Characterization of Selective Ubiquitin and Ubiquitin-Like Protease Inhibitors Using a Fluorescence-Based Multiplex Assay Format

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ABSTRACT

The reversible conjugation of ubiquitin and ubiquitin-like (UbL) proteins to protein substrates plays a critical role in the regulation of many cellular pathways. The removal of ubiquitin from target proteins is performed by ubiquitin proteases also known as deubiquitylases (DUBs). Owing to their substrate specificity and the central role ubiquitylation plays in cell signaling pathways, DUB are attractive targets for therapeutic development. The development of DUB inhibitors requires assays that are amenable to high-throughput screening and provide rapid assessment of inhibitor selectivity. Determination of inhibitor selectivity at an early stage of drug discovery will reduce drug failure in the clinic as well as reduce overall drug development costs. We have developed two novel assays, UbL-Enterokinase light chain and UbL-Granzyme B, for quantifying ubiquitin and UbL protease activity. In our quest to discover and characterize novel chemical entities, we have combined these assays with a previously developed assay in a multiplex format. This multiplex format allows for the detection of three distinct protease activities simultaneously, in a single well. We have demonstrated that the multiplex format is able to distinguish between selective and nonselective protease inhibitors. Specifically, we have used this assay format to characterize P022077, a selective ubiquitin-specific protease 7 inhibitor discovered at Progenra.

INTRODUCTION

Ubiquitin is a small protein that is covalently conjugated to specific lysine residues in target proteins, thereby regulating the target protein's function, localization, or stability.[1–3] In addition to ubiquitin, several ubiquitin-like (UbL) proteins have been described. SUMO (small ubiquitin-like modifer) is the founding member of the UbL protein family; others include ISG15 (interferon stimulated gene 15) and NEDD8 (neural precursor cell expressed developmentally down-regulated protein 8).[4] The reversible conjugation of ubiquitin and UbL proteins to target proteins regulates a plethora of cellular processes. All of these proteins utilize a similar enzymatic process for conjugation to target proteins. With respect to ubiquitin, the C-terminal glycine residue of the mature ubiquitin protein is conjugated to the ε-amino-group of lysine residues via a multistep, multienzyme pathway.[5] This results in attachment of a single ubiquitin moiety to the target protein, which is referred to as mono-ubiquitylation. Mono-ubiquitylation of target proteins can alter their function and/or localization.[6,7] Additional ubiquitins can be attached to the first ubiquitin, forming poly-ubiquitin chains. These chains can be formed via several lysine residues present in ubiquitin. Extension of poly-ubiquitin chains from all the lysines in ubiquitin has been observed in cells.[8,9] The best characterized poly-ubiquitin chains are those built via lysine 48 of ubiquitin. Lysine 48-linked chains target proteins for degradation by the ubiquitin proteasome.[10] The exact function of these various chains on target proteins is an area of intense interest and recent research suggests that chains that are not lysine 48-linked may also target proteins for degradation.[11]

Interestingly, of the UbL proteins, only SUMO has been demonstrated to form chains *in vivo*.[12,13] SUMOylation of proteins does not target proteins for degradation, but functions primarily to regulate the activity and localization of proteins, most notably transcription factors.[14,15] NEDDylation of proteins regulates the activity of substrates and is essential for the activation of the SCF family of ubiquitin E3 ligases, exemplifying the interactions among the various ubiquitin/UbL pathways.[16,17]

The conjugation of ubiquitin or UbL proteins can be reversed by a family of proteases collectively referred to as deubiquitylases (DUBs).[18] This family of enzymes can be further subdivided into five classes: ubiquitin C-terminal Hydrolase; Ubiquitin-specific protease (USP); JAMM (JAB1/MPN/Mov34 motif metalloprotease); Machado-Josephin Domain containing protease; and Ovarian Tumor related domain containing protease proteases.[18,19] Bioinformatic analysis of the human genome has identified ~100 genes

ABBREVIATIONS: AMC, 7-amino-4-methylcoumarin; DMSO, dimethyl sulfoxide; DUB, deubiquitylase; EK_L, enterokinase light chain; FAM, carboxyfluoroscein; GZMB, granzyme B; HTS, high-throughput screening; IEPD, tetrapeptide, isolevcine-glutamic acid-proline-aspartic acid; ISG15, interferon stimulated gene 15; NBD C_6-HPC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; NEDD8, neural precursor cell expressed developmentally down-regulated protein 8; NEM, N-ethylmaleimide; PLA_2, phospholipase A_2; RFU, relative fluorescence unit; SNR, signal:noise ratio; SUMO, small ubiquitin-like modifier; TAMRA, carboxytetramethylrhodamine; UbL, ubiquitin-like; USP, ubiquitin-specific protease; VS, vinyl sulfone.

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encoding DUBs.[19] Dysregulation of isopeptidases has been linked to a variety of disease states.[20,21] It is clear that DUB activity is responsible for regulating many vital cell functions.[22] Additionally, researchers have begun to identify viral and bacterial proteins that can cleave ubiquitin from ubiquitylated proteins and many of these enzymes have a direct impact on the viability and/or virulence of the pathogenic agent.[23–25] For these reasons there is a growing effort to target these enzymes for therapeutic development. The discovery and characterization of selective isopeptidase inhibitors requires robust assay systems for quantifying enzyme activity and rapidly characterizing inhibitors.

Most current assays for DUB activity employ hydrolysis of small molecule adducts such as coumarin and rhodamine from the C-terminus of ubiquitin leading to an increase in fluorescence.[26,27] However, most DUBs recognize poly- or mono-ubiquitylated protein complexes and not a small molecule attached to the C-terminus of ubiquitin or UbL. To address this disparity we developed a novel assay featuring ubiquitin attached via a peptide bond to an enzyme, rendering the enzyme inactive. After specific hydrolysis of the protein-ubiquitin bond, the enzyme is activated.

Previously, we reported an assay based upon the fusion of ubiquitin to the N-terminus of phospholipase A_2 (PLA_2).[28,29] This technology has been extended in scope, and here we describe two novel assay platforms for measuring the enzymatic activity of ubiquitin/UbL proteases. These assays involve the fusion of ubiquitin or UbL to the N-terminus of enterokinase light chain (EK_L) or granzyme B (GZMB). The newly developed reporters offer a number of benefits. For example, the SUMO3-EK_L fusion protein can be efficiently cleaved by sub-picomolar concentrations of SENP2core. Moreover, these assays can be combined with the ubiquitin-PLA_2 reporter system in a multiplex format, which has allowed us to rapidly determine the substrate specificity of DUBs. Specifically, we have discovered that USP21 can cleave both ubiquitin and ISG15 conjugates *in vitro*. In addition we have used the multiplex enzyme format to characterize two novel isopeptidase inhibitors, PR-619 and P022077. The novel reporter systems will play an important role in the characterization of ubiquitin/UbL isopeptidases and the development of selective isopeptidase inhibitors as potential therapeutic agents.

EXPERIMENTAL SECTION

General Reagents

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Tetrapeptide, isolevcine-glutamic acid-proline-aspartic acid (IEPD)-7-amino-4-methylcoumarin (AMC) was purchased from EMD Biosciences (San Diego, CA) and prepared in dimethyl sulfoxide (DMSO). 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD C_6-HPC) was purchased from Invitrogen (Carlsbad, CA) and prepared in 100% ethanol. PR-619 was purchased from LifeSensors (Malvern, PA) and prepared in DMSO. NEDD8-vinyl sulfone (VS) and NEDD8-AMC were purchased from Boston Biochem (Boston, MA). Chromatography columns and media were purchased from GE Healthsciences (Piscataway, NJ). P022077 is an analog of P5091.[30] The EK_L substrates I (QXL520-DDDDKGSK-FAM) and II (QXL570-DDDDKGSK-TAMRA) were procured from Anaspec (Fremont, CA).

Recombinant Protein Production

Ubiquitin-PLA_2, SUMO3-PLA_2, USP7, SENP1core, SENP2core (catalytic domain of SENP2), deneddylase 1 (DEN1),and PLPro were prepared as previously described.[28] Bacterial expression plasmids encoding hexaHis-tagged USP21 and the fusions of ubiquitin/UbL with the active fragment of EK_L were generated by standard molecular biology techniques (primers listed in *Supplementary Figure S1*; Supplementary Data are available online at www.liebertonline.com/adt). The ubiquitin/UbL-EK_L reporter fusion proteins were expressed and purified using methods similar to those previously described for EK_L.[31] A fusion of SUMO3 and GZMB was generated in the pP-sec-SUMO3 vector (LifeSensors) using standard molecular biology techniques. Stable *Pichia pustoris* transformants were generated according to the manufacturer's instructions. Six isolated clones were evaluated for expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot using anti-GZMB (AbD Serotec, Oxford, United Kingdom). Scale-up production was accomplished by inoculating 5 mL of BMGY until an OD_{600} of ~10 was reached. Cultures were centrifuged, resuspended in 500 mL of BMMY, and grown at 30°C with shaking at 250 rpm for 24 h. Cultures were supplemented with methanol to 1% for 48 h. The supernatant was extensively dialyzed against phosphate-buffered saline and SUMO3-GZMB was purified by nickel chromatography using standard techniques.

Enzyme Assays

UbL-EK_L assays. Unless stated otherwise, recombinant isopeptidase was mixed with UbL-EK_L and EK_L substrate I to final concentrations of 20, 50, and 20 nM, respectively, in a total volume of 100 µL in a well in a black-walled 96-well plate (Greiner Bio-One, Monroe, NC). All dilutions were performed in isopeptidase assay buffer (20 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, and 2 mM β-mercaptoethanol). The increase in fluorescence intensity over time was determined on a Perkin Elmer Envision fluorescence plate reader with excitation and emission filters corresponding to the fluorescence resonance energy transfer peptide utilized. Unless stated otherwise, net relative fluorescence units (RFUs) were determined by subtracting the blank RFU value (20 nM EK_L substrate I or 100 nM EK_L substrate II in isopeptidase assay buffer) from each data point. SENP2core, SUMO3-EK_L sensitivity experiments were performed by mixing 0–100 fM SENP2core with 50 nM SUMO3-EK_L, and 100 nM EK_L substrate I in a total volume of 100 µL as above.

SUMO3-GZMB assays. Unless stated otherwise, recombinant isopeptidase was mixed with SUMO3-GZMB, and IEPD-AMC to final concentrations of 20 nM, 50 nM, and 5 µM, respectively, in a total volume of 100 µL in a well in a black-walled 96-well plate (Greiner Bio-One). All dilutions were performed in isopeptidase assay buffer (20 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, and 2 mM β-mercaptoethanol).

The increase in fluorescence intensity over time was determined on a Perkin Elmer Envision fluorescence plate reader with excitation and emission maxima of 360 and 460 nm, respectively. Unless stated otherwise, net RFUs were determined by subtracting the blank RFU value (5 μM IEPD-AMC in isopeptidase assay buffer) from each data point. SENP2, SUMO3-GZMB concentration dependence experiments were performed as above by mixing 0–200 nM SENP2 core with 50 nM SUMO3-GZMB, and 5 μM IEPD-AMC in a total volume of 100 μL.

Multiplex Assay Format

In general, the multiplex assays included 100 nM SUMO3-GZMB, 5 μM IEPD-AMC, 30 nM ubiquitin-PLA_2, 10 μM NBD C_6-HPC, 100 nM NEDD8-EK_L, and 1 μM EK_L substrate II. In the case of USP21, NEDD8-EK_L was replaced with ISG15-EK_L. Fluorescence was measured at the following pairs of wavelengths: ex360/em460, ex460/em538, ex540/em590, and reported deSUMOylase, DUB, and deISGylase activities, respectively. Signal:noise ratio (SNR) was calculated with the following equation: $(RFU_{\text{with enzyme}} - RFU_{\text{minus enzyme}})/(StdDev_{\text{with enzyme}} + StdDev_{\text{minus enzyme}})$. Z′ value is calculated as $1 - (3/SNR)$. Signal:background was calculated as $RFU_{\text{with enzyme}}/RFU_{\text{minus enzyme}}$.

Isopeptidase Inhibition Assays

Dose ranges of isopeptide inhibitors were prepared in 100% DMSO and 2 μL aliquots were added to 96-well plates in triplicate. Eighty microliters of isopeptidase was added to the wells individually or as a mixture of three enzymes and incubated at room temperature for 30 min. About 20 μL of Reporter-fusion and reporter substrate was then added to all of the wells. Final concentrations of 10 mM N-ethylmaleimide (NEM) and 0.5%(v/v) DMSO were used as controls for 100% and 0% inhibition. Percent inhibition was calculated using the following equation $[1 - ((RFU_{\text{Compound}}l - RFU_{NEM})/(RFU_{DMSO} - RFU_{NEM}))] \times 100$. Data were plotted in Prism and IC_{50} values were determined using a sigmoidal dose–response (variable slope) model.

RESULTS AND DISCUSSION

SUMO3-EK_L and SUMO3-GZMB as Novel Protease Reporters

Previously, we reported the development of a novel ubiquitin/UbL endopeptidase assay utilizing a linear fusion of ubiquitin/UbL to the N-terminus of PLA_2.[28] PLA_2 requires a free N-terminus for activity, and fusion of ubiquitin/UbL to the N-terminus results in inactivation of the enzyme. Incubation with an ubiquitin/UbL isopeptidase results in cleavage of the α-peptide bond between the ubiquitin/UbL and PLA_2 resulting in the activation of PLA_2, which can be measured using a quenched fluorescent substrate of PLA_2.[28,29]

In addition to PLA_2, EK_L and GZMB require a free N-terminus for enzymatic activity.[31–33] We generated plasmids encoding SUMO3 fused to the N-terminus of the active forms of EK_L and GZMB and expressed and purified the fusion proteins. As predicted, these fusion proteins were catalytically inactive. To determine if these fusions could be cleaved by isopeptidases, we incubated them with the core catalytic domain of the deSUMOylase SENP2. Upon activation, EK_L will cleave an internally quenched fluorescence resonance energy transfer peptide resulting in an increase in fluorescence. As shown in *Figure 1*, incubation of SENP2 with SUMO3-EK_L and the EK_L substrate resulted in a dramatic increase in fluorescence due to SENP2 mediated cleavage of the α-peptide bond between SUMO3 and EK_L. In a similar manner, incubation of SUMO3-GZMB with SENP2 resulted in the activation of GZMB, which cleaved its substrate IEPD-AMC, leading to the release of AMC fluorophore and a concomitant increase in AMC fluorescence. In both cases the rate at which fluorescence increased depended on SENP2 concentration, demonstrating that these assays can be used to quantify deSUMOylase activity. The SUMO3-EK_L system can reach a plateau within the time frame of this assay, whereas the SUMO3-GZMB signal remains linear throughout the course of the experiment. This is most likely caused by the difference in the amount of reporter substrate used in these assays. The EK_L substrate is present at only 20 nM, whereas the GZMB substrate is present at 5 μM.

Given the high sensitivity of the SUMO3-EK_L reporter system (*Fig. 1A*), we further investigated the minimal concentration of SENP2 core this assay could reliably detect. We incubated sub-picomolar dose ranges of SENP2 with SUMO3-EK_L and EK_L substrate I and measured the change in fluorescence for 3 h. *Figure 1C* demonstrates that the SUMO3-EK_L assay exhibited a significant increase in signal with concentrations of SENP2core as low as 0.1fM.

Subsequently, we prepared linear fusion proteins consisting of alternative ubiquitin/UbL proteins fused to the N-terminus of EK_L including ubiquitin, ISG15, and NEDD8. Each of these fusions could be activated by incubation with proteases recognizing the cognate ubiquitin/UbL (*Supplementary Fig. S1*). Taken together, these data indicate that UbL-EK_L and SUMO3-GZMB are suitable reporters for measuring protease activity.

Method for Measuring Ubiquitin/UbL Protease Activity in a Multiplex Format

The generation of multiple ubiquitin/UbL protease reporter systems allowed us to investigate the potential for combining these assays for use in a multiplex format (*Fig. 2*). Due to the spectral overlap of EK_L substrate I with the PLA_2 substrate NBD C_6-HPC, we switched to an alternative substrate for EK_L (EK_L substrate II) with excitation and emission maxima of 540 and 590 nm, respectively. The lack of spectral overlap of the fluorophores utilized is shown in *Supplementary Figure S2*. Before demonstrating that these reporter systems could be measured simultaneously in a single well, we optimized the concentrations of the assay components to yield a high SNR for each isopeptidase/reporter combination (data not shown). Subsequently, we combined 100 nM of each reporter (SUMO3-GZMB, ubiquitin-PLA_2, and NEDD8-EK_L) with appropriate concentrations of their substrates (*Fig. 3A*). When all three UbL isopeptidases were added, an increase in fluorescence intensity was observed corresponding to the activation of each reporter substrate. To verify that these signals were dependent upon the presence of the cognate isopeptidase, we repeated this experiment including, in addition to the complete reaction containing all three ubiquitin

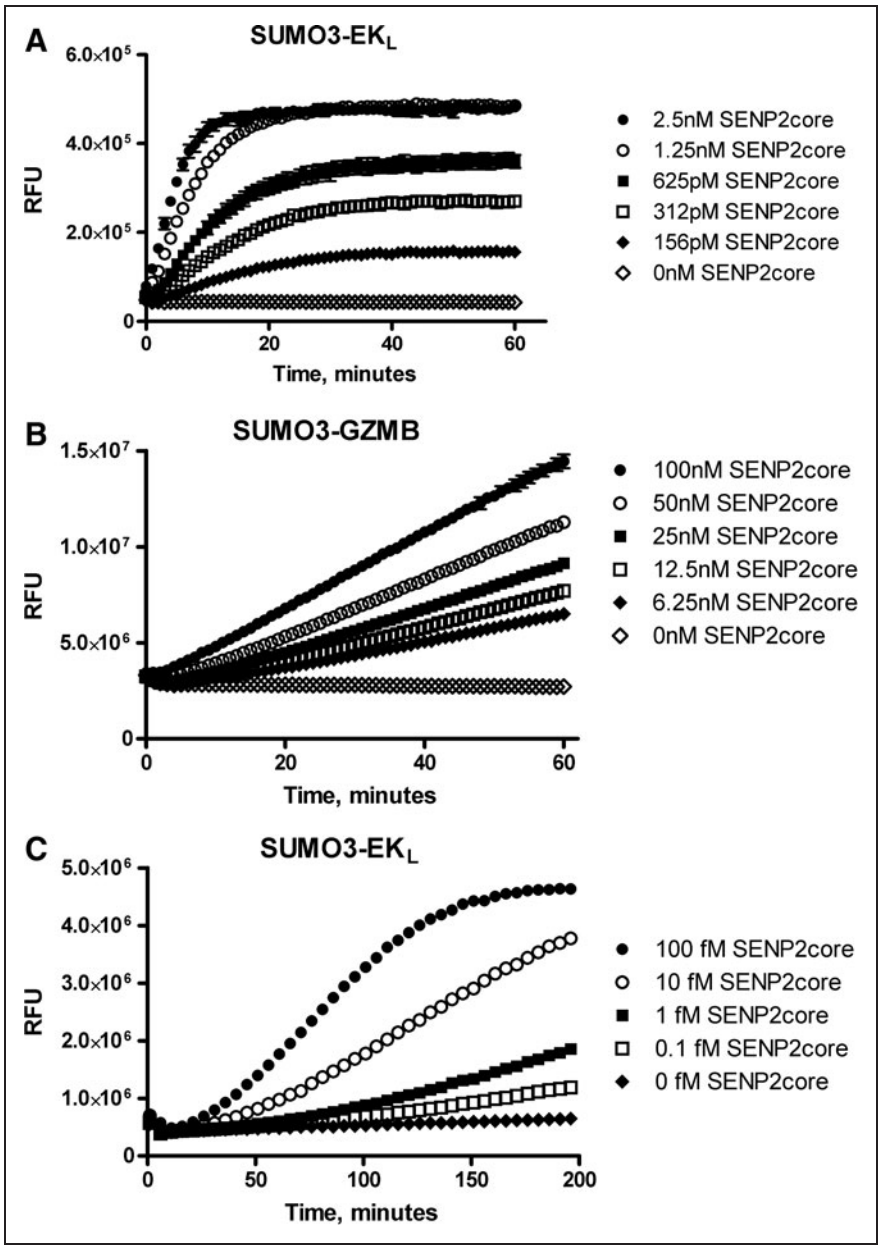

Fig. 1. Detection of SENP2core protease activity with SUMO3-GZMB and SUMO3-EK$_L$. **(A)** Increasing concentrations of SENP2core (0 nM ◇, 156 pM ◆, 312 pM □, 625 pM ■, 1.25 nM ○, 2.5 nM ●) were incubated with 100 nM SUMO3-EK$_L$, 20 nM of EK$_L$ substrate I. The data presented are means ± SEM of triplicate wells. **(B)** Increasing concentrations of SENP2core (0 nM ◇, 6.25 nM ◆, 12.5 nM □, 25 nM ■, 50 nM ○, 100 nM ●) were incubated with 100 nM SUMO3-GZMB and 5 μM IEPD-AMC. The data presented are means ± SEM of triplicate wells. **(C)** Sub-picomolar concentrations of SENP2core (0 fM ◆, 0.1 fM □, 1 fM ■, 10 fM ○, 100 fM ●) were incubated with 500 nM SUMO3-EK$_L$ and 200 nM EK$_L$ substrate I. Data shown are representative mean data from three independent experiments. AMC, 7-amino-4-methylcoumarin; EK$_L$, enterokinase light chain; GZMB, granzyme B.

isopeptidases, controls lacking one of the isopeptidases (*Fig. 3A*). *Figure 3B* shows that the signal generated by NEDD8-EK$_L$ was completely dependent on the presence of DEN1. Similar results were obtained when omitting the other isopeptidases (*Fig. 3A*). To further validate this system for high-throughput screening (HTS), we calculated the SNR and Z′ value for each reporter system in this multiplex format. Each reporter yielded a Z′ >0.5 and an SNR >10, indicating that the assay format is robust and reproducible (*Fig. 3C*). To confirm that additional combinations of reporters were useable in a HTS multiplex format, we also validated the multiplex format utilizing SUMO3-GZMB, ubiquitin-PLA$_2$, and ISG15-EK$_L$ (data not shown). We predict that additional combinations of reporter systems will be possible depending on the experimental design and the availability of suitable substrates.

Characterization of DUB Activity Using Multiplexed Format

Bioinformatic analysis has successfully identified many genes that are predicted to express ubiquitin and UbL isopeptidases. Characterizing the substrate specificity of novel isopeptidases is an important first step in determining the physiological role of the proteases. We have already demonstrated that USP7, SENP2, and DEN1 generate signals only with their cognate UbLs, Ubiquitin, SUMO3, and NEDD8, respectively. In our

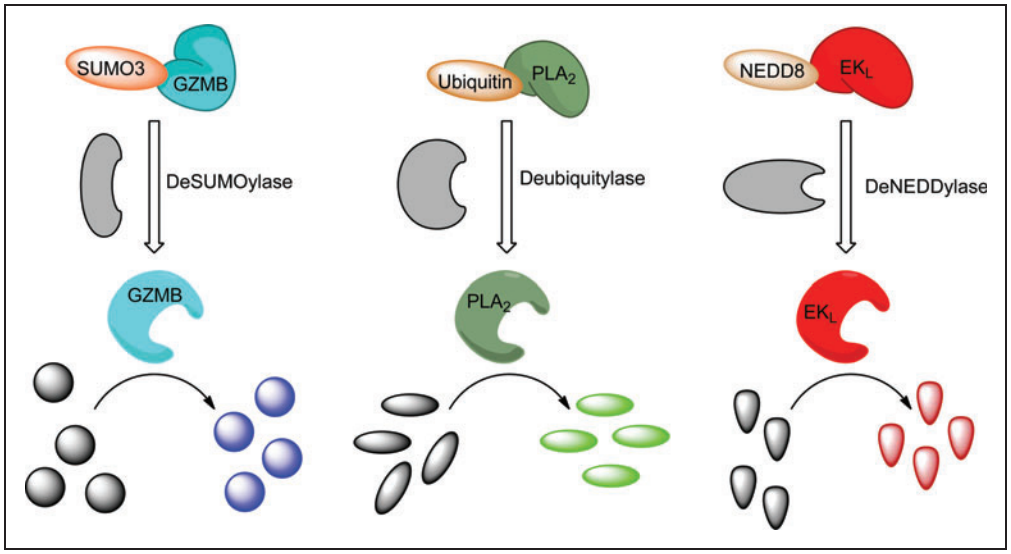

Fig. 2. Schematic representation of multiplexing format. To measure the activity of all three reporters in a single well, three isopeptidases are added to SUMO3-GZMB, ubiquitin-PLA2, and NEDD8-EK_L. Each well contains GZMB substrate (ex340/em460), PLA_2 substrate (ex460/em538) and EK_L substrate II (ex540/em590). PLA_2, phospholipase A_2. Color images available online at www.liebertonline.com/adt

efforts to characterize the USP class of enzymes, we expressed and purified USP21 and tested the activity of 100 nM of USP21 with SUMO3-GZMB, ubiquitin-PLA_2, and ISG15-EK_L and the respective substrates of these reporter enzymes in our multiplex format. For standardization purposes we included a control isopeptidase for each reporter and set the signal generated by the control isopeptidase to 100%. Control isopeptidase concentrations were selected based on similar rates of increasing fluorescent intensity, thus standardizing the assays between different UbLs. As expected, USP21 exhibited a robust signal with Ubiquitin-PLA_2 and negligible activity with SUMO3-GZMB (*Fig. 4A*). However, in addition to the DUB activity, USP21 also exhibited a modest deISGylase activity (*Fig. 4A*) demonstrating the utility of the multiplex assay format to rapidly discover novel substrate specificities of isopeptidases. To confirm this result we incubated increasing concentrations of USP21 with ISG15-EK_L and EK_L substrate I and observed dose dependent increases in USP21 activity (*Fig. 4B*). Taken together, these data report a novel deISGylase activity of USP21 and demonstrate that the multiplex assay format can be used to determine the substrate selectivity of ubiquitin/UbL proteases.

Characterization of Novel Isopeptidase Inhibitors Using Multiplexed Format

Ubiquitin isopeptidases are potential targets for therapeutic intervention. To both identify and develop isopeptidase inhibitors, it is necessary to rapidly screen novel chemical entities versus several related enzymes. The multiplex assay format described here would be predicted to accelerate screening and characterization of novel chemical entities by allowing scientists to test their effects on multiple enzymes simultaneously. To test this hypothesis we studied two small molecule isopeptidase inhibitors, PR-619 and P022077. PR-619 is a broadly active ubiquitin/UbL isopeptidase inhibitor and P022077 is an analog of the recently discovered USP7 inhibitor P5091.[30] Additionally, we tested the selective deNEDDylase activity-based probe, NEDD8-VS. This probe covalently modifies the active site cysteine residue of deNEDDylases rendering the enzyme inactive. Initially, we determined the IC_{50} of these compounds versus SENP2, USP7, and DEN1 individually (*Table 1*). Subsequently, dose ranges of PR-619 were preincubated with SENP2, USP7, and DEN1 simultaneously before addition of all three reporters and their substrates and measurement of enzyme activity (*Fig. 5A*). From these experiments it is clear that PR-619 is a nonselective isopeptidase inhibitor with IC_{50} values ranging from 4 to 16 μM for the three enzymes tested. In contrast, NEDD8-VS selectively inhibited DEN1 in both the singleplex and multiplex formats (*Table 1*). As seen in *Figure 5C*, P022077 had negligible activity versus DEN1 and SENP2core over the concentration range tested, but inhibited USP7 with an IC_{50} of 8 μM. Comparison of the singleplexed and multiplexed data in *Table 1* clearly demonstrates that the multiplex format does not dramatically alter the IC_{50} values or the selectivity of these inhibitors, validating the use of this multiplex format for the characterization of isopeptidase inhibitors.

CONCLUSIONS

The rapidly expanding field of ubiquitin research requires the development of novel technologies that will enable researchers to efficiently decipher the physiological function of the regulatory enzymes in this pathway. Ubiquitin and UbL isopeptidases are key regulators in multiple cellular signaling cascades and they represent a novel class of target for therapeutic development.[20,34,35] We have described two new reporter systems, UbL-EK_L and UbL-GZMB. These reporter fusions are efficiently cleaved by ubiquitin/UbL proteases and are useful for quantifying activity. Additionally, by selecting reporter enzyme substrates that generate fluorescent signals in nonoverlapping discreet wavelength ranges, one can combine them with the UbL-PLA_2 reporter system in a multiplex format. This multiplexed format affords the measurement of three distinct protease activities simultaneously in a single well. We have demonstrated the utility of multiplexing enzyme assays for the characterization of enzyme specificity. In addition, we confirmed that USP21 is a DUB and simultaneously uncovered a previously unreported deISGylase activity of this enzyme. To date, only one other mammalian isopeptidase, USP18, has been shown to function as a deISGylase. The multiplex format is not able to

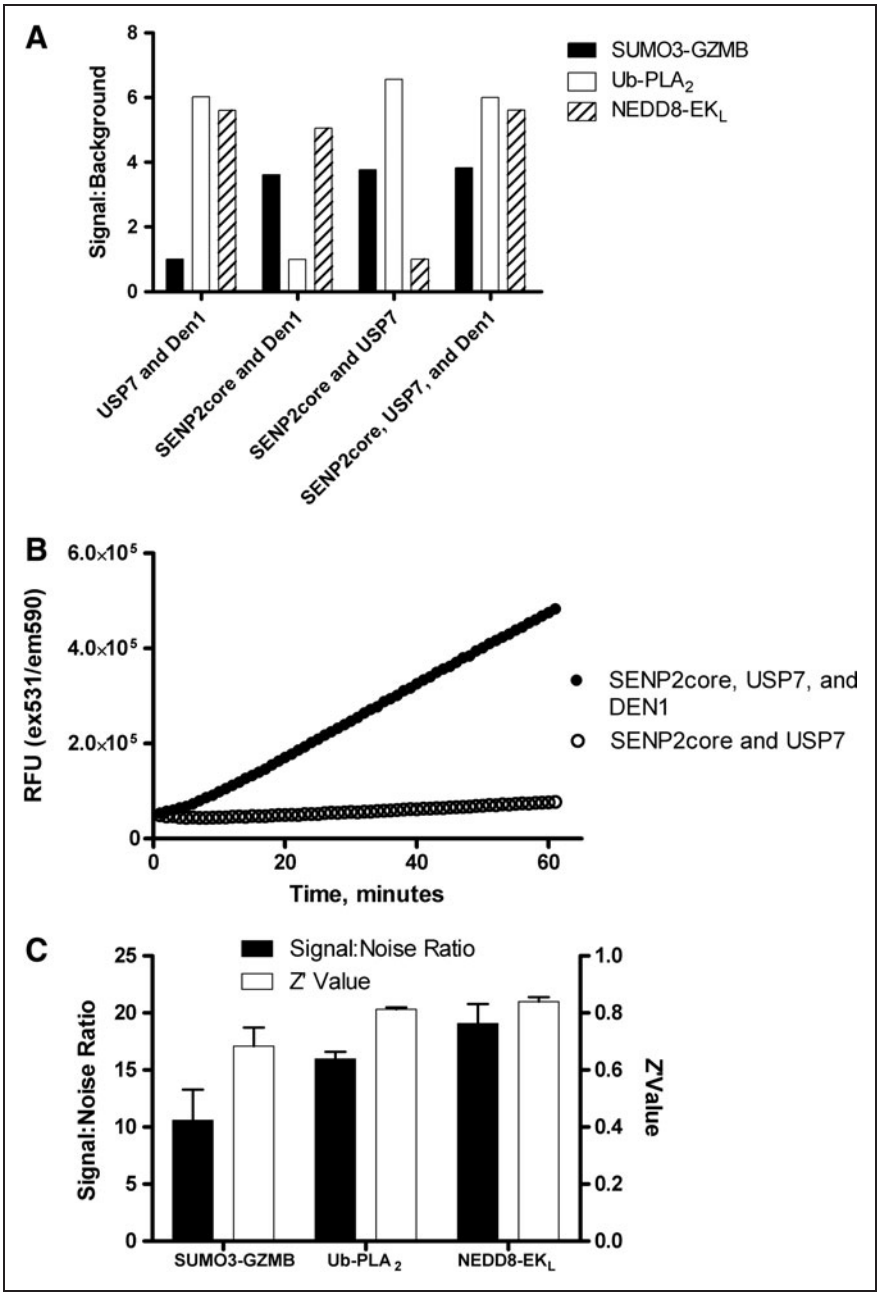

Fig. 3. Detection of ubiquitin/ubiquitin-like protease activity in a multiplex assay format. **(A)** SUMO3-GZMB, ubiquitin-PLA_2, and NEDD8-EK_L were incubated with either the complete mixture containing SENP2core, USP7, and DEN1, or a mixture lacking one of the three isopeptidases. Fluorescence intensity was measured following a 60-minute incubation and signal:background was calculated. Representative data from one of three independent experiments are shown. Bars represent the signal:background generated by the SUMO3-GZMB reporter (solid bars), ubiquitin-PLA_2 reporter (open bars), or the NEDD8-EK_L reporter (crosshatched bars) **(B)** A cocktail of isopeptidases containing USP7 and SENP2core with (closed circles) or without (open circles) DEN1 was added to wells containing SUMO3-GZMB, IEPD-AMC, ubiquitin-PLA_2, NBD C_6-HPC, NEDD8-EK_L, and EK_L substrate II. Fluorescence intensity (ex531/em590) was measured as described in the Materials and Methods section. Error bars represent standard error of $n = 3$ values. **(C)** Utilizing the data from B, the signal:noise ratio (solid bars) and Z′ ratio (open bars) were calculated as described in Materials and Methods. The data shown are the average of three independent experiments, and the error bars represent the standard error of these three values. NBD C_6-HPC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; USP, ubiquitin-specific protease.

determine absolute catalytic efficiencies of isopeptidases and additional studies are needed to fully characterize the activity of USP21. However, it is the clear that the multiplex format will facilitate the rapid initial characterization of novel ubiquitin/UbL proteases.

The therapeutic potential of targeting the ubiquitin/UbL pathways has been established with the FDA approval of the ubiquitin proteasome inhibitor, bortezomib/Velcade®, for the treatment of multiple myeloma.[36] Recently, Millennium developed a selective NEDD8 E1 activating enzyme inhibitor, MLN4924, that prevents the conjugation of NEDD8 to SCF E3 ligases.[37] This inhibitor is currently being developed as a novel cancer therapeutic. In addition to these targets, ubiquitin and UbL isopeptidases are considered potential drug targets. One major concern in the development of isopeptidase inhibitors is selectivity within a protease family. In the case of ubiquitin isopeptidases it will be advantageous to determine the selectivity of an inhibitor at an early stage to rapidly exclude nonselective inhibitors from the development process. To address this issue we have

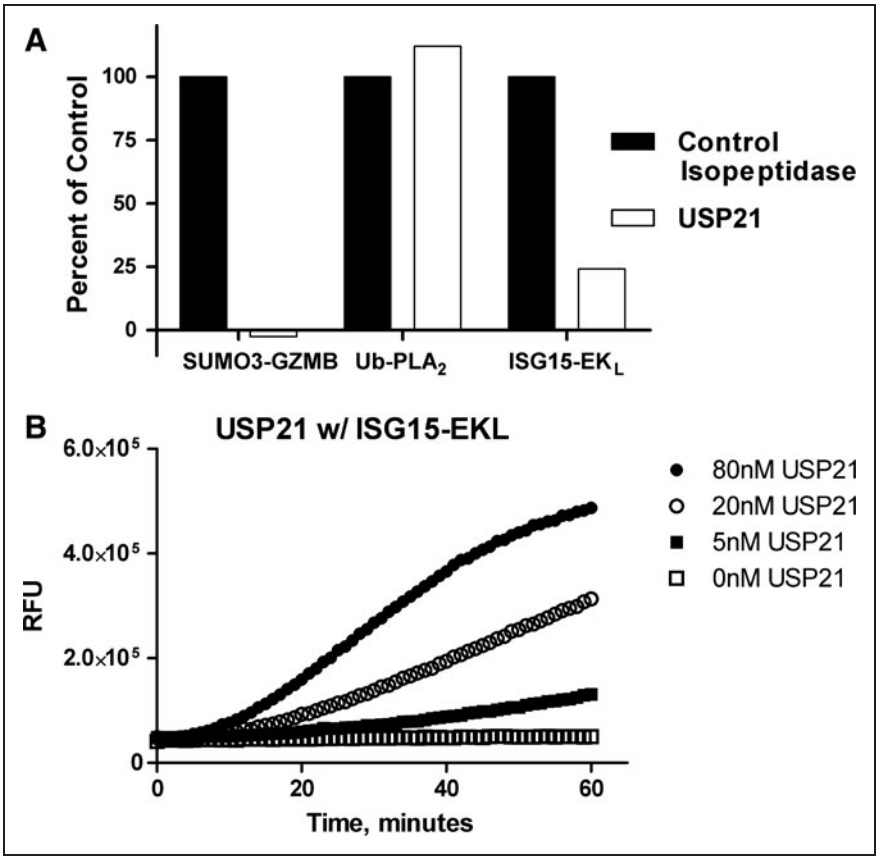

Fig. 4. USP21 is a deISGylase as well as a deubiquitylase. **(A)** 100 nM USP21 was incubated with 100 nM SUMO3-GZMB, 5 μM IEPD-AMC, 100 nM ubiquitin-PLA_2, 20 μM NBD C_6-HPC, 100 nM SUMO3-EK_L, and 1 μM EK_L substrate II. Fluorescence intensity was measured after a 60-minute incubation. USP21 deSUMOylase, deubiquitylase, and deISGylase activity were reported relative to control deSUMOylase (20 nM SENP2 core), deubiquitylase (20 nM USP7), and deISGylase (20 nM PLPro) activity, respectively (solid bars). The signal generated by USP21 is represented by open bars. Representative data from three independent experiments is shown. **(B)** Various concentrations of USP21 (0 nM □, 5 nM ■, 20 nM ○, 80 nM ●) were incubated with 50 nM ISG15-EK_L and 20 nM EK_L substrate I.

established a multiplex assay format that allows for the simultaneous measurement of the protease activities of three distinct isopeptidases. We have employed this multiplex format to characterize the selectivity of ubiquitin/UbL isopeptidase inhibitors. Both the singleplex and the multiplex formats report the same potencies for the inhibitors (*Table 1*). This multiplex assay format is amenable to HTS providing researchers with an initial determination of inhibitor selectivity during primary screens of compound libraries. It is likely that this approach will speed the discovery of selective ubiquitin/UbL isopeptidase inhibitors for further development as potential therapeutic

Table 1. IC_{50} Values for Various Isopeptidase Inhibitors

	IC_{50} (μM)					
	SENP2core		USP7		DEN1	
	Singleplexed	Multiplexed	Singleplexed	Multiplexed	Singleplexed	Multiplexed
NEM	2100 ± 40	2200 ± 80	1300 ± 200	1000 ± 120	1580 ± 40	2000 ± 50
PR-619	26 ± 8	23 ± 7	5.8 ± 2.2	4.3 ± 1.2	6.6 ± 3.4	9.0 ± 3.0
P022077	>50	>50	8.6 ± 2.0	7.8 ± 3.1	>50	>50
NEDD8VS	NT	>0.5	NT	>0.5	0.17 ± 0.01	0.21 ± 0.06

IC_{50} values in μM for four inhibitors with three ubiquitin/UbL isopeptidases as determined in either singleplexed or multiplexed assay formats. PR-619 and NEM are broad-spectrum cysteine protease inhibitors, whereas P022077 and NEDD8-VS are selective USP7 and deNEDDylase inhibitors, respectively. Data represent mean and standard deviation of three independent experiments.

NEM, N-ethylmaleimide; UbL, ubiquitin-like; USP, ubiquitin-specific protease; VS, vinyl sulfone.

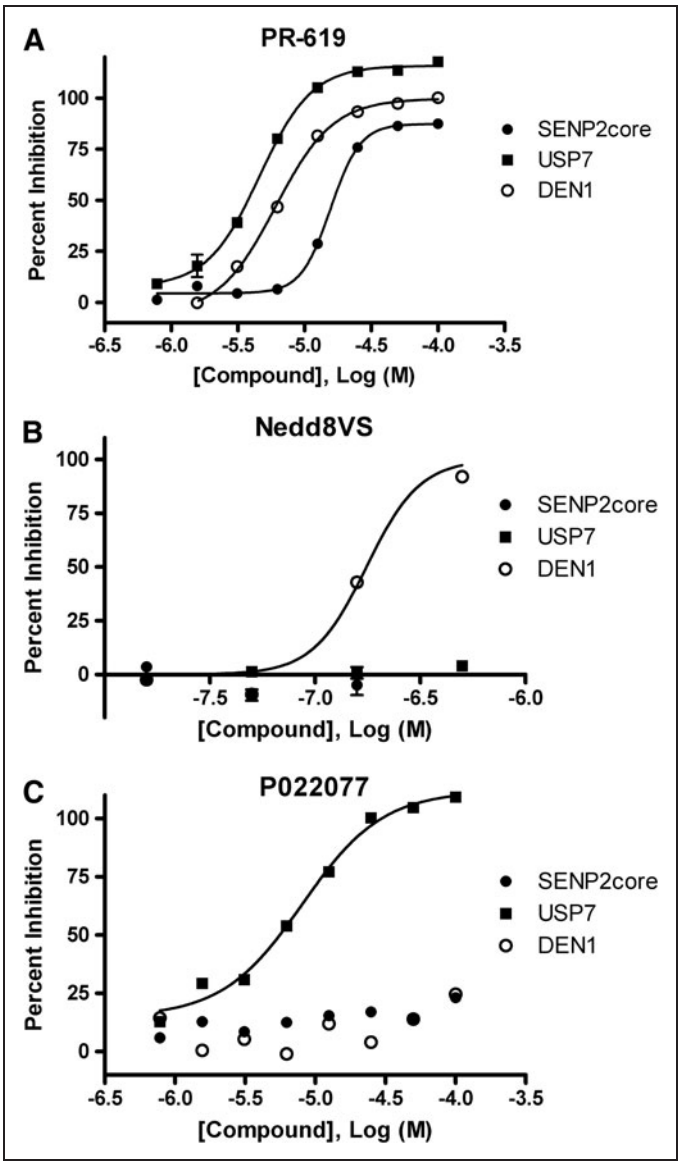

Fig. 5. The multiplex format has the utility to identify selective isopeptidase inhibitors. **(A)** Various concentrations of PR-619 were incubated with 100 nM SENP2core, 20 nM USP7, and 20 nM DEN1. After 30 min, 100 nM SUMO3-GZMB, 5 μM IEPD-AMC, 100 nM ubiquitin-PLA_2, 20 μM NBD C_6-HPC, 100 nM NEDD8-EK_L, and 1 μM EK_L Substrate II were added. Fluorescence intensity was measured after 45 min at the appropriate wavelengths and the percent inhibition for each enzyme was calculated. Percent inhibition of each isopeptidase (SENP2core ●, USP7 ■, DEN1 ○) is shown plotted versus log of the molar concentration of compound. IC_{50} values were calculated using GraphPad Prism and are shown where appropriate. **(B)** A range of concentrations of NEDD8-vinyl sulfone were tested as described above. **(C)** The selective USP7 inhibitor, P022077, was tested as above.

agents as well as tool compounds for studying the biological significance of a particular protease.

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DISCLOSURE STATEMENT

No competing financial interests exist.

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