HHS Public Access

Author manuscript

Drug Target Rev. Author manuscript; available in PMC 2016 January 27.

Published in final edited form as:

Drug Target Rev. 2015 ; 2(3): 60–64.

Ubiquitin-Specific Proteases as Druggable Targets

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The Ubiquitin Proteasome System (UPS)

In much the same way as kinases and phosphatases attach and remove, respectively, phosphate groups from proteins to modulate their activity, there are a series of enzymes (E1, E2, E3) that add one or more ubiquitins onto a protein, as well as enzymes that remove them (deubiquitinases), thereby regulating their activity, location and/or rate of degradation [1]. Ubiquitin is a 76 amino acid protein that is added onto lysines in the target protein through the C-terminal residue of ubiquitin: one can be added (monoubiquitination) or as many as ten can be added (polyubiquitination). For polyubiquitinated proteins these can be linear or branched chains of ubiquitin, with the complexity of branching reminiscent of the complexity of protein glycosylation [1, 2]. Ubiquitin contains seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63), and the N-terminal methionine that can be ubiquitinated. Common linkages include isopeptide bonds through the K48 and K63 on ubiquitin. K48 polyubiquitinated proteins are often targeted to the proteasome for protein degradation and recycling of the ubiquitin [1]. Ubiquitination of a protein can also control its activity/function, such as K63 linkages that regulate DNA damage response or cell signaling [1, 3].

Interest in the ubiquitin-proteasome system (UPS) as a target for the treatment of disease, such as cancer, neurodegeneration and autoimmune disease, has increased steadily since the approval of the proteasome inhibitors bortezomib and carfilzomib [4]. These drugs are used to treat hematological malignancies, such as multiple myeloma and mantle cell lymphoma. As yet this drug class has not been approved for solid tumors. Over time, resistance has begun to be observed for this class as well as side effect concerns, raising interest in targeting enzymes upstream of the UPS, such as the deubiquitinases and the E3 ligases, which may offer the possibility of more selectivity and fewer side effects [5].

Deubiquitinases (DUBs)

Deubiquitinases (DUBs) are upstream of the proteasome and have drawn interest as drug targets. The approximately 100 DUB enzymes can be grouped into five main classes, comprising the cysteine proteases ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), and Machado-Joseph domain proteases (MJDs) and the metalloproteases JAB1/MPN/MOV34 (JAMM) [6]. The USPs are

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the largest family of DUBs, with approximately 56 members in humans, and are the focus of this review. In addition to the study of USPs as targets for drug discovery, there is much basic biology yet to be uncovered for this class of enzymes. Questions of substrate specificity, DUB redundancy and linkage selectivity have yet to be fully addressed for the majority of this enzyme class. To date both linkage selective DUBs, such as Cezanne which is specific for Lys11 linkages [7], and non-selective DUBs, such as USP2 which can cleave K48, K63 and linear, have been identified [6, 8–11]. As has been seen for the kinase field [12], there is likely room for both selective and nonselective inhibitors as drugs and tool compounds.

The catalytic site of USPs contain a triad with a catalytic cysteine and nearby histidine and asparagine/aspartate to help poise the cysteine for nucleophilic attack. In addition to a USP domain various USPs have additional domains, such as ubiquitin-like domains and zinc-finger domains [6]. Additionally, several of the USPs function as complexes, such as USP1/UAF1, USP12/UAF1/WDR20 and USP46/UAF1/WDR20 [13, 14]. Several USPs have crystal structures reported in the PDB, including USP2 (PDB ID 2HD5), USP5 (PDB ID 3IHP), USP7 (PDB ID 4M5W), USP14 (PDB ID 2AYN), CYLD (PDB ID 2VHF), and USP21 (2Y5B). USPs in an analogous way to kinases also seem to have active and inactive conformations with active conformations observed upon ubiquitin binding, although also like kinases not every USP has been observed in both conformations [6, 15].

Assay Technologies to Interrogate DUBs

In order to identify DUB inhibitors, DUB substrates and DUB inhibitor selectivity, a variety of assay reagents have been identified and utilized in high-throughput screening (HTS) campaigns as well as lower throughput gel and western blot experiments (Figure 1) [16–18]. The higher throughput methods generally involve an increase in luminescence or fluorescence upon cleavage that can be monitored on a plate reader [16]. Commonly used reagents are ubiquitin linked to a fluorophore through a linear linkage, such as Ub-AMC (Ub-7-amino-4-methylcoumarin) and Ub-Rhodamine110 (Figure 1A), which have been used for screening various USPs including USP1 (PubChem Assay Identifier (AID) 504865), USP2 (PubChem AID 493170) and USP14 (PubChem AID 449747). More recently, reagents have been created that contain an isopeptide linkage between a di-ubiquitin (Di-Ub) to more closely mimic the most common *in vivo* Ub linkage. One example of this type of assay involves using an internally quenched fluorescent reagent in which one Ub has a fluorophore and the other has a quencher that quenches the fluorophore when the two are in close proximity but not once the Di-Ub is cleaved (Figure 1B) [8]. Another method that has been utilized represents a coupled enzyme system. In one format, called Ub-Chop2, the ubiquitin is linked to an enzyme that is only active when released and thereby can produce a fluorescence enzyme product (Figure 1C); Ub-Chop2 has been used to identify inhibitors of USP2 in a large-scale screen (PubChem AID 463254). Additionally, having these reagents with different fluorescence excitation and emission wavelengths can help mitigate compound interference, which can be particularly problematic in the blue spectral region [19], such as for Ub-AMC, by allowing for orthogonal assay development. Lastly, when aminoluciferin is tethered to the DUB substrate Z-RLRGG it is not luminescent but upon cleavage by a DUB, its luminescence can be measured by a luminometer (Figure 1D), a

technique that was used for USP8 [20]. All of these reagents can be read out in kinetic mode which can help minimize assay interference from compound fluorescence. Additional methods for identifying DUB substrates, DUB inhibitors and DUB linkage preferences include mass spectrometry based methods and protein microarray methods [17, 18, 21].

Lower throughput methods that are important for confirmation of hits rely primarily on a change in mass upon Di-Ub cleavage or DUB binding. Ubiquitin aldehyde or ubiquitin vinyl sulfone are reagents that form covalent irreversible linkages to DUBs upon binding, leading to observable mass changes to the DUBs (Figure 1E). These have been visualized in western blots with antibodies to the individual DUB, such as USP1, or to ubiquitin, or to a tag, such as HA, placed on the ubiquitin, HA-ubiquitin vinyl sulfone [22]. Additionally, Di-Ub can be cleaved by a DUB and this new product can be visualized on a gel as a band with half the molecular weight of the substrate (Figure 1F), a technique that has been used for USP1 [23]. Tetra-ubiquitin molecules are becoming increasingly available that can also be used for this purpose. Having a diversity of assay technologies is important for identifying and validating inhibitors and studying the USP family.

USPs as Drug Targets

While the USP family as a whole is still widely unexplored, selected USPs have been the focus of intense research efforts both to uncover their physiological roles and to identify inhibitors [24, 25], with some of the highlights to date described below.

USP1 deubiquitinates PCNA and FANCD2, which are proteins important for DNA repair pathways and the Fanconi Anemia pathway, respectively [3]. USP1, which is active as a complex with UAF1 [13], can deubiquitinate PCNA, which upon DNA damage is ubiquitinated and thereby recruits translesion DNA polymerase eta. ML323 was developed as an inhibitor of USP1/UAF1 after a high-throughput screen using Ub-Rhodamine110 assay [26]. Additional compounds, pimozide, GW7647 and C527 were found to be sub μM inhibitors [22, 23].

USP2, found *in vivo* as the alternately spliced USP2a and USP2b, has a large number of identified substrates important in cancer including cyclin D1, MDM2, and fatty acid synthase (FAS) [27–29]. Cyclin D1, which can be overexpressed in cancer, was degraded in response to USP2-targeting siRNA leading to inhibition of cell cycle progression in cancer cells but not normal cells [28]. USP2 can also deubiquitinate MDM2, thereby destabilizing p53 [29]. USP2a was shown to help protect prostate cancer cells from apoptosis by deubiquitinating the antiapoptotic proteins MDM2 and FAS, which can be overexpressed in cancer [27, 30]. Indeed 44% of prostate tumors tested had overexpression of USP2a. 2-cyano-pyrimidines and -triazines have been identified as inhibitors of USP2 and UCH-L3 [31].

USP5 has been implicated in suppression of both p53 and FAS levels in melanoma cells. An inhibitor EOAI3402143, which had been optimized from WP1130 [32], was shown to recapitulate USP5 knockdown results and block melanoma growth in a mouse model [33]. Inhibition was able to overcome resistance to BRAF-targeting kinase inhibitors in melanoma [33]. This compound also inhibits USP9x, a drug target discussed below [34–36].

USP7/HAUSP (herpes virus-associated ubiquitin-specific protease) deubiquitinates MDM2 (an E3 ligase; also called HDM2), thereby destabilizing p53, and can be targeted by small molecule inhibitors [37]. Additional roles for USP7/HAUSP include deubiquitination of the tumor suppressors PTEN (phosphatase and tensin homologue deleted in chromosome 10) and FOXO4 (Forkhead box O), which favors their localization to the cytoplasm versus the nucleus limiting their transcriptional activity [38, 39]. It should be noted that PTEN is also deubiquitinated by USP13 [40]. USP7/HAUSP is overexpressed in cancer, such as prostate cancer. Inhibitors of USP7 include HBX 41,108 and P22077, which were identified from HTS campaigns [37, 41, 42].

USP8 (UBPY) knockdown in gefitinib-resistant NSCLC but not gefitinib-sensitive NSCLC leads to cell death, potentially providing an avenue to pursue when resistance to EGFR receptor kinase inhibitors develops [20]. This knockdown effect could be recapitulated with a USP8 inhibitor (9-ethyloxyimino-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile) [43] that showed efficacy in a mouse xenograft model. USP8 had previously been shown to be involved in EGFR degradation [44]. Additionally, USP8 mutations cause Cushing's Disease, a disease caused by pituitary corticotroph adenomas hypersecting adenocorticotropin (ACTH) [45]. The mutations cause a decrease in binding to 14-3-3 protein, increased cleavage of USP8 and an increase in DUB activity, which leads to increased recycling of EGFR increasing the levels of ACTH [45].

USP9x plays an important role in stabilizing beta-catenin [35], MCL1 [36] and SMAD4 [34], a component of the TGFβ signaling pathway. Each of these proteins is in its own right a target for cancer therapy. USP9x is one of the DUBs inhibited by WP1130 [32].

USP12 is active as a complex USP12/UAF1/WDR20 and has several substrates identified to date including the androgen receptor, which is important in prostate and some breast cancers [14, 46]. To date only one compound has been reported to inhibit USP12/UAF1/WDR20 and that is GW7647, which also inhibits USP1 [23, 47].

USP14 is associated with the proteasome and is one of the DUBs responsible for ensuring that ubiquitin is recycled rather than degraded by the proteasome, contributing to ubiquitin homeostasis. An inhibitor of USP14 was identified and shown to enhance the activity of the proteasome [48]. Inhibition of USP14 lead to accelerated proteasomal degradation of proteins involved in neurodegenerative diseases.

USP28 has a role in stabilizing the oncoprotein c-myc as discovered in a shRNA screen [49], but also is implicated in other functions that may lead to side effects [5]. For example, USP28 is important for the DNA damage response [50]. USP28 is highly expressed in colon and breast carcinomas and has been implicated in conferring stem-cell-like traits to breast cancer cells [51].

Perspective

Deubiquitinases (DUBs) have been a subject of increased interest of late as a potential novel drug target class, in turn spurring efforts to develop relevant activity assays. Most reagents used for DUB assays contain one Ub linked to a fluorophore or luminescent compound by a

linear linkage or two Ubs linked by an isopeptide linkage but polyubiquitinated proteins can have as many as 10 Ubs linked together in linear or branched conformations [1, 9]. Having additional assay reagents, such as tetraubiquitin, with > 2 ubiquitins as well as branched Ubs may be important for further understanding Dub linkage selectivity. It will be interesting to see the impact on HTS campaigns of utilizing the different available assay reagents to understand whether specific inhibitors exist targeting the different linkages and poly-ubiquitin reagents.

Screening a large panel size was important to obtain an accurate assessment of inhibitor selectivity for kinases [12], and it will be interesting to see whether the same will hold for DUB families, such as the USPs, or whether a small panel will accurately represent overall selectivity. There is a need for the development and execution of HTS assays and the discovery of molecule probes for all of the ~56 USPs in order to understand their biology. Once a critical mass of inhibitors are available, a broad selectivity profile will be important in order to understand whether highly selective modulators across the whole DUB class can be obtained as is the case for some kinase inhibitors, such as lapatinib for EGFR, which is selective across the whole kinome [12]. A given DUB, such as USP2, can have multiple substrates that it can deubiquitinate, so in addition to polypharmacology from inhibitors that hit more than one DUB, even a DUB-selective inhibitor may impact multiple cellular proteins and processes due to multiple substrates acted on *in vivo*. Additionally, redundancy within the USP family is already being observed with multiple DUBs impacting MDM2, p53, FAS and PTEN [27, 29, 33, 37, 38]. Thus, the DUB field seems poised for rapid development: in much the same way as the approval of Gleevec sparked fervor into the kinase field [52], having a DUB inhibitor approved would be expected to do the same for the deubiquitinase field.

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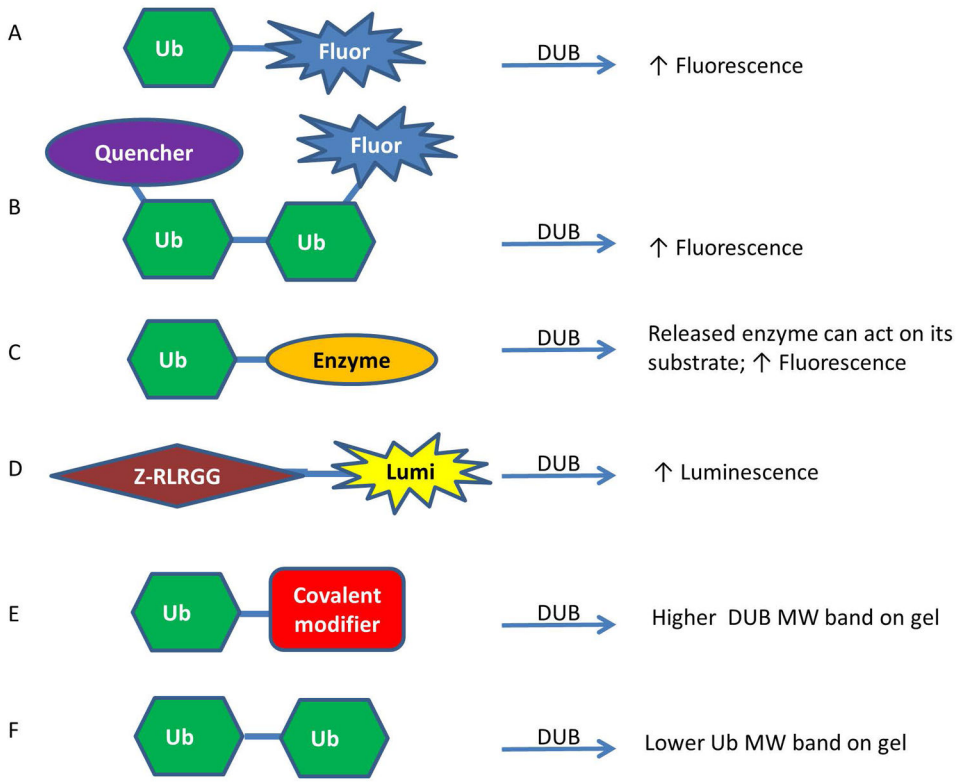

Figure 1. Representative assay formats used to study DUBs

A) Ubiquitin linked to fluorophore that fluoresces upon cleavage. B) Internally quenched fluorescence Di-Ub that fluoresces upon cleavage. C) When cleaved, the enzyme becomes active and can act on its substrate and form a fluorescent product. D) A generic DUB substrate can be tethered to a proluminescent molecule that becomes luminescent only when cleaved. E) Upon binding a DUB, the Ub is covalently attached and the MW shift can be monitored by gel assay. F) Di-Ub cleavage can be monitored by gel assay. Fluor=fluorophore; Lumi=luminescent compound; Ub = ubiquitin.