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# Targeting the ubiquitin-proteasome system for cancer therapy

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## Summary

The ubiquitin-proteasome system plays a critical role in controlling the level, activity, and location of various cellular proteins. Significant progress has been made in investigating the molecular mechanisms of ubiquitination, particularly in understanding the structure of the ubiquitination machinery and identifying ubiquitin protein ligases, the primary specificity-determining enzymes. Therefore, it is now possible to target specific molecules involved in the ubiquitination and proteasomal degradation to regulate many cellular processes such as signal transduction, proliferation and apoptosis. In particular, alterations in ubiquitination are observed in most, if not all, cancer cells. This is manifested by destabilization of tumor suppressors, such as p53, and overexpression of oncogenes such as c-Myc and c-Jun. In addition to the development and clinical validation of proteasome inhibitor Bortezomib in myeloma therapy, recent studies have demonstrated that it is possible to develop inhibitors for specific ubiquitination and deubiquitination enzymes. With the help of structural studies, rational design, and chemical synthesis, it is conceivable that we will be able to use “druggable” inhibitors of the ubiquitin system to evaluate their effects in animal tumor models in the not-so-distant future.

### Keywords

Molecule targeting; ubiquitin; proteasome; cancer therapeutics

## The ubiquitin system

Ubiquitination is catalyzed by the sequential action of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin protein ligase), which leads to the conjugation of ubiquitin to the ε-amino group of a lysine residue in target proteins (Figure 1) (1). Under certain circumstance, ubiquitin can also be conjugated to N-terminal or even non-lysine residues of proteins (2,3). Interestingly, the formation of polyubiquitin chain on certain proteins requires an additional factor (E4) (4), whereas mono-ubiquitination of protein that contains a ubiquitin-binding domain may occur in the absence of E3 (5). Additionally, there is a family of small ubiquitin-like modifiers (Ubls), including SUMO, Nedd8, FAT10, and ISG15. They can be conjugated to lysine residues of specific target proteins through mechanisms parallel to but distinct from that of ubiquitin (6). While SUMO can be conjugated to a variety of substrate proteins, other Ubls appear to have a limited number of targets. Besides affecting many cellular activities directly, these modifications also regulate ubiquitination at multiple levels (7,8). Intriguingly, RING protein Hdm2 can

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function as ligase for both ubiquitin and Nedd8 (9). RING protein Topors can act as an E3 for both ubiquitin and SUMO (10).

Although it is generally believed that there is only one E1 (UBE1) in human cells, recent studies have shown that both UBE1L2 and Uba6 can activate ubiquitin (11–13). Since they only transfer ubiquitin to particular E2s, it is likely that UBE1L2 and Uba6 may regulate a subset of ubiquitinations or modulate ubiquitination at specific organs. There are more than 40 putative E2s in mammalian cells, and each E2 may work with many different E3s to promote modification of target proteins with ubiquitin or Ubls (14). While ubiquitin E1 can act with many E2s for ubiquitination, E1s for SUMO, Nedd8, and ISG15 have dedicated E2s and only work with Ubc9, Ubc12, and UbcH8, respectively (6). There are more than 600 proteins in human cells that bear the E3 signatures, HECT domain or RING and RING-like motifs (14). The highly conserved ~350-residue HECT domain contains the conserved Cys that accepts activated ubiquitin from E2 and subsequently transfers it to target proteins (15). The RING and RING-related motif-containing proteins can function as E3s or as a component of multi-subunits E3. In both situations, the RING motif plays a critical role in binding of E2s, although it may also be involved in stabilizing the E3-substrate complex and transferring of ubiquitin to substrate (16,17). Finally, ubiquitination is a reversible process. The deubiquitinating enzymes (DUBs) are involved in releasing ubiquitin from precursors, proofreading ubiquitin-protein conjugates, removing ubiquitin from target proteins, and preventing accumulation of ubiquitin chains in the proteasome (18). Under certain circumstances, the activity of DUBs appears to be the major mechanism for change of protein ubiquitination status.

Ubiquitination affects many cellular processes, from gene transcription and DNA repair to cell cycle and apoptosis. While polyubiquitination often tags proteins for proteasomal degradation, monoubiquitination alters activity, intracellular location, and interaction of target proteins (19). Further analysis revealed that polyubiquitin chains could be formed using one of the seven lysines in ubiquitin. Besides the K48-linked polyubiquitin chain that is associated with proteasomal degradation, K63-linked polyubiquitination is also formed in cells and is involved in DNA repair, DNA replication, and signal transduction processes (20,21). It is conceivable that the functional differences between different forms of ubiquitination result from their binding with different ubiquitin-binding domains in cells (22).

## Dysfunction of the ubiquitin-proteasome system and cancer

### Alteration of E3s

**Hdm2 and p53—**The level of p53 is mainly regulated through ubiquitination-mediated degradation (23). A number of RING-containing E3s, including Hdm2, COP1, Pirh2, Topors, and CARPs, can ubiquitinate p53 (24,25). However, Hdm2 appears to be the major regulator, as the lethality of Hdm2 deficiency can be rescued by the loss of p53 (23). The E3 activity of Hdm2 towards p53 is significantly enhanced by heterodimerization with MdmX (9,26). It has been shown that amplification of the Hdm2 gene was present in approximately 10% of tumors (24), most of them retain wild-type p53. Many tumor cells also express higher level of Hdm2 without amplification of the genes (27). In addition, the amplification and/or overexpression of MdmX have been found in ~10% of diverse tumors and in 65% of retinoblastomas (24,28). Viruses may also utilize ubiquitination to disarm the p53 system. A well-documented example is that E6 of oncogenic HPV forms a complex with the HECT domain E3 E6-AP to ubiquitinate p53 and promote its degradation (29). These findings are consistent with the notion that dysfunction of the p53 system is required for tumors to develop.

**$SCF^{Skp2}$ and $SCF^{Fbw7}$—**p27 is an inhibitor of cyclin-dependent kinases (CDK) (30,31). Many investigations have found decreased expression but not mutation of p27 in various tumors (32). In fact, the level of p27 has been used as a negative prognostic marker in the diagnosis and treatment of various cancers. Since phosphorylated p27 (threonine-187) is recognized and ubiquitinated by E3 $SCF^{Skp2}$ for proteasomal degradation (33), overexpression of F-box protein Skp2 correlates with a reduced p27 level in all tumors examine (34). Furthermore, enforced expression of Skp2 in mice promotes tumor formation (35), suggesting that increased expression of Skp2 plays an important role in tumor cells. Interestingly, another complex E3 $SCF^{Fbw7}$ mainly recognizes and ubiquitinates phosphorylated oncoproteins including cyclin E, c-Myc, c- Jun, and Notch (36). Both Fbw7 mutations and mutations of its target genes, which often result in alteration of the sites recognized by Fbw7, are observed in a wide spectrum of human cancers (37). Therefore, targeting the SCF E3s should focus on specific F-box proteins.

**VCB-Cul2-VHL—**VHL is the substrate-recognition component of the cullin-based ubiquitin ligase VHL E3 (also known as VCB-Cul2) (38–40). The best-known target of VCB-Cul2-VHL is HIF-α, the unstable subunit of the hypoxia-inducible transcription factor family (HIF). Under normal oxygen condition, HIF-α is hydroxylated at the conserved proline residue, which result in its recognition by VCB-Cul2-VHL, leading to ubiquitination and degradation (41). Mutations of VHL prevent HIF-α ubiquitination and cause elevation of HIF, which in turn transactivates the expression of genes involved in adaptation to low oxygen, including vascular endothelial growth factor and erythropoietin. The importance of HIF in VHL-associated tumors is clearly demonstrated by the finding that the lack of HIF-1β or down regulation of HIF-α is sufficient to suppress the pathological changes attributed to deficiency of VHL (42). Therefore, restoring VHL E3 activity and targeting HIF and HIF-responsive genes could be promising therapies for this type of cancer.

**E3s encoded by cancer susceptibility genes—**The breast and ovarian cancer susceptibility gene *BRCA1* encodes a RING-containing protein implicated in familial breast cancers and a significant portion of sporadic breast cancers (43,44). The heterodimer of BRCA1 and another RING-containing protein BARD1 acts as an E3 for autoubiquitination and ubiquitination of multiple substrates (45,46). It has been shown that the E3 activity is required for the tumor suppressor function of BRCA1 and mutations that abolish the E3 activity are observed in tumor cells (46). Intriguingly, recruitment of BRCA1 to the damaged DNA site is mediated by RAP80, a polyubiquitin chain-binding protein (47). Therefore, ubiquitination acts as both an activator and effector of BRCA1 function.

Fanconi anemia (FA) is a rare genetic disorder characterized by aplastic anemia, chromosomal instability, and cancer susceptibility (48). Genetic and biochemical analysis of complementation groups have indicated that 8 FA proteins (FANC-A, B, C, E, F, G, L, M) constitute a nuclear complex possessing E3 activity that, in response to DNA damage, mediates the mono-ubiquitination of FANCD2 (49,50). The ubiquitinated FANCD2 is then localized in nuclear foci with proteins involved in DNA repair, including BRCA1, BRCA2, FANCN, and RAD5. Thus, FA proteins appear to function as signal transducers and important regulators in the DNA damage response network (51). It is therefore not unexpected that, in addition to their association with FA syndrome and breast cancers, alterations of the FA proteins have also been observed in a wide variety of human cancers (52).

## Dysregulation of deubiquitinating enzymes

Familial cylindromatosis is an autosomal dominant predisposition to multiple tumors of the skin appendages. Genetic studies led to the identification of cylindromatosis tumor

suppressor gene (CYLD) that encodes a DUB (53). CYLD can remove the K63-linked polyubiquitin chain from adapter molecule TRAF2 and prevent it from activating IκB kinase. Therefore, loss of CYLD leads to enhanced activation of NFκB in response to many immunological and inflammatory signals (54–56). CYLD is also able to deubiquitinate Bcl-3 and prevent it from entering nucleus, where Bcl-3 can interact with NFκB family members (p50 and p52) to activate the transcription of NFκB target genes (57). These results raise the possibility that inhibition of NFκB activation could be an effective therapy for cylindromatosis.

### Summary

Giving the presence of more than 500 E3s, approximately 100 DUBs and 70 F-box proteins in human cells, it is likely that the alterations of the ubiquitin system in cancer identified at present is only a tip of iceberg (Table 1). Furthermore, changes of substrates that affect the ubiquitination process may also contribute significantly to the dysfunction of the ubiquitin process. This is clearly illustrated by the studies of c-Myc, whose level is elevated in many cancers (58). In addition to deregulated expression, stabilization of c-Myc has been found in multiple leukemia cell lines and patients. At least in some lymphomas, the stabilization is due to mutations that prevent the phosphorylation of T58, which appears to be required for the ubiquitination and proteasomal degradation of c- Myc. T58 mutation is also present in v-Myc and likely contributes to its oncogenic ability. Therefore, modulating or targeting the ubiquitin system could be an effective means to fight cancers even when the initial defects are not resided in the system.

## Targeting the ubiquitin-proteasome system

### Proteasome as the target of chemotherapy

Several generations of proteasome inhibitors have been identified and developed, including peptide aldehydes, peptide boronates, peptide epoxyketones, and β-lactones. The tripeptide aldehydes such as MG-132 are cell-permeable proteasome inhibitors and have been widely used as important probes for the studies of the ubiquitin-proteasome system. However, they also inhibit non-proteasomal proteases and display metabolic instability *in vivo*. The dipeptide boronates including Bortezomib are much more potent and specific for the chymotrypsin-like activity of proteasome. Many studies have demonstrated that Bortezomib preferentially kills various tumor cells *in vitro* and in animal models. Furthermore, it has been successfully used as an effective therapeutic for myeloma in the clinic (59). Bortezomib is also being tried in the clinic for the treatment of a number of other tumors, alone or in combination with radio- and chemotherapy (60). However, it is not well understood at present why blocking the “non-specific” proteasomal degradation results in the differential killing of tumor cells. It appears that inhibition of the NFκB pathway, promotion of ER stress-induced apoptosis, induction of p53-dependent apoptosis, and disruption of the regulation of cell cycle-regulating proteins are involved in the selective killing of tumor cells by Bortezomib (61). It is likely that the relative importance of these mechanisms depends on the molecular pathogenesis of particular tumors. Lactacystin is a potent inhibitor of proteasomal proteases likely through forming intermediate clasto-lactacystin β-lactone (62). A number of synthetic and natural analogues of lactacystin are also being explored as potential therapeutic agents (63). Of these, the marine-derived salinosporamide A (NPI-0052) appears to be particularly interesting because it is orally bioactive and possesses unique specificity and high potency (63).

### Targeting E3: inhibiting Hdm2-mediated p53 ubiquitination

Two types of strategies have been employed to specifically inhibit Hdm2-mediated p53 ubiquitination. Based on the structure of p53 and Hdm2 interaction, a number of peptide

derivatives and small molecules, including Nutlins, RITA, MI-63, and SyI-155, have been developed to block the binding of p53 to Hdm2 (Figure 2) (64,65). Treatment of cells with them resulted in accumulation of p53 and killing of tumor cells in culture. Some of these inhibitors are effective in inhibiting the growth of inoculated tumors in nude mice and are well tolerated by the animals. It is likely that clinical trials with these inhibitors could be carried out soon.

An alternative approach to prevent Hdm2-mediated p53 ubiquitination is by inhibiting the ubiquitin ligase activity of Hdm2. We have carried out several rounds of high throughput screening to identify small molecules that inhibit the autoubiquitination of Hdm2 *in vitro*. One family of compounds, HLI98s, are able to prevent Hdm2-mediated p53 ubiquitination in *vitro* and in cells (Figure 2) (66). These compounds also increase the level of p53 in cells and preferentially kill transformed cells retaining wild-type p53. However, their limited solubility in aqueous solution prevents further studies to assess whether these compounds can inhibit tumor growth in animal models. Recently, we have identified a more potent and water-soluble homolog of HLI98s (named HLI373) and a Hdm2 inhibitor from natural products (sempervirine) (65,67). It is interesting to further examine whether these compounds have anti-cancer activities in animal tumor models.

### Inhibition of E1 to kill cancer cells and beyond

While screening for compounds that inhibit the autoubiquitination of Hdm2, we identified a cell-permeable inhibitor of the ubiquitin E1 named PYR-41 (4[4-(5-nitro- furan-2-ylmethylene)-3, 5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester) (68). Among the cellular consequences of exposure of cells to PYR-41 is increased levels and activity of p53. This correlates with its ability to preferentially induce apoptosis in transformed cells expressing wild-type p53, suggesting that the inhibitor may be a novel therapeutic for cancer. As expected, PYR-41 also blocks the non-degradative functions of ubiquitination such as IL-1 or TNFα-induced phosphorylation of IκBα. This can be attributed to its inhibition of TRAF6 ubiquitination and phosphorylation of IκBα as well as ubiquitination of IκBα itself (68). Therefore, PYR-41 may also be a potent anti-inflammation agent when applied locally.

## Conclusions

The ubiquitin system has emerged as the focus of molecular targeting in developing cancer therapeutics (Table 2). Because E3 plays a major role in determining the specificity of ubiquitination, it is regarded as a preferred target for therapeutic intervention. With the development of Nutlins, it appears that blocking the interaction between E3s and their substrates can be an effective strategy to inhibit the degradation of specific proteins (69). However, this approach depends on a clear understanding of the structural basis of E3-substrate interactions, which are not readily available for most of the E3s, and is more effective when the interaction involves limited contact and a deep “pocket”. Thus, we have been interested in targeting the activity of ubiquitin ligase. Although it is still debatable whether it is possible to specifically inhibit activities of RING E3s due to their structural similarity, we have shown that HLI98s and analogs are selectively inhibitors of Hdm2 activity (66).

While E3s are not conventional enzymes, E1s for ubiquitin or Ubls, E2s, and proteases in proteasome are classic drug targets. Inhibition of these enzymes affects nonspecifically the regulation and degradation of many proteins, most notably Hdm2 and p53 (Figure 2). Nevertheless, proteasome inhibitor Bortezomib has been used successfully in the treatment of certain cancers (59). Our studies with E1 inhibitors, which target an even greater range of cellular processes than proteasome inhibitors and differentially kill tumor cells that retain wild-type p53 (68), indicating that transformed cells are more sensitive to the interruption of

the ubiquitin-proteasome system. It is also worth noting that geldanamycin and derivatives, which are being evaluated in patients with advanced cancers in the clinic, target Hsp90, a chaperone that affects the folding and function of many proteins (70,71). Thus, while specificity is the foundation of target therapies, drugs that have more general effects on protein function and fate may be used successfully as cancer therapeutics.

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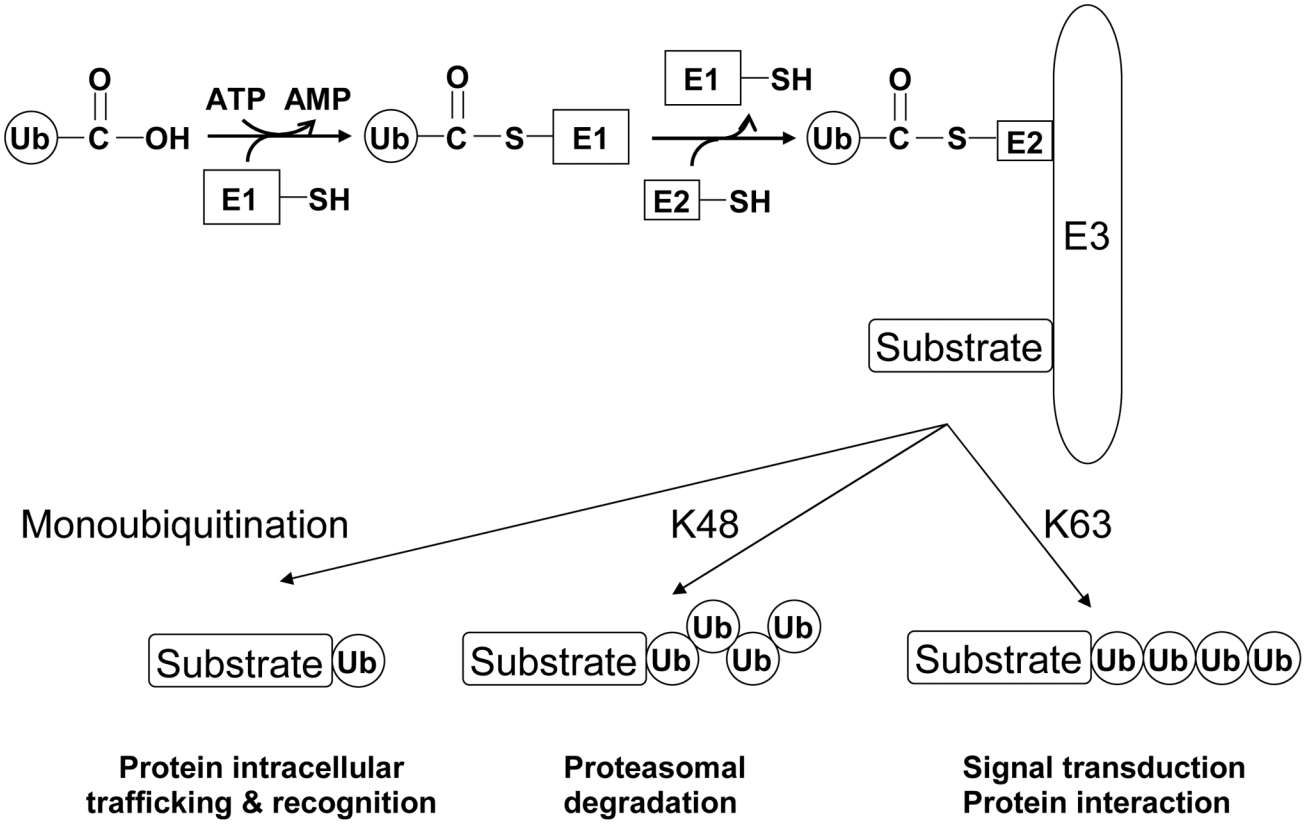


**Figure 1.**

The ubiquitination cascade. Ubiquitin (Ub) is activated by E1 and conjugated to the active Cys of E1 through thioester bond. Activated Ub is then transferred to E2 that can bind with E3. An RING-containing E3 facilitates the transfer of activated Ub to substrate from E2 directly, whereas a HECT domain E3 forms thioester bond with activated Ub and then transfer it to substrate. Monoubiquitination of target protein enable its recognition by many Ub-recognizing domains in cells, leading to alteration of protein activity and location in cells. Formation of K48-linked polyubiquitin chains on substrate proteins result in their degradation in proteasomes. Formation of K63-linked polyubiquitin chains are involved in cellular processes such as signal transduction and DNA repair.

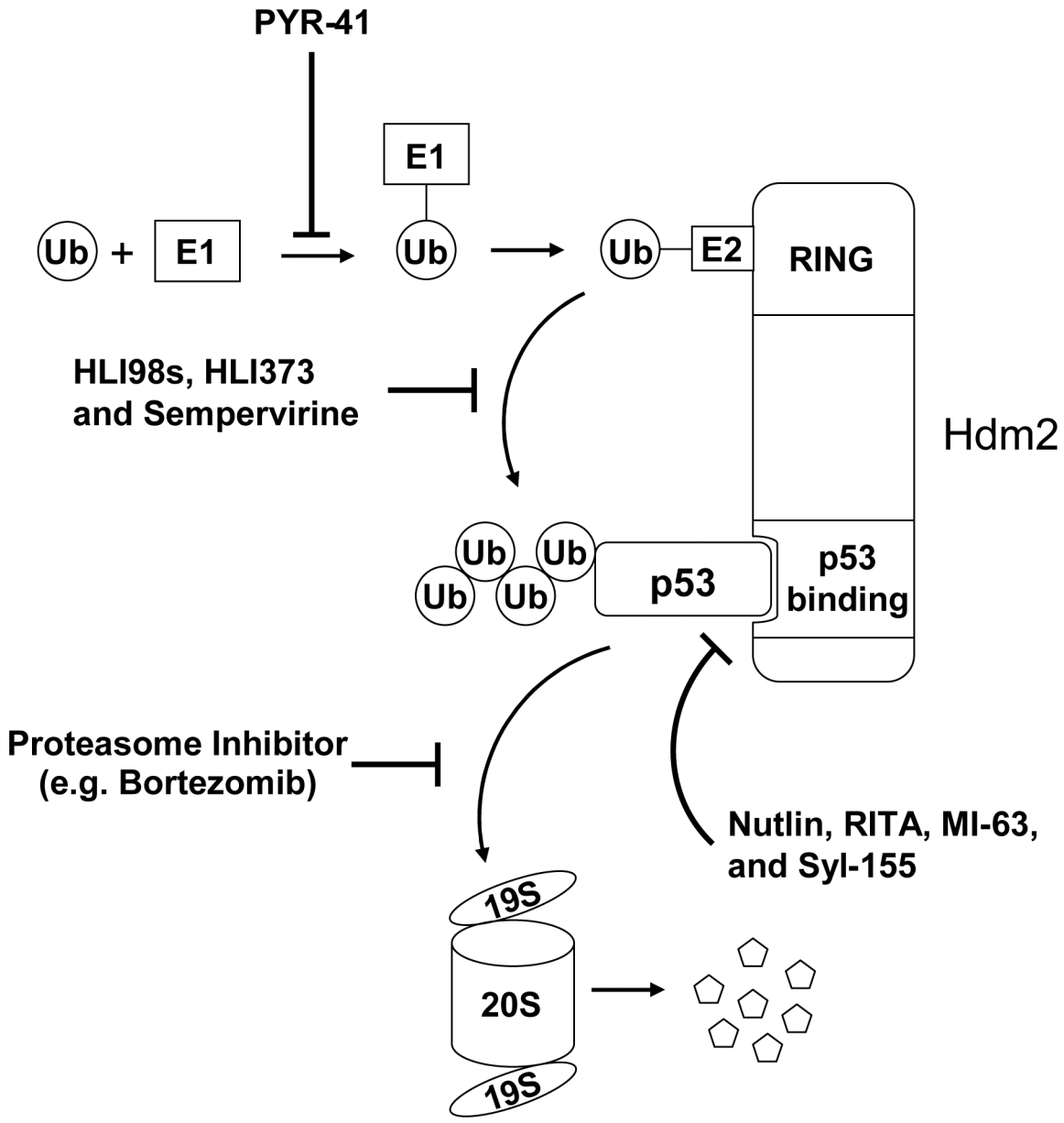


**Figure 2.**
Inhibitors that block the Hdm2-mdeiated ubiquitination and proteasomal degradation of p53.

**Table 1**

Aberrations of the ubiquitin ligases in human cancers

| Protein | Physiological Function | Pathological Change | Molecular Mechanism |
|---|---|---|---|
| Hdm2 | RING finger-containing E3 for p53 | Overexpression | Suppression of p53 activation in response to oncogenic stimuli |
| E6-AP | HECT domain-containing E3 for p53 | Activation by HPV encoded E6 | Suppression of p53 activation in response to oncogenic stimuli |
| VHL | Substrate-binding component of complex E3 VCB-Cul2-VHL | Loss of function mutation | Increased expression of critical VCB-Cul2 substrates e.g. HIF-1α |
| Skp2 | Substrate-binding component of complex E3 SCF-Skp2 | Overexpression | Down regulation of substrates, including Cdk inhibitor $p27^{kip1}$ |
| Fbw7 | Substrate-binding component of complex E3 SCF-Fbw7 | Loss of function mutation | Upregulation of substrates, including cyclin E, c- Myc, and c-Jun |
| BRCA1 | RING finger-containing E3 | Loss of function mutation | Impairment of cellular DNA repair response |
| FANCs | Complex E3 for the monoubiquitination of FANCD2 | Loss of function mutation | Impairment of cellular DNA repair response |
| CYLD | Deubiquitinating enzyme | Loss of function mutation | Reduction of the inhibition on ubiquitin-dependent NFκB activation |

## Table 2

Inhibitors of the ubiquitin-proteasome system

| Target | Inhibitors | Biological Effects |
|---|---|---|
| Ubiquitin E1 | PYR-41 | Inhibiting the activation of ubiquitin and blocking the initiation of ubiquitination |
| Hdm2 activity | HLI98s, HLI373 Sempervirine | Inhibiting the E3 activity of Hdm2, leading to accumulation and activation of p53 |
| Hdm2/p53 interaction | Peptide derivatives, Nutlins, RITA, MI- 63, Syl-155 | Blocking the recognition of p53 by Hdm2, resulting in accumulation of p53 |
| DUBs | Cyclopentenone PGs of the J series | Preventing proteasomal degradation of ubiquitinated proteins |
| Proteasome recognition | Ubistatins | Blocking the degradation of proteins conjugated with K48-linked polyubiquitin chains |
| Proteasome degradation | Peptide aldehyde, epoxyketone, and boronate; β-Lactone | Preventing proteasomal degradation of ubiquitinated proteins |