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Strategies for the Identification of novel inhibitors of deubiquitinating enzymes

Seth J Goldenberg, **Jeffrey L McDermott**, **Tauseef R Butt**, **Michael R Mattern**, and **Benjamin Nicholson**
Progenra Inc, 271A Great Valley Parkway, Malvern PA 19355, USA www.progenra.com

Abstract

Dysregulation of the ubiquitin-proteasome system (UPS) has been implicated in a wide range of pathologies including cancer, neurodegeneration, and viral infection. Inhibiting the proteasome has been shown to be an effective therapeutic strategy in humans; yet toxicity with this target remains high. Deubiquitinating enzymes (DUBs) represent an alternative target in the UPS with low predicted toxicity. Currently, there are no DUB inhibitors that have entered the clinic. To address this situation, Progenra has developed a novel assay to measure the proteolytic cleavage of ubiquitin or UBL (ubiquitin like protein) conjugates such as SUMO, NEDD8 or ISG15 by isopeptidases. Here we will discuss current platforms for detecting DUB inhibitors and underline the advantages and disadvantages of the approaches.

Keywords

Ubiquitin; deubiquitylase; deSUMOylase; deISGylase; deNEDDylase; isopeptidase; high-throughput screening

Introduction

The conjugation of Ubiquitin (Ub) and Ubiquitin-like (UBL) proteins is an important regulatory mechanism that is widespread in many biological processes [1]. The assorted diseases associated with these pathways make the pathway enzymes particularly interesting for therapeutic targets. The UPS has been validated with the approval of the proteasome inhibitor bortezomib for the treatment of multiple myeloma; however, significant toxicities were seen during clinical trials suggesting the need for more selective targets [2]. Ub- and UBL-isopeptidases represent a unique set of drug targets in the UPS that are responsible for removing ubiquitin and UBLs, such as, SUMO, NEDD8, and ISG15, from target proteins, thus affecting the targets fate [3]. To develop therapeutic agents that target isopeptidases we developed a readily quantifiable novel isopeptidase assay platform that is suitable for high-throughput screening (HTS). The assay platform consists of Ub or UBL fused to the reporter enzyme phospholipase A_2 (PLA_2). Isopeptidase activity releases PLA_2 that cleaves its substrate generating a signal that is linear with isopeptidase concentration and is able to discriminate DUB, deSUMOylase, deNEDDylase and deISGylase activities. The assay can be successfully employed to screen for inhibitors of isopeptidases.

Therapeutic targets in the Ubiquitin Proteasome System (UPS)

The approval of the proteasome inhibitor bortezomib (velcade) for the treatment of multiple myeloma validated targeting of the ubiquitin-proteasome pathway (UPS) for the treatment of cancer [4]. However, extended treatment with bortezomib is associated with toxicity and drug resistance, limiting its efficacy [2]. In contrast, therapeutic strategies that target specific

aspects of the ubiquitin-proteasome pathway upstream of the proteasome, would be predicted to have lower toxicity. While activating enzymes (E1) and conjugating enzymes (E2) are upstream of the proteasome one must be aware of the consequences of targeting them, as disruption of the E1 leads to cell cycle arrest [5] and E2s have been shown to be required for development [6]. Targeting the ubiquitin activating enzyme may be predicted to affect too many cellular functions for it to be tolerated by normal cells; yet targeting the Nedd8-activiating enzyme for inhibition has been reported to be successful in pre-clinical studies [7]. The mechanism of action is most likely through inactivation of the cullin-based E3 ligases, many of which play a crucial role in cell cycle checkpoints whose disruption would have a more immediate effect on rapidly dividing cancer cells.

E3 ligases, with only a limited number of substrates represent attractive drug targets in the UPS. One of the most interesting E3 targets is the Skp1-Cul1-Roc-Fbox Protein complex (SCF). The SCF complex consists of many variable F-box adaptor proteins each of which target only a few substrates for ubiquitination [8]. Two therapeutically relevant Fbox proteins are Skp2 [9] and β-TRCP [10], which play key roles in cell cycle progression. However, to inhibit these proteins one must disrupt a protein-protein interaction, which is considered a more difficult target than an enzymatic target.

Isopeptidases belong to five subfamilies that have been identified to date. Four of these five families are cysteine proteases, which have been shown to be good therapeutic targets. The ubiquitin C-terminal hydrolases (UCH); ubiquitin-specific proteases (UBP/USP); Machado-Joseph Domain (MJD); and ovarian tumor related (OTU) isopeptidases are cysteine proteases while the JAB1/MPN/Mov34 metalloenzyme (JAMM) motif dubs are Zn metaloproteases. 90 putative DUBs have been identified with 79 most likely being functional. There are also many UBL-isopeptidases that are good therapeutic targets [3,11].

The role of DUBs and ULPs in disease

Several isopeptidases have been implicated in disease [12], in particular cancer (refer to Table 1). For example, ubiquitin-specific protease 7, also known as herpesvirus-associated ubiquitin-specific protease (USP7/HAUSP), regulates the ubiquitination state of the RING-finger E3 ligase Mdm2 (and its human homolog Hdm2) [13]. Hdm2 targets the tumor suppressor p53 for ubiquitination and facilitates its degradation by the proteasome [14,15]. Many other RING-finger E3 ligases are capable of auto-ubiquitination; Hdm2 is no exception and auto-ubiquitinates resulting in its own proteolytic degradation [16]. However, Hdm2 also ubiquitinates p53 resulting in degradation of p53 via the proteasome. Initially, USP7 was believed to primarily deubiquitinate p53, increasing the level of p53 [17]. However, more recent genetic and biochemical studies have found that with respect to p53 and Hdm2, the primary target of USP7 is Hdm2 [13,18]. These data were corroborated by structural biology studies which revealed that Hdm2 and p53 recognize the tumor necrosis factor-receptor associated factor (TRAF) domain of USP7 in a mutually exclusive manner, but Hdm2 binds to the TRAF domain with a higher affinity than p53 [19].

While DUBs have received the most attention, proteases that deconjugate UBLs from their target proteins have also been linked to various pathophysiologies, as they are critical to cellular localization, transcriptional regulation, signal transduction pathways, and the regulation of some ubiquitin E3 ligases [20-24].

Current assay systems for DUBs and UBL-isopeptidases

Many assays currently in use rely on cleavage of linear Ub-fusions, which can be produced in *E.coli* (tetra-Ub, Ub-CEP52, Ub-GSTP1, Ub-DHFR, Ub-PESTc, etc.) or synthesized chemically [25-27]. For small scale analysis of isopeptidase activity, reaction products are

analyzed by gel electrophoresis, or are selectively precipitated and analyzed by liquid scintillation spectrometry. Gel-based procedures are labor intensive and expensive, and while scintillation counting approaches are quantitative and allow processing of larger numbers of samples than gel-based assays, they require centrifugation and recovery of supernatant. For HTS, a fluorogenic substrate, Ub-AMC (Ub-7-amino-4-methylcoumarin), has been employed in some cases, as well as a similar substrate, the tetrapeptide z-LRGG-AMC, which mimics the carboxyl terminus of ubiquitin [28]. A limiting factor with both of these fluorescent substrates is the fact that this small adduct cannot be hydrolyzed efficiently by the largest class of DUBs, Ub-specific protease (UBP/USP) class enzymes. Moreover, the excitation wavelength of Ub-AMC is in the UV range, which is known to excite a number of screening compounds and give rise to up to 20% false positives [29]. Fluorescence Resonance Energy Transfer (FRET) has also been developed for HTS screens [30]. Both AMC and FRET, however, suffer from the need for specialized custom reagents and equipment, as well as from difficulty in adapting to a multi-well plate format from which the endpoints can be read directly. In many of these approaches, expensive double-labeled or tagged substrates must be generated specifically for each assay (refer to Table 2 for comparison of current technologies).

An improved paradigm for detection of DUB and UBL-isopeptidase inhibitors, the Ubiquitin like protein-Phospholipase A_2 (UBL-PLA_2), Reporter Assay

While isopeptidases have been of interest for some time to our knowledge no compounds have entered clinical trials. One reason for this could be the assays employed in drug discovery campaigns. All of the previously described platforms are based on Ub linked to small chemical adducts and are not related to the physiologic target of most isopeptidases, mono- or poly-ubiquitin fused to a protein. To fulfill the need for a convenient and physiologically relevant assay that is suitable for high throughput screening, Progenra has developed an isopeptidase assay, based on the observation that most USPs can hydrolyze linear Ub fusions (α-NH bond) as well as ε-NH_2-isopeptide linkages, that exploits the requirement of certain proteins for a free N-terminus to be active. This assay can be used for either DUBs or ULPs.

The UBL-PLA_2 assay is based on the concept that PLA_2 requires a free amino terminus to be catalytically active. PLA_2 cleaves phospholipids to produce lysophospholipids and free fatty acids and requires a free N-terminus for catalytic activity [31]. When PLA_2 is fused to a UBL it is inactive and cannot cleave it substrate. When a DUB or other UBL isopeptidase is present it cleaves the UBL from PLA_2, freeing PLA_2 to act on its substrate. There are a number of commercial substrates for PLA_2 including the fluorescent phospholipid 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β-BODIPY C_5-HPC) and 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3- phosphocholine (NBD C_6-HPC). The fluorescence response produced by these substrates occurs over a wavelength range that is better suited for drug discovery than that employed in the Ub-AMC assay, and it is amplified by the coupling of DUB catalytic activity to PLA_2, resulting in enhanced sensitivity. Importantly, the Ub/ UBL-PLA_2 fusion proteins represent more physiological substrates than the short carboxy terminal adducts exemplified by the commercially available AMC or TR-FRET reagents.

Future of DUB Therapies

To our knowledge, no DUB inhibitors have entered clinical trials. However, modulation of the DUB CYLD pathway with aspirin has been shown to be therapeutically viable in humans [32,33]. Progenra's proprietary assay technology has been used by multiple groups to screen more than 100,000 compounds to date with that number expected to double by the

end of 2008. Due to the increasing screening that is being performed against isopeptidases by ourselves and other researchers we anticipate that multiple DUB inhibitors will enter the clinic in the near future.

Abbreviations

(DUBs)	deubiquitinases
(HTS)	high-throughput screening
(PLA_2)	phospholipase A_2
(Ub)	Ubiquitin
(UBL)	Ubiquitin-like
(UPS)	Ubiquitin Proteasome System
(SCF)	Skp1-Cul1-Roc-Fbox Protein complex
(UCH)	ubiquitin C-terminal hydrolases
(UBP/USP)	ubiquitin-specific proteases
(MJD)	Machado-Joseph Domain
(OTU)	and ovarian tumor related
(JAMM)	JAB1/MPN/Mov34 metalloenzyme
(TRAF)	tumor necrosis factor-receptor associated factor
(UBP/USP)	Ub-specific protease
(Ub-AMC)	Ub-7-amino-4-methylcoumarin

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Table 1

Isopeptidases implicated in various diseases

Pathology	**DUBs**
Cancer	USP2a [34], USP7 (HAUSP) [13], CYLD [35], UCH-L1 [36], USP6 (Tre-2) [37],USP20 (VDU2) [38], USP8 (UBPY) [39], STAMBP (AMSH) [40]
Neurodegeneration	USP14 [41], Ataxin 3 [42], UCH-L1 [43]
Hematological	USP1 [44], DUB-1,DUB-2 [45]
Viral infection	$UL36^{USP}$ [46], HMWP (pUL48) [47], PLP2 [48]
Bacterial infection	SseL [49], ElaD [50]

Table 2

Comparison of current DUB assays.

Platform	Summary	Advantages	Disadvantages
Ub-AMC	Fluorogenic substrate fused to ubiquitin that only fluoresces once it is cleaved from ubiquitin by an isopeptidase.	Sensitive reporter for the four enzymes in the UCH family of DUBs. Ub, SUMO, NEDD8, ISG15-AMC reagents commercially available	Excitation at 340nm is an unfavorable wavelength for drug discovery. A poor substrate for USPs such as USP2 core
Lanthascreen (TR-FRET)	Time resolved FRET based assay. The LanthaScreen™ DUB Substrate consists of an N-terminal YFP fusion of ubiquitin and a C-terminal extension of a terbium labeled cysteine residue. In the presence of a DUB, the Tb labeled C-terminal extension is cleaved from the substrate, resulting in a decrease in the TR-FRET signal.	Less susceptible to compound interference than AMC assay format. Sensitive reporter for the four enzymes in the UCH family of DUBs	Loss of signal assay. A poor substrate for USPs such as USP7 ISG15 Lanthascreen reagent not commercially available
Ub-PLA_2	UBL-PLA_2 consists of a linear fusion that is available for isopeptidase cleavage. Following isopeptidase activity, PLA_2 is free to act on its substrate giving a readily quantifiable fluorescent response.	More physiologically relevant substrate for most isopeptidases. Generates a robust signal within one hour. Fluorophors excited outside the UV range. Ub, SUMO, NEDD8, ISG15-PLA_2 reagents commercially available	Relative to assays with a small adduct at the carboxy terminus of ubiquitin, Ub-PLA_2 is a less sensitive reagent for measuring activity of the four enzymes in the UCH family.