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Review Article

Small molecules that target the ubiquitin system

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Eukaryotic life depends upon the interplay between vast networks of signaling pathways composed of upwards of 10^9–10^{10} proteins per cell. The integrity and normal operation of the cell requires that these proteins act in a precise spatial and temporal manner. The ubiquitin system is absolutely central to this process and perturbation of its function contributes directly to the onset and progression of a wide variety of diseases, including cancer, metabolic syndromes, neurodegenerative diseases, autoimmunity, inflammatory disorders, infectious diseases, and muscle dystrophies. Whilst the individual components and the overall architecture of the ubiquitin system have been delineated in some detail, how ubiquitination might be successfully targeted, or harnessed, to develop novel therapeutic approaches to the treatment of disease, currently remains relatively poorly understood. In this review, we will provide an overview of the current status of selected small molecule ubiquitin system inhibitors. We will further discuss the unique challenges of targeting this ubiquitous and highly complex machinery, and explore and highlight potential ways in which these challenges might be met.

The ubiquitin system: a brief introduction

Ubiquitination is indispensable for the survival of all known eukaryotic cells and is vital for most if not all cellular processes [1,2]. The system is composed of a cascade of enzymes that reversibly catalyze the covalent attachment of ubiquitin, an 8.5 KDa protein module, at a defined position on specific protein substrates. Although it is best known for triggering protein degradation via the ubiquitin–proteasome system (UPS) [3], it also plays fundamental roles in a broad range of phenomena such as the DNA damage response, protein localization, intracellular signaling, autophagy, and endocytosis [4–9]. Ubiquitination is carried out sequentially by three specialized enzyme classes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligating enzyme). Deubiquitination is mediated by deubiquitinating enzymes (DUBs) that cleave ubiquitin from the substrate protein or trim ubiquitin moieties from a polyubiquitin chain (Figure 1) [10]. The balance between ubiquitination and deubiquitination is tightly coupled and is a critical determinant of protein levels and activity.

A definitive link between aberrant ubiquitin signaling and disease

Several lines of evidence support the notion that dysregulation of ubiquitination is closely associated with multiple human diseases, including numerous cancer types [11–13], cardiovascular disease [14], viral diseases [15–18], neurodegenerative disorders [19], and congestive heart failure [20]. Disruption of normal (de-)ubiquitination can result via many distinct, though not mutually exclusive, mechanisms. Firstly, components of the ubiquitin machinery can directly suffer missense mutations or small deletions, exemplified by PARKIN E3 ligase mutations, which cause a familial form of Parkinson's disease [21]; BRCA1-associated protein 1 (BAP1, a UCH family member) mutations that are found in many cancer types including melanoma, mesothelioma and renal cell carcinoma [22]; mutations in USP7, USP8, USP9X that are associated with neurological disorders [23], Cushing disease [24,25], and developmental disorders [26], respectively; mutations of STAMBP, which encodes a JAMM family

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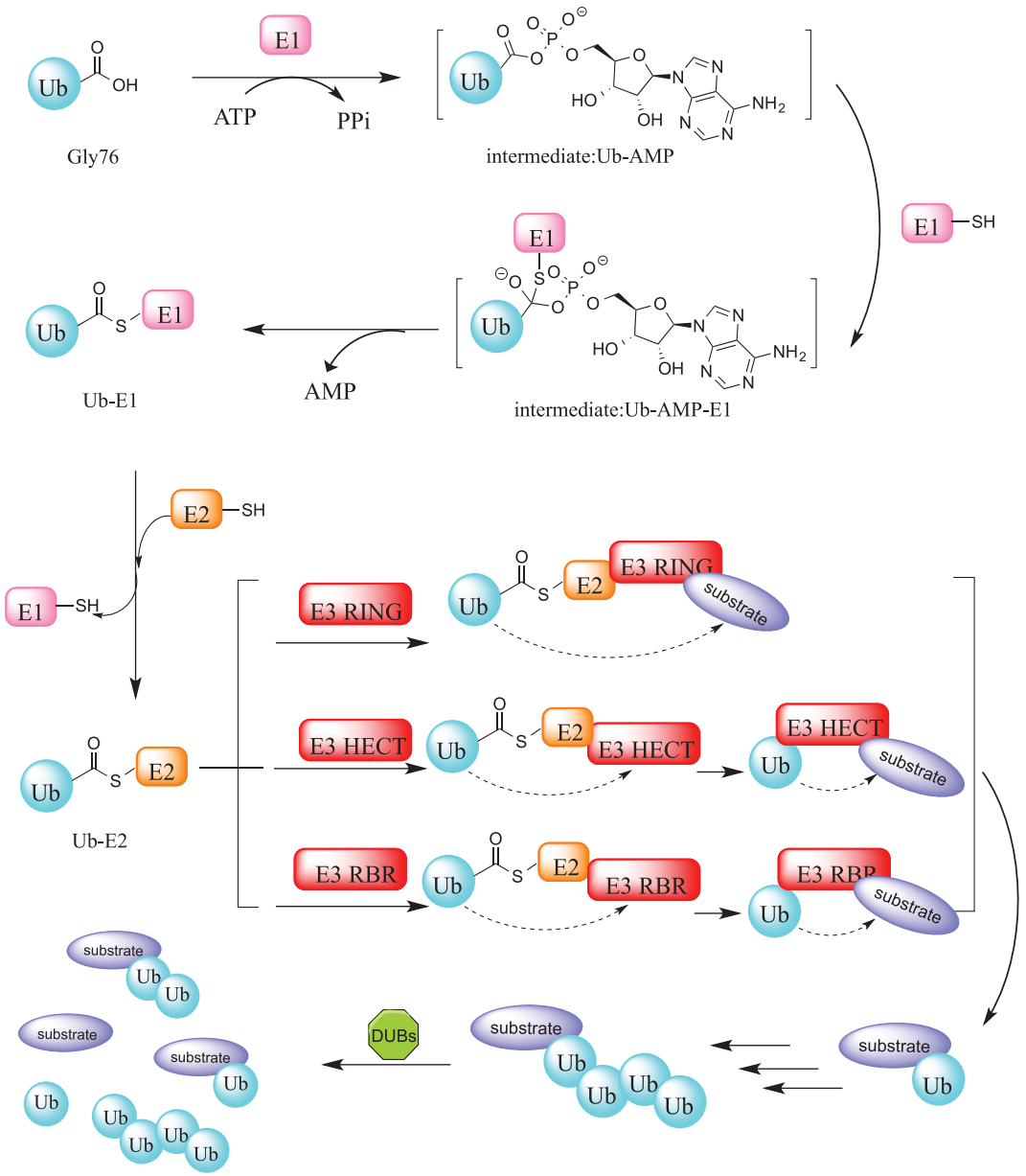

Figure 1. The ubiquitination and deubiquitination cascade.

Protein ubiquitination is initiated by the E1 enzyme that catalyzes the adenylation of the C-terminal glycine residue of Ub, forming Ub-AMP. This phosphoester bond subsequently undergoes a nucleophilic substitution involving the sulfhydryl group of the E1 active site cysteine residue. Following this, Ub is transferred to an E2 enzyme active site cysteine residue through a trans thioesterification reaction that yields an activated thioester-linked E2-Ub complex. Finally, an E3 ligase catalyzes the transfer of Ub from the E2 enzyme to a target substrates creating an isopeptide bond between the C-terminal glycine of ubiquitin and the substrate lysine. Depending on the particular class of E3 ligase, this reaction is stepwise (HECT or RBR E3s) or direct without the formation of intermediates (RING E3s). The ubiquitination process is reversed by DUBs through cleavage of ubiquitin from the substrate.

DUB, can lead to microcephaly–capillary malformation syndrome [27]. Secondly, perturbation of the ubiquitin machinery can also be caused by gene rearrangements, illustrated by the TRE17/USP6 translocation that is causally linked to aneurysmal bone cysts [28]. Thirdly, the expression of DUBs and E3 ligases have been found to be up-regulated or lost in a wide variety of tumors: gene amplification of the RING-type E3s MDM2 or

MDMX, exerts oncogenic effects in primary colorectal cancers [29] and sarcomagenesis [30]; ubiquitin-dependent degradation of AMP-activated protein kinase (AMPK), which controls cellular energy status, is mediated by the cancer-specific MAGE-A3/6-TRIM28 ubiquitin ligase [31]; overexpression of USP28 stabilizes the MYC proto-oncogene in colon and breast carcinomas [32].

The vast majority of cellular proteins are subject to ubiquitination and the net effect of corrupting ubiquitin signaling is that the levels and the activity of target proteins are either augmented (gain of function) or suppressed (loss of function). It is not surprising that these changes can play a major role in disease. In this light, the ubiquitin system potentially offers an inroad to the development of novel targeted treatments. In the case of cancer, for example, the past decade has witnessed a shift away from traditional chemotherapies towards precision treatments that specifically target tumor cells and thus result in more limited cytotoxicity in healthy tissues. This approach has largely focused on the design of kinase inhibitors and whilst there have been improvements in patient outcomes, the promised revolution has thus far failed to completely materialize. The two principal reasons for this are the acquisition of resistance and the fact that this class of inhibitors is limited in range since they were designed only for so-called 'druggable' targets. In the ensuing sections, we will discuss how either inhibiting parts of the ubiquitin system or, by contrast, actually harnessing the destructive power of the ubiquitin machinery, could each be a powerful solution to circumvent these problems and potentially hasten a new dawn for targeted therapies.

Therapeutic targeting of the ubiquitin machinery

A first question to consider is, what represents an optimal target? The overall architecture of the ubiquitin apparatus can be conveniently viewed as being composed of the following: the proteasome, E1, E2, and E3 enzymes that catalyze ubiquitination, the DUBS that mediate deubiquitination, and the ubiquitin molecule itself. Related to this, are two further questions, specifically, what is the optimal means for achieving inhibition, e.g. small molecules, peptides, antibodies, RNA-based reagents? And, what particular biochemical phenomenon is targeted: inhibition of activity by direct binding to and disruption of a specific regulatory domain/function; blocking regulatory protein–protein interactions? The therapeutic potential of this pathway is now being exploited and many UPS modulators have now entered the clinic (Table 1). There are many peptide-based inhibitors of the proteasome, such as Carfilzomib (see Table 1). Recently, microRNAs have been shown to modulate the UPS. By example, miR-137 has been reported to target Mib1 (an ubiquitin ligase), which controls neuronal maturation [53]. Both the messenger RNA and protein levels of USP25 have been shown to be regulated by direct binding of miR-200c to the 3′-untranslated region of USP25 [54]. This raises the possibility that RNA-based regulation of the UPS could serve as a potential future therapeutic method. However, to date, the majority of UPS inhibitors are cell permeable small molecules (see Table 1).

Whilst inhibitors of the proteasome and of the E1 enzyme have shown efficacy, an obvious drawback of such compounds is that they influence generically a large number of proteins/cellular networks which renders them toxic. To address this problem, in recent years efforts have focussed on identifying inhibitors of specific E3 ligases or DUBs. Here, we would like to update the very recent development of selected small molecule inhibitors against components of the ubiquitin system. A comprehensive description of earlier compounds has been provided elsewhere [1,55–58].

Targeting E3 ligases

The human proteome encodes more than 600 E3 ligases that fall into three main groups: the really interesting new gene (RING) class comprising ∼600 members, the homologous to E6AP C terminus (HECT) class which includes at least 28 members and the RING between RING (RBR) class encompassing 14 members [59–61]. A detailed ubiquitin taxonomy, specifically, a precise mapping of the enzyme to substrate, is currently lacking, however, the sheer number of ligases points strongly a high degree of specificity. This suggests that selective blocking of E3 ligase function would not result in the levels of toxicity associated with inhibition of the proteasome, ubiquitin or the broader spectrum E1- or E2-enzymes. Below we discuss recent advances in the development of such inhibitors.

RING-type E3 ligase inhibitors

TRAF6 is an E3 ligase known for its critical role in many immune signaling pathways. The molecule C25-140 (**1**, Table 2 was identified in a high-throughput screen designed to identify compounds that disrupt the TRAF6–Ubc13 protein–protein interaction [62]. Although C25-140 was also shown to inhibit cIAP1 [62], the

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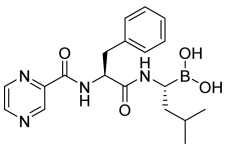
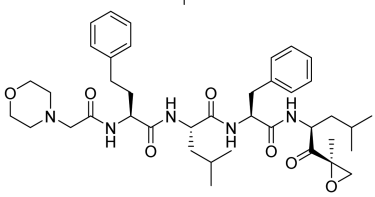
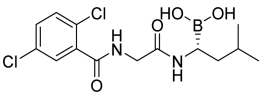
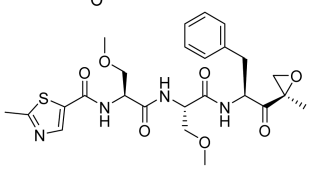
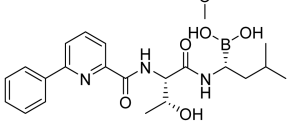
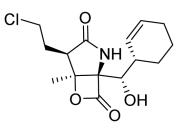
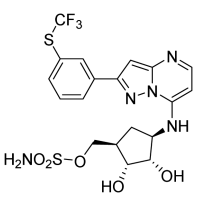
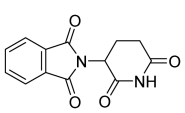
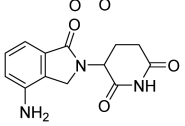
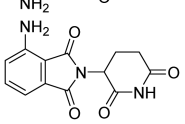
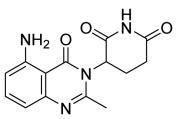
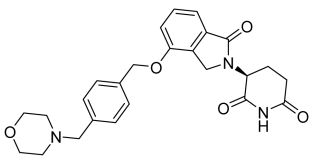
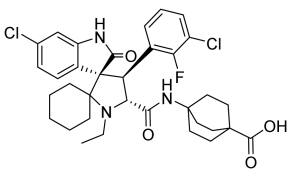
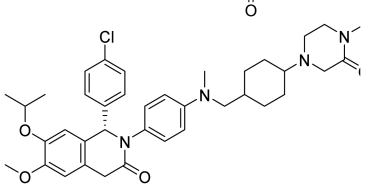
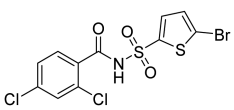
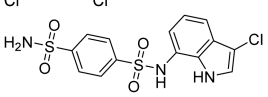
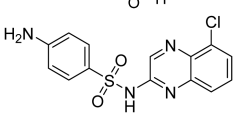
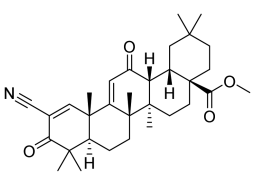
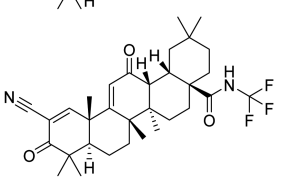
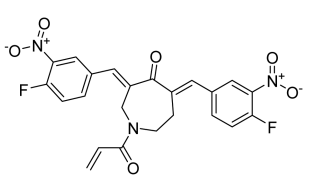
Table 1. Proteasome, E1, E3, or DUB modulators in clinic use or in (pre)-clinical development Part 1 of 2

Target	Compound ID	Structure	Highest clinical stage and status[1]	Reference
Proteasome inhibitors				
proteasome	Bortezomib		FDA approved	Teicher et al. [33]
	Carfilzomib		FDA approved	Kim and Crews [34]
	Ixazomib		FDA approved	Kupperman et al. [35]
	Oprozomib		Phase 1/ Phase 2, completed	Chauhan et al. [36]
	Delanzomib		Phase 1/ Phase 2, terminated	Piva et al. [37]
	Marizomib		Phase 3, recruiting	Potts et al. [38]
E1 modulator				
UAE (Uba1)	TAK-243		Phase 1, completed	Milhollen et al. [39] Hyer et al. [40]
E3 modulators				
CRBN	Thalidomide		FDA approved	Ito et al. [41]
	Lenalidomide		FDA approved	Krönke, et al. [42, 43]
	Pomalidomide		FDA approved	Fischer et al. [44]

Continued

Table 1. Proteasome, E1, E3, or DUB modulators in clinic use or in (pre)-clinical development Part 2 of 2

Target	Compound ID	Structure	Highest clinical stage and status[1]	Reference
	Avadomide		Phase 1/ Phase 2, active	Rasco et al. [45]
	Iberdomide		Phase 1/ Phase 2, recruiting	Bjorklund et al. [46]
MDM2	APG-115		Phase 1/ Phase 2, recruiting	Rasco et al. [47]
	CGM097		Phase 1, active	Holzer et al. [48]
Cul4-DCAF15	Tasisulam		Phase 3, terminated	Han et al. [49]
	Indisulam		Phase 2, completed	
	NSC-339004		Phase 2, Completed	
Keap1	Bardoxolone methyl		Phase 3, recruiting	Gross et al. [50]
	Omaveloxolone		Phase 2, completed	Lynch et al. [51]
Deubiquitinase inhibitors				
USP14 > UCH37; many other DUBs inhibited	VLX1570		Phase 1/ Phase 2, prematurely ended	Paulus et al. [52]

[1]Data from ClinicalTrials.gov and EU Clinical Trials Register.

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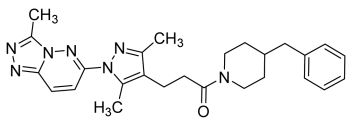
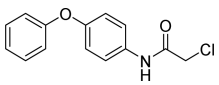
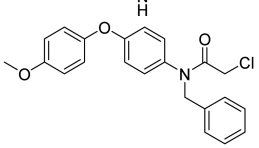
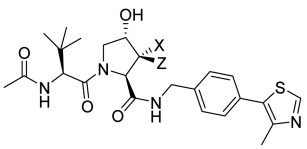
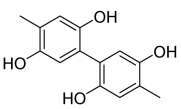
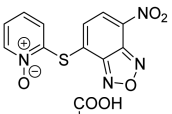
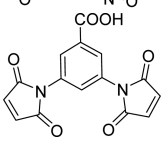
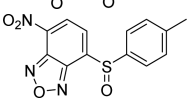
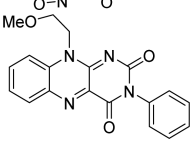
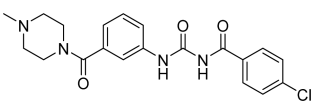
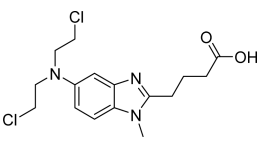
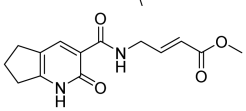
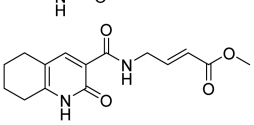
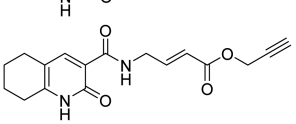
Table 2. Recently reported structures of small molecule inhibitors of E3 ligases

Target	Compound ID	Structure	Reference
TRAF6	**1**, C25-140		Brenke et al. [62]
RNF4	**2**, TRH 1-23		Ward et al. [63]
	3, CCW16		
VHL	**4a**: X = F, Z = H **4b**: X = H, Z = F		Testa et al. [64]
WWP2	**5**		Watt et al. [65]
	6		
	7		
	8		
	9		
SMURF1	**10**, HS-152		Tian et al. [66]
HOIP	**11**, Bendamustine		De Cesare et al. [67]
	12		Johansson et al. [68]
	13		
	14		

relatively greater selectivity of this compound was revealed by its inability to block multiple other E3 ligases including RING-type (e.g. cIAP1 and MDM2) and HECT-type (e.g. ITCH and E6AP) enzymes. Indeed, it has been argued that the identified off-target activity of C25-140 could prove to be beneficial for the treatment of autoimmune and inflammatory diseases since illicit TNFα signaling has been shown to underlie the development of these diseases [62]. RNF4 is an E3 ubiquitin ligase that promotes ubiquitination and subsequent proteasomal degradation of SUMOylated proteins [69,70]. TRH 1-23 (**2**, Table 2) is a promising RNF4 inhibitor that was recently identified in an activity-based protein profiling (ABPP) screen. An analog of TRH 1-23, named CCW-16 (**3**), was found to be a more a potent RNF4 inhibitor, exhibiting an IC_{50} of 1.8 μM [63].

Von Hippel–Lindau (VHL) E3 Ubiquitin Ligase is one of the few E3 ligases, including cereblon, VHL, MDM2, and cIAP, that have been successfully exploited in targeted protein degradation strategies [71,72]. Recognition of hydroxyproline (Hyp) by VHL is highly stereoselective and conformation-dependent. Fluoro-hydroxyproline (F-Hyp) containing compounds (**4a**, **4b**) have been reported to be VHL Ligands with higher binding affinity and improved metabolica stability compared with the parent Hyp-containing ligand. The ligand **4a** has been employed successfully in novel VHL-based PROTAC reagents [64] (see section targeted protein degradation).

HECT-type E3 ligase inhibitors

High-throughput screening of small molecule libraries [65] has yielded many compounds (see Table 2, structures **5–9**) that show promise as potentially therapeutically relevant WWP2 E3 ligase inhibitors (IC_{50} values are in the low μM range). The sites of interaction between inhibitor and target have been determined by NMR, which may facilitate the process of refining these WWP2 inhibitors. A potent SMURF1 small molecule inhibitor named HS-152 (**10**) was discovered using a cell-based high-throughput screening campaign [66]. This compound blocks the catalytic activity of SMURF HECT domains thereby abrogating SMURF1-mediated SMAD1 degradation as well as SMURF1-mediated RHOA/RHOB degradation.

RBR-type E3 ligase inhibitors

A label-free MALDI-TOF mass spectrometry approach was used to test 1430 FDA approved drugs for their ability to specifically inhibit E3 ligases representative of the three different enzyme classes. By this means, bendamustine (**11**, Table 2), a chemotherapeutic reagent commonly used in the treatment of chronic lymphocytic leukemia and lymphomas, was found to inhibit HOIP with an IC_{50} of 6.4 μM, while it did not significantly affect the activity of either MDM2 or ITCH [67]. A fragment-based covalent ligand screen, with the aim of identifying inhibitors that covalently target the active site cysteine residue of HOIP, has highlighted three selective small molecules (see Table 2, compounds **12–14**) as promising HOIP inhibitors, which displayed limited off-target activity against other RBR or HECT E3 ligases [68].

Targeted protein degradation

Whilst selective inhibition of the activity of specific E3 ligases could prove to be of significant therapeutic value, a potentially revolutionary innovation currently gaining widespread attention takes an entirely different approach. Rather than inhibiting E3 ligases, targeted protein degradation, including proteolysis targeting chimeras (PROTACs) [73], immunomodulatory drugs (IMiDs) [74], and specific and nongenetic IAP-dependent protein erasers (SNIPER) [75,76], harnesses the destructive power of the host cell ubiquitin machinery to eliminate unwanted disease-causing proteins in a targeted manner. Since a detailed account, this technology is beyond the scope of this review, we refer readers to many recent reviews that comprehensively summarize recent progress in this field [77–84].

Targeting deubiquitination

The human proteome contains ∼100 DUBs belonging to two distinct enzyme classes: the cysteine proteases and the metalloproteases. The cysteine protease class comprises six different enzyme families: ubiquitin-specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado-Joseph (Josephin) domain-containing proteases (MJDs), motif interacting with Ub-containing novel DUB family (MINDY) [85], and zinc finger containing ubiquitin peptidase 1 (ZUFSP/ZUP1) [86–89]. The metalloprotease enzyme class consists of a single family of enzymes: JAB1/MPN/MOV34 (JAMMs). DUBs strip protein substrates of ubiquitin thus rescuing them from degradation via the proteasome. Accordingly, inhibiting DUBs, in common with the PROTAC technology, can selectively eliminate targets by rendering them

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susceptible to proteasomal degradation. Numerous small molecule DUB inhibitors have been reported, however, until recently, only few of them showed selectivity [90].

USP7

The ground-breaking work on ubiquitin-specific protease (USP) 7 inhibitors represents a paradigm for the discovery and characterization of highly selective small molecule inhibitors of DUBs. USP7 or HAUSP (herpesvirus associated ubiquitin-specific protease) is best known for its role as a regulator of the tumor suppressor p53 [91,92] although it has many other reported targets. USP7 deubiquitinates murine double minute-2 (MDM2), an E3 ligase of p53, resulting in p53 degradation. Consequently, it would be expected that a USP7 inhibitor would promote stabilization of p53 leading to suppression of cell growth and/or apoptosis that may be beneficial for the treatment of certain cancers. The 9-chloro derivatives of amidotetrahydroacridine, HBX19818 (**15a**) and HBX28259 (**15b**) (see Figure 2), have been reported to be irreversible inhibitors of USP7 that function via nucleophilic attack of catalytic cysteine residues mediated by the reactive chloride moiety and resulting in covalent linkage to the protein [93]. HBX 19818 was found to specifically inhibit USP7 but not a panel of different DUBs under physiological conditions. This compound was shown to inhibit HCT116 proliferation and to induce apoptosis in a dose-dependent manner. Interestingly, HBX 28364 (**16**), which lacks the basic alkyl amine side chain of HBX 19818, does not show any inhibitory activity. Crystallographic analysis of USP7-inhibitor complexes has enabled structure-guided optimization of potent and highly specific USP7 inhibitors, and mainly three binding regions have been unmasked to date (red, blue, and orange in Figure 2B) [94]. P5091 (**17**, Figure 2) was initially discovered as a dual USP7/USP47 inhibitor [95], which causes apoptosis of multiple myeloma cells and prolonged survival of transplanted mice (using xenograft tumor models) [96]. Further optimization of P5091 led to the discovery of P50429 (**18**) and P22077 (**19**) that inhibit the proliferation of HCT116 cells [95] and neuroblastoma growth [97], respectively. A combination of solution NMR and mass spectrometry studies, revealed that P22077 and P50429 bind irreversibly to the catalytic cysteine C223 of USP7 (red in figure 2B). This interaction induced a conformational switch in the enzyme associated with an active site rearrangement [98]. Medicinal chemistry optimization of a fragment yielded the small molecules GNE6640 (**20**) and GNE6776 (**21**) shown in Figure 2. This class of allosteric inhibitors has been demonstrated to bind to the 'palm' region of the USP7 catalytic domain thereby impeding ubiquitin binding (orange, Figure 2B) [99,100]. NMR-based saturation transfer difference (STD) experiments showed that these compounds bind to a novel functional site 12 Å away from the catalytic cysteine residue [100]. These molecules potently inhibited the activity of USP7 (IC50 values of 1.34 μM and 0.75 μM, respectively) and did not inhibit the activity of a panel of 36 DUBs at concentrations of 100 μM, which is convincing evidence of high target selectivity [99]. Cocrystal structures have further shown that GNE6640 and GNE6776 interact with acidic residues that commonly promote hydrogen-bond interactions with Lys48 of ubiquitin. A group of pyrimidinone analogs (**22–30**) [101–104], reported by several laboratories independently, have been shown to bind to the same catalytic region that is normally occupied by the C-terminal tail of ubiquitin (blue structure, Figure 2B). One of these compounds (**30**, Figure 2) strongly inhibited the proliferation of several cancer cell lines with equal or greater efficacy compared with known MDM2 antagonists (IC50 = 6 nM). All of these highly selective inhibitors bind non-covalently to USP7, with the exception of FT827, which harbors a vinylsulfonamide moiety that extends to the catalytic triad and covalently binds to the active cysteine [103].

Other DUBs

Ubiquitin-specific protease 14 (USP14), a proteasome associated DUB, has been reported to be involved in tumorigenesis in many cancer types. It also plays an important role in neurodegenerative disorders. IU1 (**31**, see Table 3) was first reported in 2010 as a selective small molecule inhibitor of USP14, which could enhance the degradation of several proteasome substrates such as tau and TDP-43 [105]. Chemical modification of IU1 resulted in IU1-47 (**32**), which is 10-fold more potent than IU1 but retained specificity for USP14. Following IU1-47 treatment, both endogenous wild-type tau and pathological mutantforms of tau (in murine primary neurons and in Human iPSC-derived neurons) could be significantly degraded, and this effect was dependent on Lys174 in tau [106]. An analog of IU1 termed 1B10 (**33**) was found recently to be more potent than IU1, and also had a better membrane permeability [107]. UCHL1, belonging to the Ubiquitin C-terminal Hydrolase subfamily, is considered to be a promising therapeutic target in neurodegenerative diseases, cancer, and liver/lung fibrosis. Very recently, several cyanopyrrolidine-based UCHL1 inhibitors, that covalently bind to the active cysteine, have been reported. Compound **34**, for example, was first reported as a UCHL1 inhibitor in a patent

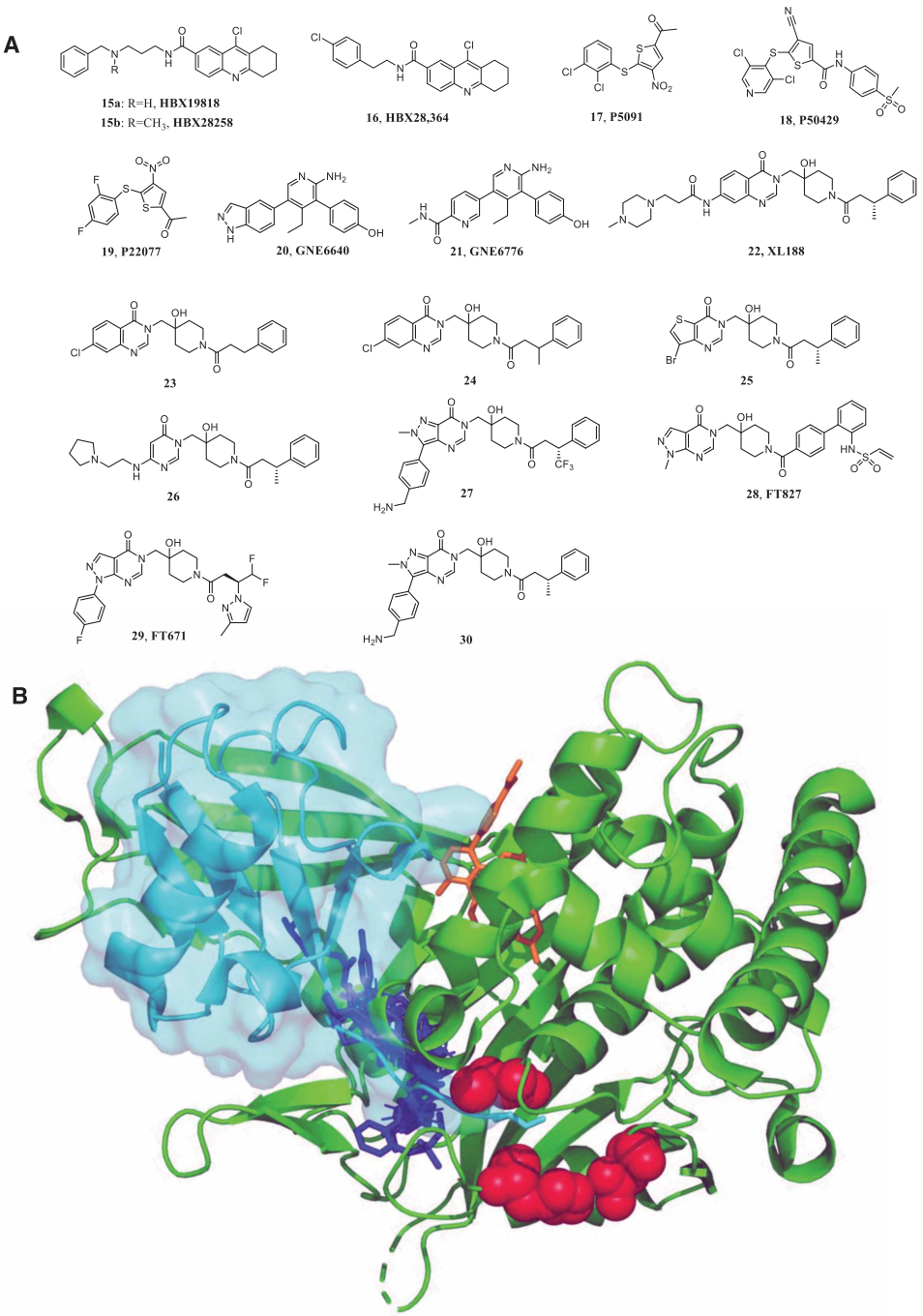

Figure 2. Small molecule inhibitors of USP7 and their representative binding domains.

(**A**) Structures of small molecule inhibitors targeting USP7. (**B**) The binding characteristics of current inhibitors of USP7. The catalytic triad of the catalytic domain is shown as red spheres. Compounds P50429 and P22077 both bind to this region. The allosteric inhibitors (GNE6640 and GNE6776, shown in orange) occupy a functional site that is 12 Å away from the catalytic cysteine. The pyrimidinone analogs (shown in blue) bind to the catalytic region that is normally occupied by the C terminus of ubiquitin (shown in cyan).

application by Mission Therapeutics [108]. But it was found to inhibit both UCHL1 and UCHL3 with IC_{50} values of 0.67 ± 1.0 μM and 6.4 ± 1.1 μM, respectively [109]. A more selective and potent UCHL1 inhibitor (**35a**) [118], as well as a structurally related activity-based probe (**35b**) [110], were reported to label UCHL1 in

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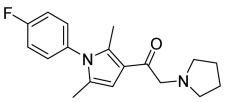
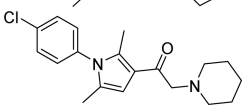
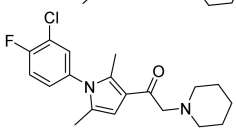
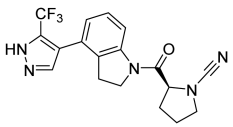
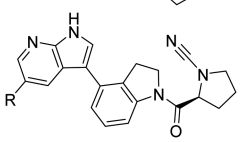
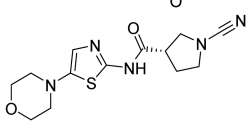
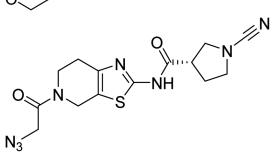
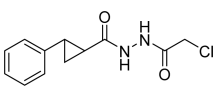
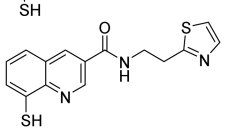
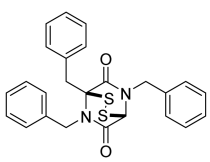
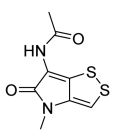
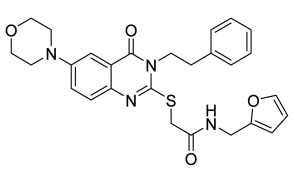
Table 3. Selected structures of currently reported small molecule inhibitors targeting DUBs

Target	Compound ID	Structure	Reference
USP14	**31**, IU1		Lee et al. [105]
	32, IU1-47		Boselli et al. [106]
	33, 1B10		Palmer et al. [107]
UCHL1	**34**		Krabill et al. [108] Kemp et al. [109]
	35a:R = H **35b**:R = CCH		Nattawadee et al. [110]
	36, 6RK73		Geurink et al. [111]
	37, 8RK64		
OTUB2	**38**, OTUB2-COV-1		Resnick et al. [112]
Rpn11	**39**, 8TQ		Perez et al. [113]
	40, Capzimin		Li et al. [114]
Rpn11, also Csn5 and AMSH	**41**, SOP11		Li et al. [115]
RPN11 and other JAMM metalloproteases	**42**, Thilolutin		Lauinger et al. [116]
STAMBP	**43**, BC-1471		Bednash et al. [117]

living cells at low micromolar concentrations, and block pro-fibrotic responses in IPF cellular models without substantial associated cytotoxicity. The cell permeable small molecules 6RK73 (**36**) and 8RK64 (**37**) were found to target UCHL1 but no other DUBs [111]. However, they did inhibit the non-DUB protein Park7 as an off-target. After chemically decorating fluorophores, such as BodipyFL-alkyne, BodipyTMR-alkyne, and Rhodamine110-alkyne, the first examples of a UCHL1-selective probe were generated and the Bodipy-labeled reagent was shown to react with UCHL1 in zebrafish embryos. OTUB2 is an ovarian tumor domain (OTU) family member that preferentially cleaves Lys63-linked polyubiquitination chains and to a lesser degree, Lys11-linked and Lys48-linked chains. Recently, the first highly selective OTUB2 covalent inhibitor named OTUB2-COV-1 (**38**, Table 3) was identified by combining a new high-throughput thiol-reactivity assay with high-throughput crystallography [112].

Rpn11 (POH1/PSMD14), contains Jab1/MPN metalloenzyme (JAMM) motif, is an essential catalytic subunit of the 19S regulatory particle of the proteasome that cleaves polyubiquitin chains from substrates prior to their proteasomal degradation [119]. Several small molecule inhibitors targeting RPN11 have recently been identified. Quinoline-8-thiol (**39**, 8TQ, see Table 3) and its derivatives were discovered by screening a library of metal-binding pharmacophores [113]. Further molecular refinement of the 8TQ scaffold yielded several compounds capable of inhibiting RPN11 at submicromolar concentrations. The 8TQ derivative Capzimin (**40**) was found to be a potent Rpn11 inhibitor (IC_{50} = 2.4 μM) [114]. Unlike classical proteasome inhibitors that target the 20S core particle, Capzimin stabilizes proteasome substrates and inhibits tumor cell proliferation including the proliferation of cells that are resistant to bortezomib. Through the use of a novel assay for monitoring proteasome-mediated protein degradation, Li et al. [115] also identified epidithiodiketopiperazines as being a new class of RPN11 inhibitors. The most promising candidate, SOP11 (**41**), quenched RPN11 protease activity by chelating the active site Zn^{2+} ion. This inhibition stabilized the levels of many substrates that would otherwise have been degraded by the proteasome in cells. However, SOP11 also inhibits the activity of other JAMM family members *in vitro*, including Csn5 and AMSH. Thiolutin (**42**), a disulfide-containing antibiotic and anti-angiogenic compound produced by Streptomyces, was reported to function as a zinc chelator that inhibits RPN11 and other JAMM metalloproteases [116]. STAMBP (or AMSH), is a K63-specific JAMM-type DUB that protects endosome cargo proteins from lysosomal degradation and also controls the levels of ubiquitination of ESCRT proteins. BC-1471 (**43**) was discovered as a specific STAMBP inhibitor (IC_{50} = 0.33 μM) that selectively blocked deubiquitination of Ub-NALP7 by recombinant STAMBP [117] but did not significantly inhibit the activity of a panel of 38 different DUBs at the concentration tested.

Targeting ubiquitin: a new mechanism

Targeting ubiquitin itself could also prove to be a valid approach to perturb ubiquitin signaling. Although not drug-like, molecules called ubistatins have been shown to bind to ubiquitin in a chemical genetic screen [120,121], although these molecules have features also found in 'frequent hitters'. An additional advance in a similar direction came through the discovery of a cyclic peptide ubiquitin binder, by using the RaPID system [122]. These peptides have been shown to efficiently trigger apoptosis in cancer cells by binding tightly and with a high degree of specificity to K48-linked Ub chains, thus disrupting their interaction with the proteasome and their subsequent degradation. This unique mode of action could open new opportunities for therapeutic intervention.

Future challenges

The last two decades, most strikingly in the area of oncology, have witnessed a shift away from broad-spectrum medicines such as traditional chemotherapies towards precision treatments that specifically target diseased cells and thus result in more limited off-target cytotoxicity. Whilst there have been some improvements in patient outcomes, the promised revolution has thus far largely failed to materialize. The UPS is integral to virtually all known eukaryotic cellular processes and in recent years it has become clear that specifically targeting (or harnessing in the case of the targeted protein degradation technology) this system could open a new front in the treatment of numerous diseases. Towards this aim, steady progress is being made, evidenced by the number of unique drugs entering the clinic. However, in order to maximize the potential of manipulating the UPS, many challenges will have to be overcome and new opportunities will need to be explored while unique dimensions of this complex pathway are being elucidated.

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Exploiting E3 ligase and DUB structural diversity

In this review, we have highlighted the potential of targeting E3 ligases and DUBs. Because each enzyme class interacts with a defined set of substrates, inhibitors of these enzymes should in principle exhibit greater specificity and reduced on-target toxicity compared with proteasome inhibitors, for example. There are, however, possible pitfalls. An obvious potential limitation relates to the fact that RING-type E3 domains and HECT domains share a high degree of structural similarity, as do the catalytic pockets of DUBs. These sites seem to be the most suited to drug development [123], but this similarity increases the probability of obtaining inhibitors that block the activity of multiple enzymes. This might not necessarily be a bad thing if the treatment is effective and unwanted toxicity is significantly lower than currently available options. One way to obtain selective inhibitors would be to target the non-catalytic regions rather than catalytic pockets. For instance, USP7 contains a central binding cleft in the N-terminal TRAF-like domain that is specifically recognized by [PA]-xx-S amino acid motifs found in p53, Hdm2, and Epstein–Barr nuclear antigen 1 [91]; the rhodanese domain of USP8 includes a ligase recognition site [124]; a non-catalytic ubiquitin-like (UBL) region of USP11 has been shown to interact with a helical motif but this site is absent from paralogs USP4 and USP15 [125]. However, current knowledge of such binding sites is still lacking for most DUBs and E3 ligases. Moreover, DUBs are flexible enzymes, that often can allosterically alternate between active and inactive conformations to regulate their activity [126,127].

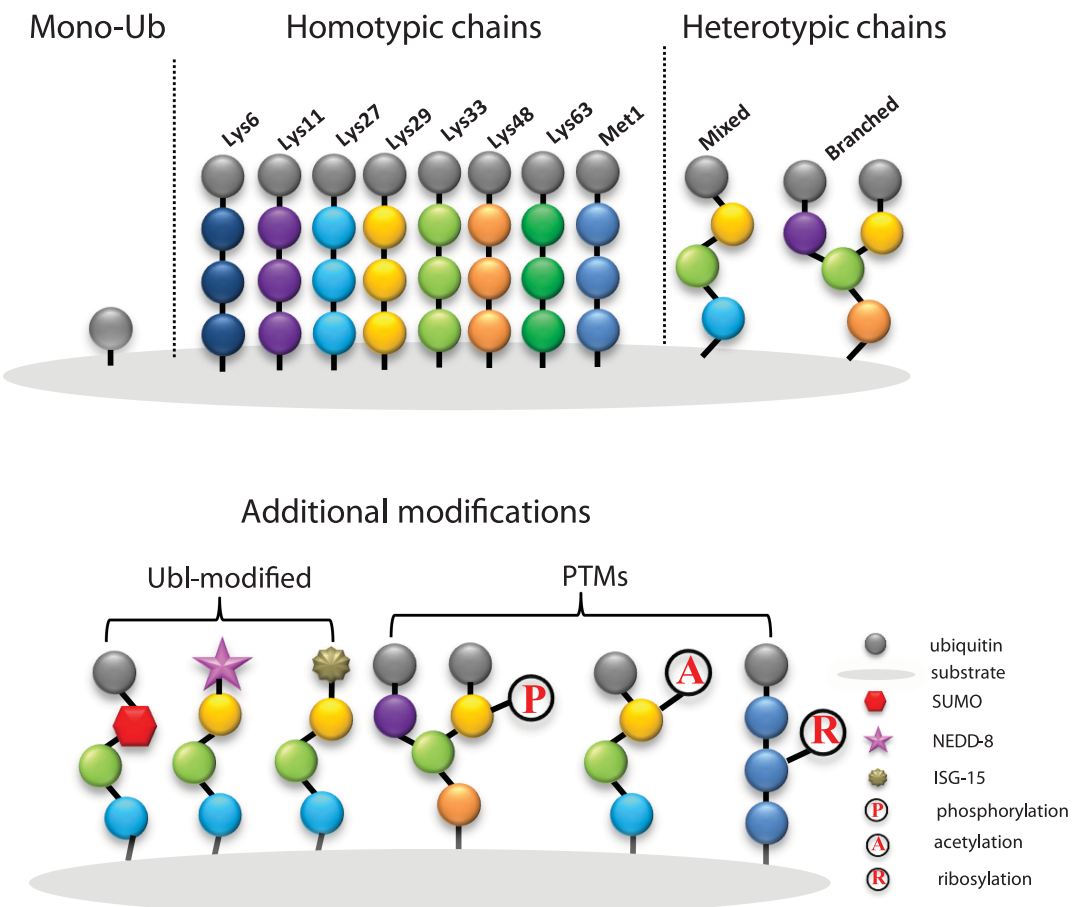

Figure 3. The diversity of ubiquitin modifications.

Monoubiquitin is the simplest modification. Eight distinct homotypic polyubiquitin chains are formed by each ubiquitin molecule linking to another via a Lys or Met1 at the same position. Heterotypic chains consist of more than one linkage type in linear or branched mode. Modifications of ubiquitin and UBL modifiers, such as SUMO, NEDD-8 or ISG-15, as well as with other PTMs such as phosphorylation (P), acetylation (A) and ribosylation generates additional levels of complexity.

Complexity of the ubiquitin code

The versatile function of ubiquitination requires the ubiquitin system to be remarkably complex. An essential feature of this complexity which is referred to as the 'ubiquitin code', consists of the diverse architecture of Ub chains, Ub chain length and post-translational modifications (PTMs) at multiple sites (Figure 3). The first layer of this complexity results from the fact that the carboxy terminus of Ub can be conjugated to any of seven lysine residues (Lys6, Lys 11, Lys 27, Lys 29, Lys 33, Lys 48, Lys 63) or the N-terminal methionine residue (Met1) on the proximal Ub moiety yielding eight homotypic polyubiquitin chains [128]. The diversity of chain length and topology determines the fate of the substrate, while K48-polyubiquitin is considered to be a signal for degradation by the proteasome [129]. Heterotypic chains and branched chains form the second layer of ubiquitination complexity. In this case, several different linkage types can be contained in one ubiquitin chain or multiple lysine sites on one ubiquitin molecule can be modified to form branched structures [127]. The huge number of distinct chain architectures, resulting from these different linkages, carry important signals in cellular signal transduction pathways [130]. It has been reported that branched chains can enhance substrate recognition by the proteasome and ultimately drive turnover of cell-cycle regulators during mitosis [131]. However, the function of heterotypic/branched chains are still poorly understood because technologies to study them *in vivo* are limited. The complexity of the ubiquitin code is further expanded through the cross-communication between ubiquitin and other PTMs. Phosphorylation [132–134], acetylation [133, 135], and more recently ribosylation [136–139] are all found on ubiquitin chains, and ubiquitin can be connected to UBL modifiers, such as small ubiquitin-related modifier (SUMO) [140], neuronal precursor cell-expressed developmentally down-regulated protein 8 (NEDD8) [141], and interferon-stimulated gene 15 (ISG15) [142]. In sum, whilst the ubiquitin code is evidently more intricate than is currently known, future approaches to manipulate the code could produce selective inhibitors of specific proteins/biological phenomenon.

Functional redundancy

Functional redundancy, that is, the tendency of one protein to compensate for the loss of function of a different protein, is a common biological phenomenon and is one the major causes of resistance to targeted treatments, particularly in oncology. Despite the very large numbers of E3 ligases and DUBs, the UPS exhibits a significant degree of functional redundancy. How can this problem be surmounted to produce clinically robust therapies? To date, a detailed ubiquitin taxonomy is absent such that there is an imprecise mapping of enzymes to the substrates they target. Producing a more comprehensive map would go some way to solving this problem by helping to define suitable combination therapies that are less susceptible to redundancy.

Conclusion

One ultimate goal for a biomedical researcher is to design therapies that effectively treat the disease, do not cause off-target toxicity and that are not susceptible to resistance. During the past decade, we have witnessed dramatic progress in ubiquitin system chemistry and biochemical research into the pathway, resulting in some knowledge of the 'ubiquitin code', and UPS enzyme function and their mechanisms of regulation. Parallel to these discoveries has been the development of an increasing number of inhibitors targeting this system, which could prove to be an efficacious and selective way to treat diseases such as cancer.

Perspectives

We are evidently still far away from having a complete picture of ubiquitin biology. In the coming years, fully deciphering the nature of the Ub code will become a priority as little is known about the biological relevance of most ubiquitin chain linkage types (such as K27-, K29-, and K33-linked polyUb chains), or additional layers of complexity of the ubiquitin code (branched and hybrid chains, mixed PTMs). In this respect, methods for unraveling the secrets of the Ub code, such as ubiquitin chain restriction analysis (UbiCRest) [143,144] and Ub-clipping technology [145], will be important. To optimize the prospects of developing E3 or DUB inhibitors for clinical use, mapping the E3-substrate and DUB-substrate relationships are urgently needed as well as structural insight into how specific substrates are recognized and how their ubiquitination is regulated in time and space and under different cellular conditions. This represents an important and at the same time very challenging task. Furthermore, developing novel screening technologies for

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inhibitor discovery is crucial as the high concentrations of reducing agents used in assays result in very high false-positive rates [146] and as a result reported Ub system inhibitors can be unreliable. With advances in bioinformatics and novel technologies for high-throughput screening and other tools (such as activity-based probes, high-throughput crystallography, and the use of mass spectrometry), the development of specific E3 and DUB inhibitors may become within reach. In addition to blocking the UPS, targeted protein degradation technology could prove to be an essential part of modern medicines armory to treat disease.

Competing Interests

H.O. holds shares in Ubiq Bio B.V.

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Abbreviations

BAP1, BRCA1-associated protein 1; DUBs, deubiquitinating enzymes; HECT, homologous to E6AP C terminus; MDM2, murine double minute-2; OTUs, ovarian tumor proteases; PROTACs, proteolysis targeting chimeras; PTMs, post-translational modifications; RBR, RING between RING; RING, really interesting new gene; SUMO, small ubiquitin-related modifier; UBL, ubiquitin-like; UCHs, ubiquitin carboxyl-terminal hydrolases; UPS, ubiquitin–proteasome system; USP14, ubiquitin-specific protease 14; USPs, ubiquitin-specific proteases; VHL, Von Hippel–Lindau; ZUFSP/ZUP1, zinc finger containing ubiquitin peptidase 1.

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