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Drug discovery in the ubiquitin regulatory pathway

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The ubiquitin system has been implicated in the pathogenesis of numerous disease states, including oncogenesis, inflammation, viral infection, CNS disorders and metabolic dysfunction. Ubiquitin conjugation and deconjugation to substrate proteins is carried out by multiple families of proteins, each with a defined role in the enzymatic cascade. This conjugation–deconjugation system parallels the kinase–phosphatase system in that both alter protein function by the addition and removal of post-translational modifiers. Our understanding of ubiquitin biology and strategies to interfere pharmacologically with the ubiquitin regulatory machinery is progressing rapidly. In light of increased interest in ubiquitin pathways as drug targets, we review the ubiquitin enzymatic cascades, highlighting therapeutic opportunities and enzymatic mechanisms. We also discuss the challenges of targeting this class of enzymes with small molecules, as well as current approaches and progress in drug discovery.

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▼ Ubiquitin (Ub) and ubiquitin-like modifiers (Ubls) comprise a family of low molecular weight polypeptides (76–165 amino acids) that function through their covalent attachment to a wide range of cellular substrate proteins (Table 1). Throughout this review, we use the abbreviations Ub/Ubl to denote members of this family of protein modifiers generically. Ubls are related in primary sequence, share between 10% and 55% sequence identity to ubiquitin, and invariably encode a glycine residue at their C-terminus. The C-terminal glycine residue forms an isopeptide bond with the ε-amino group of one-or-more lysine residues within the target protein. Each Ub/Ubl directs distinct sets of biological consequences and each requires distinct conjugation and deconjugation machinery. Moreover, the same Ub/Ubl can produce various effects depending on whether a monomer or an isopeptide-linked multimeric chain is attached to the substrate. For example, multi-ubiquitylation can induce protein degradation, whereas mono-ubiquitylation affects protein localization. Table 1 summarizes the biochemical and biological features of Ub/Ubl family members.

Ubiquitin conjugation and deconjugation

The ubiquitylation and de-ubiquitylation cycle

The Ub/Ubl conjugation cascade is initiated by an activating enzyme (E1) that activates the C-terminal glycine residue of the Ub/Ubl by a two-step catalytic process (Figure 1). First, hydrolysis of ATP results in the formation of an acyl-adenylate intermediate, linking AMP with the C-terminal carboxyl group of the Ub/Ubl. Second, the adenylated carbonyl group of the C-terminal residue is transferred to the catalytic cysteine in the E1, thereby generating an E1–Ub or E1–Ubl thioester linkage. Following the E1 activation step, the Ub/Ubl is transferred to the active site cysteine of the carrier protein (E2). Subsequently, the ligase enzyme (E3) catalyzes the transfer of the Ub/Ubl from the E2 thioester intermediate to an isopeptide linkage with a selected substrate. Progressive rounds of E3-promoted ubiquitin ligation result in the generation of a multimeric ubiquitin chain. In the final step of the cycle, de-ubiquitylating enzymes (DUB) hydrolyze the isopeptide bond to counterbalance the conjugation machinery and replenish the Ub/Ubl pool. Additional details about the ubiquitin conjugation–deconjugation process are given later in this article and in a recent and comprehensive review [1].

Enzymatic diversity and modularity: strength in numbers

Genome mining efforts have identified at least 530 human genes that encode enzymes

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Table 1. The family of ubiquitin and ubiquitin-like modifiers

Modifier	Size	Identity	C-terminus	Mono/multi	Lysine	Function
Ubiquitin	76	100%	LRLRGG 76	Multi	K48	Proteasome degradation
				Multi	K63	Signal transduction
				Mono		Protein trafficking
NEDD8	81	54.3%	LALRGG 76	Mono		Cullin regulation
ISG15	165	17.0%	LRLRGG 157	Mono		Signal transduction
SUMO1	101	13.9%	QEQTGG 97	Mono		Localization
				Multi	K16[a]	
SUMO2	95	12.6%	QQQTGG 93	Mono		Localization
				Multi	K11[a]	
SUMO3	103	11.7%	QQQTGG 92	Mono		Localization
				Multi	K11	Inhibits Aβ production [68]
APG12	140	10.7%	KSQAWG 140	Mono		Autophagy
APG8	125	9.5%	KSQAWG 120	Mono		Autophagy

[a]Predicted SUMO polymerization sites based on sequence alignment.
Abbreviations: APG, autophagy; NEDD, neural precursor cell expressed, developmentally downregulated; SUMO, small ubiquitin-like modifier.

responsible for Ub/Ubl conjugation and deconjugation (Table 2). Many of these genes encode multiple splice variants, thereby increasing the diversity of enzyme families regulating Ub/Ubls. There are a multitude of E3s, reflecting their role as specificity determinants, an intermediate number of E2s, and few E1s, which are redundant to multiple pathways. The Ub/Ubl system is modular, as each E2–E3 pair and DUB recognizes a distinct set of cellular substrates. For example, the E2–E3 pair UbcH7–E6AP ubiquitylates p53, whereas the E2–E3 pair UbcH7–c-Cbl ubiquitylates Syk kinase (Ubc; Ub carrier protein). This example illustrates that the same E2 in conjunction with different E3s recognizes distinct substrates.

E1: Ub/Ubl activating enzymes

E1s comprise either a single polypeptide or a heterodimer that contains a Thif domain (typically containing a nucleotide binding region), a Uba domain and a catalytic cysteine [2]. There are 13 human genes (Table 2) that encode one-or-more of these motifs. Evidence to date suggests that each E1 activates a single Ub/Ubl; conversely, each Ub/Ubl is activated by a single E1. For example, the E1 monomer Uba1 activates ubiquitin, whereas the E1 dimer Uba3–APPBP1 activates the Ubl NEDD8.

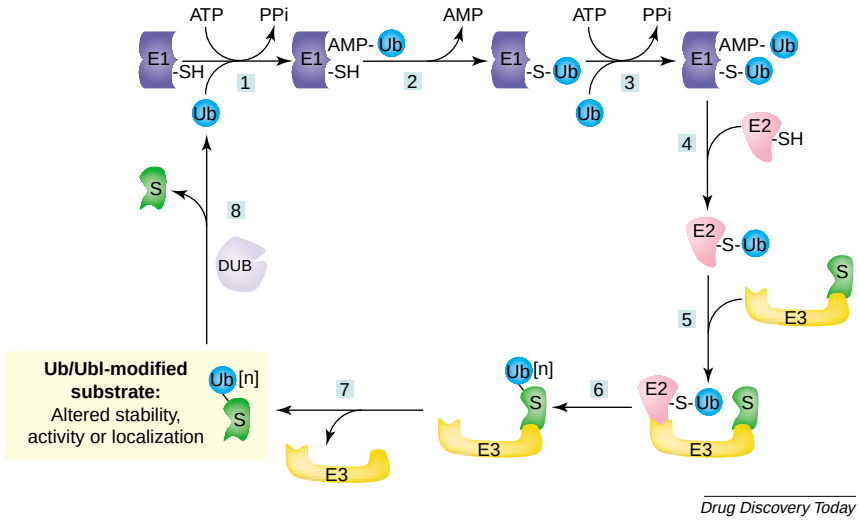

Figure 1. The ubiquitin (Ub) or ubiquitin-like (Ubl) pathway enzymatic cascade. The Ub/Ubl conjugation cascade (steps 1–6) is initiated with the hydrolysis of ATP by E1, and formation of a Ub- or Ubl-AMP intermediate (step 1). The Ub/Ubl is then transferred to the E1 active site cysteine generating an Ub–E1 or Ubl–E1 thioester bond (step 2). A second Ub/Ubl is charged by a second round of ATP hydrolysis (step 3), resulting in transfer of the thioester-linked Ub/Ubl from E1 to E2 active site cysteine (step 4). The Ub–E2 or Ubl–E2 thioester intermediate then interacts with an E3 to which a specific substrate is bound (step 5). This complex promotes the formation of an isopeptide linkage between the Ub/Ubl and the substrate (step 6). Progressive rounds of ubiquitylation (steps 5 and 6) result in the formation of multimeric isopeptide-linked chains (indicated by Ub[n]). Subsequently, Ub- or Ubl-modified substrates are released from the E3 (step 7). These modified substrates exhibit altered stability, activity or localization. De-ubiquitylating enzymes (DUBs) reverse this reaction by hydrolyzing Ub/Ubls from the substrate (step 8). Abbreviation: S, substrate.

Table 2. Ubiquitin or ubiquitin-like enzyme diversity[a]

Class	Sub-class	Genes	Isoforms[b]
Ub or Ubl		11	11
E1		13	15
E2	Ubc	37	63
	Uev	12	14
E3	RING	270	438
	PHD	86	137
	HECT	28	43
	U-box	7	13
DUB	USP	63	97
	ULP	7	11
	JAMM	12	18
	UCH	4	10

[a]Hidden Markov modeling and Gibb's sampling was used to search SMART, GenBank and other genomic databases to identify putative Ub or Ubl regulatory molecules.
[b]Isoform number includes total number of alternatively spliced transcripts that give rise to distinct protein products.
Abbreviations: DUBs, de-ubiquitylating enzymes; E1, Ub/Ubl-activating enzyme; E2, Ub/Ubl-carrier protein; E3, Ub/Ubl-protein ligase; HECT, homologous to E6-AP carboxyl terminus; JAMM, Jab1/MPN domain metalloenzyme; PHD, plant homeodomain; RING, really interesting new gene; SUMO, small Ubl modifier; Ub, Ubiquitin; Ubcs, ubiquitin carrier proteins; Ubl, ubiquitin-like; UCH, ubiquitin C-terminal hydrolases; Uevs, ubiquitin enzyme variants; ULP, Ubl-specific protease; USP, Ub-specific proteases.

E2: Ub/Ubl carrier proteins

The E2s typically comprise a single polypeptide and are defined by the presence of a Ubc domain. There are 49 E2 genes in the human genome (Table 2). The majority of E2s (37) have a conserved catalytic cysteine involved in thioester bond formation with Ub/Ubl. However, 12 E2 variants (Uevs) lack the catalytic site cysteine and can act by forming heterodimers with E2s containing functional active sites. These heterodimeric E2s exhibit unique biochemical activities. For example, the Ubc13–Uev1 heterodimer promotes the formation of lysine-63-linked multi-ubiquitin chains that regulate cell-signaling molecules [3,4], but not the formation of canonical lysine-48-linked Ub chains involved in protein degradation.

E3: Ub/Ubl protein ligases

E3s are the primary determinant of substrate specificity and represent the largest and most diverse class of Ub/Ubl regulatory enzymes. The human genome encodes 391 potential E3s, as defined by the presence of HECT, RING finger, PHD or U-box domains (Table 2). These domains mediate the interaction of the E3 with the E2 (see [5] for review). E3s encompass a broad spectrum of molecular architectures ranging from large multimeric complexes (e.g. anaphase promoting complex or APC), in which E2 binding, substrate recognition, and regulatory functions reside in separate subunits, to relatively simple single component enzymes (e.g. MDM2 or murine double minute) in which all necessary functions are incorporated into one polypeptide.

De-ubiquitylating enzymes

The 77 human genes encoding DUBs comprise several cysteine or metalloprotease families. These enzymes either process Ub/Ubl precursors, remove Ub/Ubl from substrates, or both. The cysteine protease family of DUBs share extensive amino acid conservation in the vicinity of their catalytic cysteine, histidine and aspartate residues, and can be classified as: (1) ubiquitin-specific proteases (USP) or ubiquitin proteases (UBP), which cleave the C-terminus of Ub/Ubl from either substrate proteins or from adjacent Ub/Ubl subunits within a multimeric chain; (2) C-terminal hydrolases (UCH), which process peptide bonds in ubiquitin precursors; or (3) SUMO (small Ubl modifier) proteases (ULP), which remove SUMO from substrate proteins and process the nascent SUMO to its mature form. USPs, UBPs and ULPs often contain large flanking motifs that recognize specific substrates [6]. The metalloprotease family of DUBs is defined by the recently described JAMM motif [7], and accounts for the de-ubiquitylating and deNEDDylating activities (removal of NEDD protein) associated with the proteasome lid and COP9 signalosome, respectively.

Consequences of ubiquitin conjugation

The consequences of Ub/Ubl conjugation are numerous and varied (Table 1). The best characterized consequence of Ub/Ubl modification is the targeting of substrates to the proteasome by lysine-48-linked multi-ubiquitin chains. This ubiquitin–proteasome pathway serves as a mechanism for the directed proteolysis of intracellular regulatory and signaling molecules, as well as for the turnover of aberrant or misfolded proteins. In contrast to lysine–48-linked ubiquitin chains, lysine-63-linked chains or monomeric ubiquitin serve to regulate signaling [8], DNA repair [9,10] and intracellular protein localization [11], but do not initiate proteasome-mediated protein turnover. Protein modification by the Ubls NEDD8, SUMO, ISG15 and Apg8/12 regulate E3 activity, protein localization, signal transduction, and autophagy, respectively.

Therapeutic opportunities

Ub/Ubls regulate diverse biological pathways and therefore promise an array of novel targets in the treatment of multiple disease mechanisms. The following sections provide examples of Ub/Ubl regulatory enzymes that act in

therapeutically relevant pathways such as oncology, inflammation, metabolism, viral disease and CNS disorders.

Oncology

The ubiquitin–proteasome system has been validated as a therapeutic target in oncology by positive clinical results with the proteasome inhibitor Velcade®. Velcade® has recently been approved for the treatment of multiple myeloma [12]. It is a dipeptidyl boronic acid inhibitor of the chymotrypsin-like activity of the proteasome [12], and probably inhibits the degradation of one-or-more factors that suppress tumor growth. However, proteasomal inhibition will affect multiple pathways in non-tumor cells, which can lead to toxicity. It follows, therefore, that selective inhibitors of specific Ub/Ubl regulatory enzymes will provide an opportunity for more selective manipulation of these pathways that might result in fewer adverse side effects.

Ub/Ubl conjugation regulates multiple pathways relevant to oncology, including cell-cycle progression, growth factor signaling, DNA repair, and apoptosis (Table 3). For example, the Skp1–Cullin1–F-box (SCF) RING finger E3 directs the multi-ubiquitylation and subsequent degradation of G1/S cell-cycle regulators p27 and cyclin E (reviewed in [13]). Mitotic progression is regulated by the anaphase promoting complex/cyclosome (APC/C), a RING finger E3 that directs the degradation of key mitotic regulators, including securin and cyclin B (reviewed in [14]). Furthermore, the steady-state levels of the tumor suppressor protein p53 are regulated by the RING finger E3, MDM2 [15], and by the HECT domain E3, E6AP [16]. Inhibition of SCF, APC or MDM2 is expected to be anti-proliferative. As evidence of their importance in oncogenic transformation and tumorigenesis, many components of the Ub/Ubl conjugation machinery are dysregulated, amplified or mutated in various cancers, and/or correlate with a poor prognosis. For example, skp2, a substrate adaptor in the SCF complex, is an oncogene that is overexpressed in cancer of the prostate, breast, and lung, among others [17–19]. As many tumor cells appear to rely on dysregulated Ub/Ubl pathways, these observations suggest the existence of a therapeutic window.

Inflammation

The Ubl system regulates general inflammatory cytokine signaling cascades, such as those leading to NF-κB activation, and more specific pathways such as those originating from the B- and T-cell receptors (Table 3). NF-κB activation is dependent on two RING finger E3s, TRAF6 and SCF. TRAF6, in conjunction with a heterodimeric E2 (Uev1–Ubc13), catalyzes the formation of lysine-63-linked ubiquitin chains, which promote IκB phosphorylation [8,20]. Phosphorylated IκB is then recognized and ubiquitylated by the SCF, resulting in IκB degradation and NF-κB activation. Hence, ubiquitylating enzymes regulate at least two steps in this crucial inflammatory pathway.

Ub/Ubls also regulate other transcription factors. The JAK–STAT pathway is potentiated by ISG15 modification of JAK1 [21], and suppressed by the removal of ISG15 by the Ubl protease, UBP43 [22,23]. UBP43 is selectively expressed in macrophages and cells of monocytic lineage, and its expression is upregulated in response to the bacterial cell-wall component, lipopolysaccharide [24]. This suggests that UBP43 could play a crucial role in the immune response to bacterial pathogens.

Metabolism

Ub/Ubl regulatory enzymes play a key role in skeletal muscle atrophy, cholesterol biosynthesis and bone metabolism (Table 3). Muscle atrophy is an important morbidity factor in diseases such as renal failure, diabetes and denervation. The E3 substrate adaptor Atrogin-1–MAFbx (muscle atrophy F-box) and the RING finger E3 MuRF (muscle ring finger) are upregulated during skeletal muscle wasting [25,26]. Consistent with these data, mice deficient in either Atrogin-1–MAFbx or MuRF showed resistance to muscle atrophy [26]. Another metabolic mechanism regulated by the Ub/Ubl pathway is the rate-limiting step in cholesterol biosynthesis, which is catalyzed by HMG-CoA reductase (HMGR). Levels of HMGR are controlled by the HMG-CoA reductase degradation (HRD) complex, which comprises the RING finger E3, Hrd3p, and the E2, Ubc7p [27,28]. Enhancing HRD activity would be an appropriate strategy for treating hypercholesterolemia [29]. Other disease areas that involve the previously mentioned RING finger E3 TRAF6 are osteoporosis and bone metastasis. Mice deficient in TRAF6 exhibit osteopetrosis or overly dense bone, owing to a defect in osteoclast differentiation or activation, resulting in decreased bone re-sorption [30].

Viral disease

Many human pathogenic viruses have evolved the ability to suppress host immune responses by downregulating key antigen-presentation molecules such as the major histocompatibility complexes (MHC), or co-stimulatory molecules such as B7 (reviewed in [31]). Kaposi sarcoma-associated virus (KSHV) has been shown to express two gene products, MIR1 and MIR2 (modulator of immune recognition), which are responsible for MHC class I internalization and degradation in the lysosome. MIR1 and MIR2 function as PHD domain E3s that catalyze ubiquitylation of the cytoplasmic tail of MHC class I molecules, resulting in their internalization and down-regulation. Importantly, other herpes viruses and poxviruses contain MIR homologs in their genomes, suggesting that this

Table 3. The ubiquitin system in disease pathways[a]

Therapeutic area	Ub regulatory protein	Class	Substrate(s)	Function	Refs
Oncology	APC	E3 (RING)	Securin, cyclin B	Mitosis	Reviewed in [14]
	UbcH10	E2			
	SCF	E3 (RING)	p27	G1/S checkpoint	Reviewed in [13]
	Skp2	SCF adaptor			
	Cdc4	SCF adaptor	Cyclin E	S Phase, DNA replication	
	Ubc12	E2 (for NEDD8)	Cullin family	Regulates SCF function	[69]
	Uba3/APPBP1	E1 (for NEDD8)			
	MDM2	E3 (RING)	p53	G1/S checkpoint	[15]
	Pirh2	E3 (RING)	P53	G1/S checkpoint	[70]
	E6AP	E3 (HECT)	p53	G1/S checkpoint	[16]
	SMURFs	E3 (HECT)	SMADs	TGF-β signaling	[71]
	CHFR	E3 (RING)	Plk1	G2/M checkpoint	[72]
	EFP	E3 (RING)	14–3-3σ	Cell cycle regulation	[73]
	Ubc8	E2			
	Ubp-M	UBP	Histone	Histone de-ubiquitylation	[74]
	IAPs	E3 (RING)	Auto-ubiquitylation	Apoptosis	[75]
	Rad5	E3 (RING)	PCNA	Damage-induced DNA repair	[76]
	Rad6	E2			
	Rad18	E2			
	Ubc13/Mms2	E2			
Inflammation	TRAF2, 5, 6	E3 (RING)	Auto-ubiquitylation	NF-κB, AP-1, c-Src/PI3K activation	[4,8,20]
	Ubc13/Uev1a	E2			
	SCF	E3 (RING)	IκB	NF-κB activation	Reviewed in [13]
	β-TRCP	SCF adaptor			
	UBP43	UBP (for ISG15)	Unknown	Negative regulator of JAK–STAT signaling	[22]
	DUB-1	UBP	Unknown	B-cell-selective cytokine-inducible DUB	Reviewed in [77]
	DUB-2	UBP	Unknown	T-cell-selective cytokine-inducible DUB	Reviewed in [77]
Metabolism	Hrd1p	E3 (RING)	HMGR	ERAD, degradation of HMGR	[27–29]
	Ubc7p	E2			
	SCF	E3 (RING)	Unknown	Skeletal muscle atrophy	[25,26]
	Atrogin-1/MAFbx	SCF adaptor			
	MuRF1	E3 (RING)	Unknown		[26]
	TRAF6	E3 (RING)	Auto-ubiquitylation	Osteoclast activation	[30,78,79]
Viral disease	MIR1/MIR2/c-MIR	E3 (PHD)	MHCI, B7.2, ICAM1	Immune evasion	Reviewed in [31]
	TSG101	E2 (variant)	Unknown	Viral budding, immune evasion	Reviewed in [80]
CNS disorders	Parkin	E3 (RING)	α-synuclein, synphilin-1, CDC rel-1, Pael-R	Misfolded-protein stress response	Reviewed in [34]
	UCH-L1	E2 (variant)	Peptide-ubiquityl amides		[38]
	APP-BP1	E1	Cullin	Neurotoxicity	[81]

[a]These examples do not constitute a comprehensive list of potential Ub- or Ubl-related therapeutic opportunities, but rather provide representative coverage of both therapeutic areas and enzyme target classes.

Abbreviations: β-TRCP, β-transducin repeat-containing protein; AP-1, activator protein-1; APC, anaphase promoting complex; APP-BP1, Alzheimer's precursor protein-binding protein-1; Cdc, cell division cycle; CHFR, checkpoint with forkhead and ring finger domains; c-Src, cellular rous sarcoma; DUB, de-ubiquitylating enzyme; E1, Ub/Ubl-activating enzyme; E2, Ub/Ubl-carrier protein; E3, Ub/Ubl-protein ligase; E6AP, E6-associated protein; EFP, estrogen-responsive finger protein; ERAD, endoplasmic reticulum-associated protein degradation; HMGR, HMG-CoA reductase; Hrd1p, HMG-CoA reductase degradation 1p; IAPs, inhibitor of apoptosis; MDM, murine double minute 2; MIR, modulator of immune recognition; Mms, methyl methanosulfate sensitive; MuRF1, muscle ring finger 1; PCNA, proliferating cell nuclear antigen; Pirh2, p53-induced protein with a RING-H2 domain; Plk1, Polo-like kinase 1; Rad, radiation sensitive; SCF, Skp1-Cul1-F-box; Skp2, S-phase kinase-associated protein 2; SMADs, similar to mother's against decapentaplegic; SMURFs, Smad ubiquitin regulatory factors; TGF-β, transforming growth factor-β; TRAF, TNF receptor associated factor; TSG, tumor susceptibility gene; Uba, Ub-activating enzyme; Ubc, Ub-carrier protein; UBP, Ub protease; UCH, Ub C-terminal hydrolase; Uev, Ub enzyme variant.

mechanism is conserved in immune evasion. Viruses can also use the host ubiquitin machinery to complete their life cycles. Tsg101 is a UEV normally involved in vesicular transport [11]. Recent data suggest that the late domains of the Gag proteins of HIV-1 and Ebola virus recruit Tsg101 to induce particle budding from the plasma membrane [32]. A short sequence in the Gag protein interacts with Tsg101 via a hydrophobic cleft, suggesting that this interaction might be amenable to small-molecule intervention [33].

CNS disorders

Dysregulation of ubiquitin-mediated protein degradation and the accumulation of aggregated neurotoxic proteins play a role in a variety of neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD). Genetic analysis of familial forms of these disorders has implicated several Ub regulatory enzymes in disease pathogenesis (reviewed in [34]). Loss-of-function mutations in the *Parkin* gene, which encodes a RING finger E3, have been linked to autosomal recessive PD [35]. A majority of these mutations resides in the RING finger domains and prevent the ubiquitylation and degradation of several substrates, including glycosylated α-synuclein [36] and Pael-R [37], both of which form neurotoxic aggregates when overexpressed. Another example of a Ub regulatory enzyme involved in Parkinson's disease is the DUB, UCH-L1. UCH-L1 is abundantly expressed in the brain (1–2%), and promotes protein degradation by liberating ubiquitin conjugates. A missense mutation (I93M) that decreases UCH-L1 protease activity was discovered in a subset of patients with autosomal-dominant PD [38]. Conversely, a reduced risk of PD was associated with a S18Y mutation [39]. This mutation, which lies outside the active site, might inhibit the formation of protein aggregates by suppressing the recently revealed lysine-63 ubiquitin ligase activity of UCH-L1 [40].

Ub/Ubl regulatory enzymes: structural biology and drug discovery

Considerable progress has been made in our understanding of E1, E2, E3, DUB structures and catalytic mechanisms. The following sections outline the structural and enzymatic properties of each class of Ub/Ubl regulatory enzyme, and emphasize how these findings can facilitate the discovery and characterization of inhibitors. Small-molecule inhibitors for each class of Ub/Ubl regulatory enzymes are outlined, in addition to their biochemical and biological mechanisms of action.

E1

The first solved structure for an E1 enzyme was that of Uba3-APPBP1. This structure has led to a model for the sequential steps of NEDD8 adenylation, thioester formation and transfer to E2 [2]. The Uba3-APPBP1 comprises two distinct domains – an adenylation domain and a catalytic cysteine domain – that flank a pair of large clefts, which provide binding sites for NEDD8 and E2. At the base of one of the clefts is an ATP-binding pocket that serves as the NEDD8 adenylation site. One approach to E1 inhibition would be to compete with ATP binding, in a similar way conceptually to that of kinase inhibitors [41]. An example of such an inhibitor is the non-hydrolysable ubiquitin-adenylate peptide analog, which is ATP competitive and inhibits the E1 with a K_i of ~50 nM [42]. The only described small-molecule inhibitor of E1 (IC_{50} ~40 μM) [43] is the mushroom metabolite panepophenanthrin; however, its mechanism of action has not yet been described.

E2

Several E2 structures have been solved, both individually [44] and in complex with binding partners [3,4,45–48]. These structures have provided insight into the architecture of the catalytic site and the nature of the interactions of E2 with Ub/Ubl [47], E1 [2], an E2 variant [3,4], E3s [45,46], and substrate [48]. All E2s exhibit a highly conserved globular fold with a shallow groove flanking the active site cysteine. An NMR-based model of the E2-ubiquitin thioester intermediate indicates that the C-terminal tail of ubiquitin is positioned in this groove [47]. Structure-based drug design should aid in the design of small molecules by exploiting the active-site groove present in E2s.

E3

Recent crystal structures of the HECT domain E3s, E6-AP [45] and WWP1-AIP5 [49], and the RING finger E3s, c-Cbl [46] and SCF [50], provide insight into the potential for targeting these E3s with small-molecule inhibitors. The elongated L-shaped structure of the E6-AP complexed with UbcH7 (E2) reveals a 41 Å gap between the E6-AP catalytic cysteine and the E2 catalytic cysteine. This observation suggests that a large conformational change is necessary to transfer ubiquitin from E2 to the E6-AP catalytic cysteine [45], a prerequisite for ubiquitin ligation to substrates catalyzed by HECT-domain E3s. Further dramatic evidence of conformational change comes from structural and mutagenesis studies implicating the hinge loop of the HECT domain E3 WWP1/AIP5, which rotates to orientate the substrate-binding domain in proximity to the E2 binding site [49]. In addition, the crystal structure of c-Cbl, a RING finger E3, suggests that significant conformational changes are required to bridge a distance of 60 Å between the E2 catalytic cysteine and the ubiquitylated substrate [46]. The requirement for conformational changes is further

highlighted by the crystal structure of the multi-subunit SCF. Similar to the RING finger E3 c-Cbl/E2 co-structure, the distance between residues of the substrate-binding site and the E2 active site cysteine is ~50 Å. It is possible that small-molecule inhibitors could exploit these conformational changes by stabilizing an inactive state via a mechanism of action similar to that of Gleevec® [51].

The E2–E3 interface provides another potential opportunity for small-molecule interference. The E6-AP and c-Cbl structure highlight a hydrophobic groove into which conserved loops of the E2 bind [45,46]. These loops contain specificity determinants for E2–E3 interactions; thus, inhibitors that recognize this interface could be designed to inhibit specific E2–E3 interactions.

Finally, targeting allosteric sites might be a viable strategy for inhibiting E3 activity. This is highlighted by the E3 Ubr1, which mediates protein degradation by the 'N-end rule' pathway. Ubr1 exists in an auto-inhibited state that can be released by dipeptides [52]. This raises the possibility that small allosteric effectors could regulate other E3s. Such effectors or their binding sites could be exploited for the development of small-molecule therapeutics.

Small-molecule inhibitors of E3s fall into two general categories: (1) inhibitors of E3–substrate interactions [53–56], and (2) ligase activity inhibitors [57–59]. Examples of E3-substrate-binding inhibitors include p53–MDM2 interaction antagonists. The co-crystal structure of a p53 peptide (residues 15–29) bound to the N-terminal domain of MDM2 (residues 17–125) reveals a deep hydrophobic cleft in MDM2 that binds a p53 amphipathic α-helix [60]. This structure was used to design benzodiazepine analogs [53] and polyaromatic compounds [56] that disrupt the p53–MDM2 interaction. One such polyaromatic compound was shown to activate the p53 pathway and induce cell death [56]. High-throughput screening is another approach that has been used to identify p53–MDM2 antagonists. This approach led to the discovery of the fungal cyclic peptide chlorofusin [54] (IC_{50} ~4.6 μM) and chalcone derivatives [55] (IC_{50} ~117 μM), which disrupt the p53–MDM2 interaction. These studies validate the E3–substrate interaction site as a target for small-molecule inhibition of substrate ubiquitylation.

The first described small-molecule inhibitors of E3 ligase activity were the mono- and bi-functional phenylarsenoxides, whose activities include inhibition of protein degradation in cell-free systems (IC_{50} ~4–20 μM) [57]. Bifunctional phenylarsenoxides react irreversibly with vicinal thiol groups and inhibit the N-end rule RING finger E3, E3α (IC50 ~53 μM). Another example of an irreversible E3 inhibitor is a tetrazole compound (Ro106–9920) that blocks the ubiquitylation of IκBα in a biochemical system (IC_{50} ~2.3 μM) [58]. In cells, Ro106-9920 blocks IκBα degradation and NFκB-dependent cytokine production (IC_{50} ~0.7 μM). In two rat models for acute inflammation, Ro106-9920 lowers TNF-α in serum, and prostaglandin E2 in lung. Importantly, compounds with three distinct chemical structures, belonging to the benzsulfonamide (IC_{50} ~12.7 μM), urea (IC_{50} ~14.2 μM) and imidazolone (IC_{50} ~3.2 μM) classes, were recently shown to inhibit the MDM2 ubiquitylation of p53 [59]. These compounds are reversible inhibitors that do not interfere with p53- or E2-binding to MDM2, suggesting that they could be acting allosterically. These compounds might work by locking MDM2 in an inactive state, taking advantage of the conformational changes required for E3 activity (see earlier).

De-ubiquitylating proteases

Crystal structures of the several de-ubiquitylating cysteine proteases bound to their Ub/Ubls, reveal that they share a common active-site geometry, while possessing divergent overall topologies [61–63]. The catalytic triad Cys-His-Asp and residues forming the oxyanion hole are conserved among de-ubiquitylating cysteine proteases [63]. The structure of the UBP HAUSP shows that ubiquitin binding realigns the catalytic cysteine and histidine to enable productive hydrogen bonding, and leads to the formation of the oxyanion hole [63]. Sequence alignments suggest that this structural rearrangement probably occurs in all UBPs [63], distinguishing them from UCHs (i.e. Yuh1), which show less-dramatic catalytic site rearrangement upon substrate binding [61].

Peptide-based strategies in combination with X-ray crystallography and modeling studies have been used successfully in the design of potent small-molecule protease inhibitors [64]. Furthermore, several cysteine protease inhibitors, including a rhinovirus 3C protease inhibitor, a caspase I/ICE inhibitor, a caspase K inhibitor and a cathepsin S inhibitor [64], are in clinical trials or preclinical development. Reported peptide inhibitors of DUBs comprise the entire ubiquitin protein, and have therefore provided little structure–activity relationship information with regard to prime and non-prime P-site preferences [65]. The only reported non-peptide small-molecule DUB inhibitor is D12-prostaglandin J2, which inhibits ubiquitin peptidase activity in cells (IC_{50} ~30 μM) and causes cellular accumulation of p53, and cell death [66]. Related compounds containing a cross-conjugated alpha,beta unsaturated dienone substituent, and two sterically accessible beta-carbons (NSC-302979, IC_{50} ~15 μM; DBA, IC_{50} ~20–40 μM), were also able to act as Michael-acceptor irreversible inhibitors of ubiquitin proteases [67]. Michael acceptors can serve as a useful starting point in the design of selective and potent DUB inhibitors.

Concluding remarks

The success of the proteasome inhibitor Velcade® in the treatment of several types of cancers demonstrates that the Ub/Ubl system provides a novel pathway for pharmacological intervention. Numerous components of the Ub/Ubl system have been validated as targets for oncology, inflammation, metabolism, viral infection and CNS disorders. Rapid progress in understanding the biochemistry of the Ub/Ubl system offers insight into strategies for interfering with these enzymes. Several small molecules have been identified that interfere with the Ub/Ubl cascade. The challenge that remains is to discover more potent and selective small-molecule inhibitors with suitable pharmaceutical properties. Importantly, because of the modularity of the Ub/Ubl pathways, and homologies within Ub/Ubl enzyme families, identification of a pharmacophore that selectively inhibits one Ub/Ubl regulatory enzyme should enable the rapid design of inhibitors of other therapeutically important members of this target class.

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