biol* 1:207-214, 1999
86. Pietenpol JA, Bohlander SK, Sato Y, et al: Assignment of the human p27Kip1 gene to 12p13 and its analysis in leukemias. *Cancer Res* 55:1206-1210, 1995
87. Kawamata N, Morosetti R, Miller CW, et al: Molecular analysis of the cyclin-dependent kinase inhibitor gene p27/Kip1 in human malignancies. *Cancer Res* 55:2266-2269, 1995
88. Slingerland J, Pagano M: Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* 183:10-17, 2000
89. Brownell JE, Sintchak MD, Gavin JM, et al: Substrate-assisted inhibition of ubiquitin-like protein-activating enzymes: The NEDD8 E1 inhibitor MLN4924 forms a NEDD8-AMP mimetic in situ. *Mol Cell* 37:102-111, 2010
90. Swords RT, Kelly KR, Smith PG, et al: Inhibition of NEDD8-activating enzyme: A novel approach for the treatment of acute myeloid leukemia. *Blood* 115:3796-3800, 2010
91. Davis RE, Brown KD, Siebenlist U, et al: Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *J Exp Med* 194:1861-1874, 2001
92. Lam LT, Davis RE, Pierce J, et al: Small molecule inhibitors of I kappaB kinase are selectively toxic for subgroups of diffuse large B-cell lymphoma defined by gene expression profiling. *Clin Cancer Res* 11:28-40, 2005
93. Ngo VN, Davis RE, Lamy L, et al: A loss-of-function RNA interference screen for molecular targets in cancer. *Nature* 441:106-110, 2006
94. Milhollen MA, Traore T, Adams-Duffy J, et al: MLN4924, a NEDD8-activating enzyme inhibitor, is active in diffuse large B-cell lymphoma models: Rationale for treatment of NF-(kappa)B-dependent lymphoma. *Blood* 116:1515-1523, 2010
95. Campbell KJ, Rocha S, Perkins ND: Active repression of antiapoptotic gene expression by ReIA(p65) NF-kappa B. *Mol Cell* 13:853-865, 2004
96. Wang M, Medeiros BC, Erba HP, et al: Targeting protein neddylation: A novel therapeutic strategy for the treatment of cancer. *Expert Opin Ther Targets* 15:253-264, 2011
97. Milhollen MA, Thomas MP, Narayanan U, et al: Treatment-emergent mutations in NAE β confer resistance to the NEDD8-activating enzyme inhibitor MLN4924. *Cancer Cell* 21:388-401, 2012
98. Xu GW, Ali M, Wood TE, et al: The ubiquitin-activating enzyme E1 as a therapeutic target for the treatment of leukemia and multiple myeloma. *Blood* 115:2251-2259, 2010
99. Yang Y, Kitagaki J, Dai RM, et al: Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. *Cancer Res* 67:9472-9481, 2007
100. Kapuria V, Peterson LF, Showalter HD, et al: Protein cross-linking as a novel mechanism of action of a ubiquitin-activating enzyme inhibitor with anti-tumor activity. *Biochem Pharmacol* 82:341-349, 2011
101. Zheng T, Wang J, Song X, et al: Nutlin-3 cooperates with doxorubicin to induce apoptosis of human hepatocellular carcinoma cells through p53 or p73 signaling pathways. *J Cancer Res Clin Oncol* 136:1597-1604, 2010
102. Lee YM, Lim JH, Chun YS, et al: Nutlin-3, an Hdm2 antagonist, inhibits tumor adaptation to hypoxia by stimulating the FIH-mediated inactivation of HIF-1 α . *Carcinogenesis* 30:1768-1775, 2009
103. Roh JL, Kang SK, Minn I, et al: p53-Reactivating small molecules induce apoptosis and enhance chemotherapeutic cytotoxicity in head and neck squamous cell carcinoma. *Oral Oncol* 47:8-15, 2011
104. Endo S, Yamato K, Hirai S, et al: Potent in vitro and in vivo antitumor effects of MDM2 inhibitor nutlin-3 in gastric cancer cells. *Cancer Sci* 102:605-613, 2011
105. Zauli G, Voltan R, Bosco R, et al: Dasatinib plus Nutlin-3 shows synergistic antileukemic activity in both p53 wild-type and p53 mutated B chronic lymphocytic leukemias by inhibiting the Akt pathway. *Clin Cancer Res* 17:762-770, 2011
106. Vassilev LT, Vu BT, Graves B, et al: In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 303:844-848, 2004
107. Ding K, Lu Y, Nikolovska-Coleska Z, et al: Structure-based design of spiro-oxindoles as potent, specific small-molecule inhibitors of the MDM2-p53 interaction. *J Med Chem* 49:3432-3435, 2006
108. Shangary S, Qin D, McEachern D, et al: Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. *Proc Natl Acad Sci U S A* 105:3933-3938, 2008
109. Shangary S, Ding K, Qiu S, et al: Reactivation of p53 by a specific MDM2 antagonist (MI-43) leads to p21-mediated cell cycle arrest and selective cell death in colon cancer. *Mol Cancer Ther* 7:1533-1542, 2008
110. Buckley DL, Van Molle I, Gareiss PC, et al: Targeting the von Hippel-Lindau E3 ubiquitin ligase using small molecules to disrupt the VHL/HIF-1 α interaction. *J Am Chem Soc* 134:4465-4468, 2012
111. Aghajan M, Jonai N, Flick K, et al: Chemical genetics screen for enhancers of rapamycin identifies a specific inhibitor of an SCF family E3 ubiquitin ligase. *Nat Biotechnol* 28:738-742, 2010
112. Orlicky S, Tang X, Neduva V, et al: An allosteric inhibitor of substrate recognition by the SCF-(Cdc4) ubiquitin ligase. *Nat Biotechnol* 28:733-737, 2010
113. Sterz J, von Metzler I, Hahne JC, et al: The potential of proteasome inhibitors in cancer therapy. *Expert Opin Investig Drugs* 17:879-895, 2008
114. Kupperman E, Lee EC, Cao Y, et al: Evaluation of the proteasome inhibitor MLN9708 in preclinical models of human cancer. *Cancer Res* 70:1970-1980, 2010
115. Zhou HJ, Aujay MA, Bennett MK, et al: Design and synthesis of an orally bioavailable and selective peptide epoxyketone proteasome inhibitor (PR-047). *J Med Chem* 52:3028-3038, 2009
116. Chauhan D, Catley L, Li G, et al: A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from bortezomib. *Cancer Cell* 8:407-419, 2005
117. Piva R, Ruggeri B, Williams M, et al: CEP-18770: A novel, orally active proteasome inhibitor with a tumor-selective pharmacologic profile competitive with bortezomib. *Blood* 111:2765-2775, 2008
118. Kim W, Bennett EJ, Huttlin EL, et al: Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* 44:325-340, 2011