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Targeting Deubiquitinases Enabled by Chemical Synthesis of Proteins

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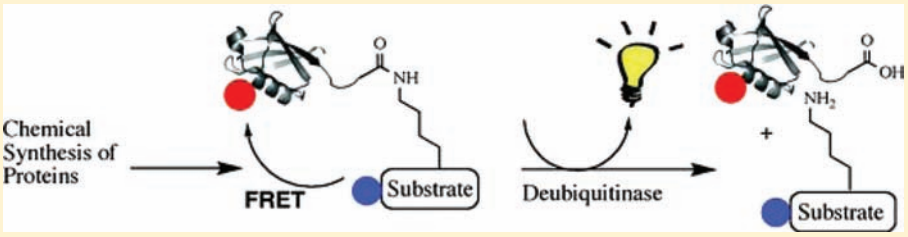
Supporting Information



ABSTRACT: Ubiquitination/ubiquitylation is involved in a wide range of cellular processes in eukaryotes, such as protein degradation and DNA repair. Ubiquitination is a reversible post-translational modification, with the removal of the ubiquitin (Ub) protein being catalyzed by a family of enzymes known as deubiquitinases (DUBs). Approximately 100 DUBs are encoded in the human genome and are involved in a variety of regulatory processes, such as cell-cycle progression, tissue development, and differentiation. DUBs were, moreover, found to be associated with several diseases and as such are emerging as potential therapeutic targets. Several directions have been pursued in the search for lead anti-DUB compounds. However, none of these strategies have delivered inhibitors reaching advanced clinical stages due to several challenges in the discovery process, such as the absence of a highly sensitive and practically available high-throughput screening assay. In this study, we report on the design and preparation of a FRET-based assay for DUBs based on the application of our recent chemical method for the synthesis of Ub bioconjugates. In the assay, the ubiquitinated peptide was specifically labeled with a pair of FRET labels and used to screen a library comprising 1000 compounds against UCH-L3. Such analysis identified a novel and potent inhibitor able to inhibit this DUB in time-dependent manner with k_{inact} = 0.065 min^{-1} and K_i = 0.8 μM. Our assay, which was also found suitable for the UCH-L1 enzyme, should assist in the ongoing efforts targeting the various components of the ubiquitin system and studying the role of DUBs in health and disease.

INTRODUCTION

The attachment of a ubiquitin (Ub) or polyubiquitin (polyUb) chain to a protein target, referred to as ubiquitination/ubiquitylation, best known for its signaling role in protein degradation, is involved in a wide range of eukaryotic cellular processes.[1] Ubiquitination involves three enzymes, E1, E2, and E3, that cooperate to link, via an isopeptide bond, the C-terminal Gly of Ub to the lysine side chain of a protein target.[1,2] Target modification can involve either monoubiquitination (i.e., the attachment of a Ub monomer) or polyubiquitination (i.e., the attachment of a Ub chain of various lengths and linkage types).[3−6] As a result, a variety of molecular events may transpire, such as regulation of protein degradation or DNA repair.

Ubiquitination is a reversible post-translational modification wherein the removal of the Ub protein is catalyzed by a family of enzymes known as deubiquitinases (DUBs).[7,8] DUBs can remove Ub or polyUb from proteins/peptides, process Ub precursors, and disassemble unanchored polyUb chains.[7,8] There are approximately 100 DUBs encoded in the human genome, which were identified by in silico efforts and activity-based profiling.[9−11] DUBs are involved in a variety of regulatory processes, such as cell-cycle progression, tissue development, and differentiation. Several DUBs have been implicated in various diseases, including neurological disorders, infectious diseases, and cancer.[11−14] Hence, it is not surprising that these enzymes are emerging as potential therapeutic targets and have encouraged the development of assays for the discovery of inhibitors for pharmaceutical needs and for studying the roles of DUBs in various biological functions.[9−11] However, despite the efforts that have been invested in targeting some of these DUBs, to the best of our knowledge, none of these strategies have identified inhibitors against any DUB that has reached advanced clinical stages. This is due to several challenges that are encountered in the discovery process, such as the absence of a highly sensitive and practically available high-throughput screening (HTS) assay to identify lead compounds against a DUB of interest.[13] One of the reasons for the lack of such an assay is related to synthetic difficulties in preparing an efficient assay that takes into consideration the relatively complex structure of the natural

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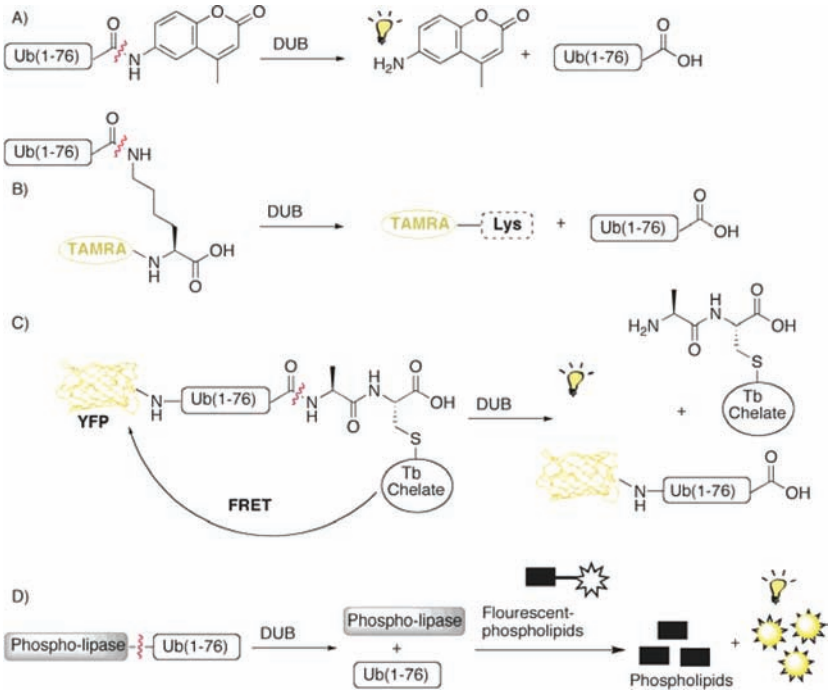
Scheme 1. Current Strategies That Are in Use as HTS Assays for DUBs



substrate, that is, ubiquitinated peptides, proteins, and Ub chains presenting the native isopeptide bond. Moreover, the complexity of the Ub signal and the involvement of different types of Ub chains, which are known to adopt a variety of conformations,[3−5] together make the development of a general assay highly challenging. Presently, an appropriate assay, adaptable to a specific target and DUB, has yet to be designed.

In efforts aimed at introducing HTS assays for the characterization of DUBs, four strategies have been developed. These attempt to overcome the absence of a highly efficient and practically available reporting system, mainly by avoiding the construction of the native isopeptide bond (Scheme 1A,C,D). The first method is based on attaching 7-amino-4-methylcoumarin (AMC) to the C-terminus of Ub (Ub-AMC), which, upon the action of DUB, releases the fluorogenic AMC unit (Scheme 1A).[15] This assay, despite its successful use for the study of DUB kinetic and inhibition,[16] suffers from several limitations, such as low sensitivity and inefficient cleavage by the largest class of DUBs, the UBP/USP class enzyme, in addition to difficulties in realizing large-scale preparations. A second strategy developed by Tirat and co-workers is based on enzymatic preparation of an assay in which TAMRA-labeled Lys was linked via the isopeptide bond to the C-terminus of Ub (Scheme 1B).[17] Although the longer wavelength of this fluorophore improved the accuracy of the assay and the isopeptide bond was maintained, the enzymatic preparation of this substrate was challenging and produced the desired products with poor yield, thus limiting its practicality. Riddle and co-workers presented a method using fluorescence resonance energy transfer (FRET) in which the N-terminal of Ub was coupled to a yellow fluorescent protein, while the C-terminus was extended by a dipeptide labeled with terbium chelate (Scheme 1C).[18] Despite some advantages of this strategy, such as a longer fluorescent lifetime and a more sophisticated and sensitive detection assay, this method is not suitable for many DUBs due to unnatural connectivity of the dipeptide to Ub. A different approach based on a phospholipase linked to Ub was also developed.[19] Upon hydrolysis by DUB, the free phospholipase acts on fluorescent phospholipid substrates to generate a fluorescent signal (Scheme 1D). However, this method cannot be used for the ubiquitin C-terminal hydrolases (UCHs) family or any other DUBs that recognize both the protein target and the proximal Ub unit.

We and other research groups have recently developed a number of methods that allow for chemical synthesis of ubiquitinated peptides,[20−23] proteins,[24,25] and Ub chains in their natural form,[26−31] thereby eliminating the need for any enzymatic machinery in preparing these bioconjugates. While such methods are becoming increasingly useful for studying the structure and function of Ub bioconjugates,[24−26,29−31] the potential of these tools for targeting the Ub system (e.g., inhibitors for DUBs and ligases) has not yet been realized. Taking advantage of our chemical methods, we report here on the design and synthesis of a practically useful FRET-based HTS assay that can detect hydrolysis of the scissile native isopeptide bond. Using this HTS assay, we identified novel and highly potent inhibitors of UCH-L3. The system was also found to be applicable for the UCH-L1 enzyme and should enable the discovery and optimization of potent inhibitors of this enzyme, as well.

EXPERIMENTAL METHODS

General Methods. *High-Performance Liquid Chromatography (HPLC).* Analytical reverse-phase HPLC was performed on a Thermo instrument (Spectra System p4000) system using Jupiter C4 column (5 μm, 300 Å, 150 × 4.6 mm). Preparative reverse-phase HPLC was performed on a Waters instrument system using a Jupiter C4 column (5 μm, 300 Å, 250 × 22.4 mm). The flow rates used were 1.2 mL/min (analytical) and 20 mL/min (preparative). The linear gradients used to elute the bound peptides were integrated from buffer A (water with 0.1% (v/v) trifluoroacetic acid [TFA]) and buffer B (acetonitrile with 0.1% (v/v) TFA).

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Mass Spectrometry. Electrospray ionization mass spectrometry (ESI–MS) was performed on a LCQ Fleet mass spectrometer (Thermo Scientific) with an ESI source.

Fluorescence. Fluorescent measurements were performed on an Infinit M200 fluorescence plate reader (TECAN).

Reagents. Resin, protected amino acids, 7-methoxycoumarin-4-acetic acid (MCA), HBTU, HOBt were purchased from Novabiochem. Bovine serum albumin was purchased from Calbiochem. HEPES, EDTA, and DMSO were purchased from Sigma. DMF was purchased in biotech grade. UCH-L3 and UCH-L1 were purchased from BostonBiochem. 2,4-Dinitrophenyl (Dnp) was purchased from AK-Scientific. Commercial reagents were used without further purification.

Solid Phase Peptide Synthesis (SPPS). SPPS was carried out manually in syringes equipped with Teflon filters, purchased from Torviq or by using an automated peptide synthesizer (CS336X, CSBIO). Coupling of the MCA fluorophore was assisted by a CEM Discover Microwave reactor.

Synthesis of Peptide **1**. Synthesis was carried out on Rink amide resin (0.59 mmol/g, 0.1 mmol scale). The peptide sequence {(Nle)FK(ivDde)TEG} was synthesized manually as follows: Amino acids and HOBt/HBTU were used in 4-fold excess, while DIEA was used in 8-fold excess to the initial loading of the resin. Fmoc deprotection was achieved by treatment of the resin using 20% piperidine (3 × 3 min). Subsequently, the free amine was coupled to MCA and assisted by microwave irradiation at 60 °C for 20 min, using 5-fold excess of MCA, HOBt, and DIC to the initial loading of the resin.

Preparation of Substrate 3. *Synthesis of Peptide* **2** *{(MCA)-p53(384–389)}-{Ub(46–76,(Asp52-Dnp))}.* Peptide **1** was treated with 5% $N_2H_4(H_2O)$ in DMF for 20 min to remove the ivDde protecting group, followed by DMF washing. This step was repeated four times to ensure complete deprotection. At this stage, an isopeptide bond was formed by coupling Fmoc-Gly-OH, followed by elongation of the remaining amino acids using a peptide synthesizer. Amino acids and HOBt/HBTU were used in 5-fold excess, while DIEA was used in 10-fold excess to the initial loading of the resin. Coupling proceeded for 1 h, with Fmoc-deprotection being achieved using 20% piperidine with 5/10/5 min cycles. Fmoc-Asp(Dnp)-OH was coupled instead of Asp52. Global deprotection and cleavage from the resin was achieved by treatment of the resin-bound peptide with 95:2.5:2.5 TFA/triisopropylsilane/water for 2 h at room temperature. The crude peptide was precipitated in anhydrous Et_2O, centrifuged, dissolved in HPLC buffer, and lyophilized. HPLC purification afforded the corresponding peptide at a ∼21% yield over two steps (∼60 mg).

Ligation of Peptide **2** *with Ub(1–45)-thioester.* Tris(2-carboxyethyl)phosphine (TCEP) (14.3 mg mL^{-1}) and 4-mercaptophenylacetic acid (MPAA) (100 mM) were dissolved in 6 M Gn·HCl, 200 mM phosphate buffer, pH ∼7.2. Peptide **2** (62.7 mg, 1.1 equiv) and Ub(1–45)-thioester (64.4 mg, 1 equiv) were added to this solution at a concentration of 2.5 mM and incubated for 2 h at 37 °C. The reaction was monitored to completion using analytical RP-HPLC and purified by preparative RP-HPLC to afford the ligation product at a ∼50% yield (∼60 mg).

Preparation of Substrate **4**. The preparation of substrate **4** was achieved as described for substrate **3** except for the following modifications: Fmoc-Asp(OtBu)-OH was coupled as Asp52 and Fmoc-Glu(Dnp)-OH was coupled instead of Glu34.

Kinetic Parameters. Forty-six microliters from a 40 nM stock solution of UCH-L3 (50 mM HEPES, 0.5 mM EDTA, 5 mM DTT, and 0.5 mg mL^{-1} ovalbumin, pH 8) was added to a Nunc 96-well black assay plate and incubated for 15 min to ensure full reduction of UCH-L3. To start the enzymatic reaction, 50 μL of various concentrations of substrate **3** (2–20 μM, 50 mM HEPES, 0.5 mM EDTA, pH 8) was added to each well. The fluorescence (E_{ex} = 325 nm, E_{em} = 445 nm) of the product {(MCA)-p53(384–389)} was measured continuously and the concentration of the product was determined using a standard curve generated using the fully digested substrate. The initial velocities (V_0) were fitted to the Michaelis–Menten equation to determine K_M, V_{max}, and k_{cat} using SigmaPlot 2000 software.

HTS for UCH-L3 Inhibitors. The Diversity Set III library obtained from the National Cancer Institute (NCI) was used for screening against UCH-L3. In each well of the 96-well black plate, we incubated 4 μL of test compound (25 μM, final concentration) with 46 μL of UCH-L3 (40 nM) for 20 min at room temperature. After adding 50 μL of substrate **3** (8 μM), the fluorescence emission intensity was measured to reveal the effect of the tested compounds on enzyme activity. To ensure reliable results, each assay plate contained an enzyme control to examine activity in the absence of DMSO (50 μL of UCH-L3, 50 μL substrate buffer), a substrate control to define the background of substrate hydrolysis (50 μL of buffer, 50 μL of substrate **3**), and a DMSO control to determine activity in the absence of any NCI compound (4 μL of DMSO, 46 μL of UCH-L3, 50 μL of substrate **3**). Compounds that exhibited ≥50% inhibition were further tested at 5 and 10 μM.

Quenching of {(MCA)-p53(384–389)} with the Inhibitors, LS1–LS6. LS1–LS6 (6.25 μM) were incubated with 1 μM of {(MCA)-p53(384–389)}. Fluorescence emission intensity was measured to ensure that the inhibitors did not quench the hydrolyzed product {(MCA)-p53(384–389)}.

Time-Dependent Inactivation of UCH-L3 by LS1. Four microliters of LS1, at various concentrations (0–4 μM), was incubated with 46 μL of UCH-L3 (20 nM) for periods of 0–9 min at room temperature, followed by addition of 50 μL of substrate **3** (8 μM). Fluorescence emission of the product {(MCA)-p53(384–389)} was measured and initial velocities (i.e., V, the initial velocity in the presence of LS1, and V_0, the initial velocity without preincubation with LS1) were determined. Plots of $\ln(V/V_0)$ against time were used to obtain inactivation rate constants (k_{obs}). To further characterize LS1, we plotted k_{obs} against LS1 concentrations and fitted the data to a nonlinear curve using SigmaPlot 2000 software to define the maximal rate of enzyme inactivation (k_{inact}) and the LS1 concentration required reaching the half-maximal rate of inactivation (K_i).

RESULTS AND DISCUSSION

1. Design and Synthesis of the HTS Assay. When designing a HTS assay for DUBs, the following features should be considered: (1) The assay substrate should mimic the naturally occurring substrate as closely as possible and ideally have the Ub linked via the isopeptide bond to the peptide/protein or comprise Ub linked to another Ub (i.e., di-Ub); (2) the rate of cleavage of the assay substrate should be similar to that of the naturally occurring substrate; (3) the assay substrate should be amenable to large-scale preparation; and (4) the assay could be adopted for use with several DUBs with some modifications. With these criteria in mind, we decided to develop a HTS assay that includes all these features and initially made use of this assay for the discovery of novel inhibitors against UCH-L3. UCH-L3 catalyzes the removal of small adducts, such as small molecules and short peptides from the C-terminus of Ub.[32−35] Recent studies have shown that UCH-L3 plays an important role in a programmed cell death, which is implicated in number of human diseases, including neurodegenerative diseases, autoimmune diseases, and cancer. For example, overexpressed and increased UCH-L3 activity has been reported in multiple types of cancer cells, suggesting that UCH-L3 activity might be required for cancer cell survival.[36,37] Therefore, inhibition of this enzyme offers a potential therapeutic strategy which has triggered the search for novel inhibitors of UCH-L3 by several groups.[16−18,38]

The general strategy of our HTS assay relies on a ubiquitinated peptide incorporating elements of a FRET-based detection system, where energy is transferred from an

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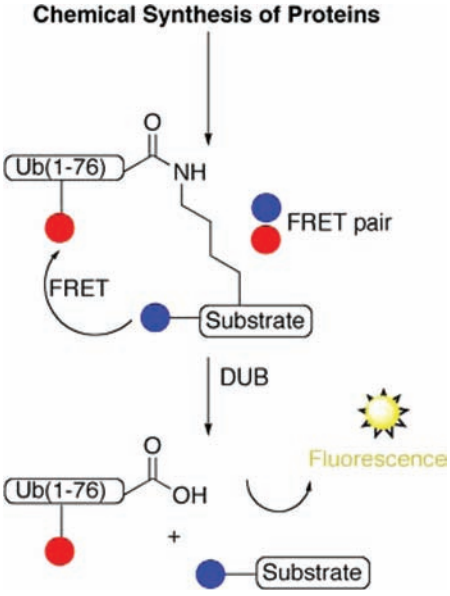
excited donor fluorophore from the peptide component to an acceptor fluorophore on Ub which serve as quencher (Scheme 2). For FRET, the fluorophore and quencher must be in a close

Scheme 2. General Strategy for FRET-Based HTS Assay for DUB



proximity (1−10 nm) and the emission spectrum of the donor should overlap with the excitation spectrum of the quencher. Upon examining the three-dimensional structure of Ub (1UBQ),[39] several possible residues were deemed as promising in term of their potential to serve as an anchoring point for the donor and quencher moieties. As shown in Figure 1, Glu34 and

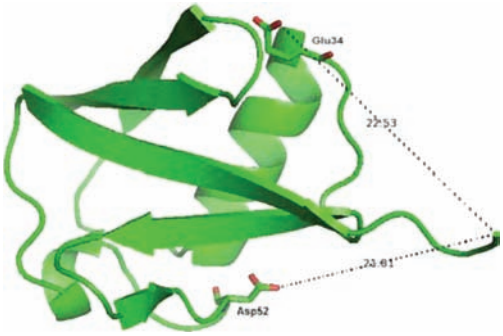


Figure 1. Highlighting the side chains of Glu34 and Asp 52 in ball and stick representation and their distances (~2.2 nm) from the C-terminus of Ub. The model of the Ub structure (1UBQ) was generated using PyMOL software.

Asp52 are located in solvent-exposed regions at a distance of ~2−3 nm from the C-terminus of Ub. In the case of a ubiquitinated peptide bearing the donor and quencher, this distance could vary slightly but is estimated to be within the desired distance for efficient FRET.

Previous studies using site-directed mutagenesis and NMR[40] revealed that Asp52 is not involved in the binding of Ub to UCH-L3, while Glu34 plays some role in substrate-enzyme recognition. Indeed, our analysis of the more recent X-ray structure of Ub-vinylmethylester with UCH-L3 supports these observations.[34] The introduction of the quencher at various positions would thus provide us with the opportunity to examine the effect of quencher location on FRET efficiency and assess assay sensitivity.

We decided to use the well-known donor−quencher pair MCA as the fluorophore and Dnp as the quencher.[41] These are two relatively small size molecules, as compared to other known large polycyclic FRET pairs. Therefore, if positioned on the appropriate residues, it is unlikely that the incorporation of MCA and Dnp would disrupt proper folding of the substrate and affect the activity of the DUB of interest. Additionally, these molecules can be easily and efficiently coupled to a variety of amino acids.

With the above design in mind, we utilized our recent synthetic tools employing only SPPS and NCL for the synthesis of ubiquitinated peptide.[23] We tested our strategy on the ubiquitinated peptide derived from p53, p53(384−389), bearing the MCA and Dnp donor−quencher pair.

The ubiquitinated p53 peptide was found, in our hands, to be an excellent substrate for UCH-L3.[23] The strategy for synthesis of this substrate modified with the donor−quencher pair is shown in Scheme 3. The branched Lys was masked orthogonally with the protecting group, ivDde, to introduce the isopeptide bond in a site-specific manner with the desired peptide being prepared employing Fmoc-SPPS. The N-terminus of the p53 peptide was coupled to the commercially available acid form of MCA to generate peptide **1**. Then, the ε-amine group of the peptide Lys residue was selectively unmasked by applying 5% hydrazine in DMF to remove the ivDde and allow for subsequent Fmoc-SPPS processing of the Ub(46−76) fragment. The Asp residue modified with the Dnp moiety[41] was incorporated instead of Asp52. Side chain deprotection and cleavage form the resin (achieved with TFA/H_2O/TIS) followed by ether precipitation, HPLC purification, and lyophilization afforded the purified peptide **2** at a 20−30% isolated yield.

Ub(1−45)-thioester was prepared employing Fmoc-SPPS, as we described previously.[42] Finally, the two fragments were ligated, via NCL,[43] to produce the desired ubiquitinated peptide {(MCA)-p53(384−389)}-{Ub(Asp52-Dnp)}, **3**, bearing the donor−quencher probe at a 50% isolated yield (Figure 2). A similar strategy was adopted to prepare the ubiquitinated peptides using Glu34 as the site of attachment of the Dnp probe to generate {(MCA)-p53(384−389)}-{Ub(Glu34-Dnp)}, **4**.

2. Fluorescence Properties of the FRET System. Next, we examined the integrity and efficiency of the two assay systems using fluorescence spectroscopy and LC−MS to follow the ability of UCH-L3 to cleave these substrates. In the fluorescence assay, we mixed the substrates individually with UCH-L3 and followed fluorescence emission (E_{ex} = 325 nm, E_{em} = 445 nm) upon release of the product {(MCA)-p53(384−389)}. A 6-fold increase in fluorescence was observed in the case of {(MCA)-p53(384−389)}-{Ub(Asp52-Dnp)}, with an initial rate of 466.6 μM s^{-1} per milligram of enzyme (Figure 3). In the case of {(MCA)-p53(384−389)}-{Ub(Glu34-Dnp)}, an increase of 3-fold in fluorescence was observed (initial rate of 333.3 μM s^{-1} per milligram of enzyme), reflecting a lower cleavage efficiency (Figure 3). These results indicate that {(MCA)-p53(384−389)}-{Ub(Asp52-Dnp)} is the preferred substrate for UCH-L3 and are consistent with the importance of Glu34 for enzymatic activity. Importantly, complete quenching of both substrates was observed prior to enzymatic

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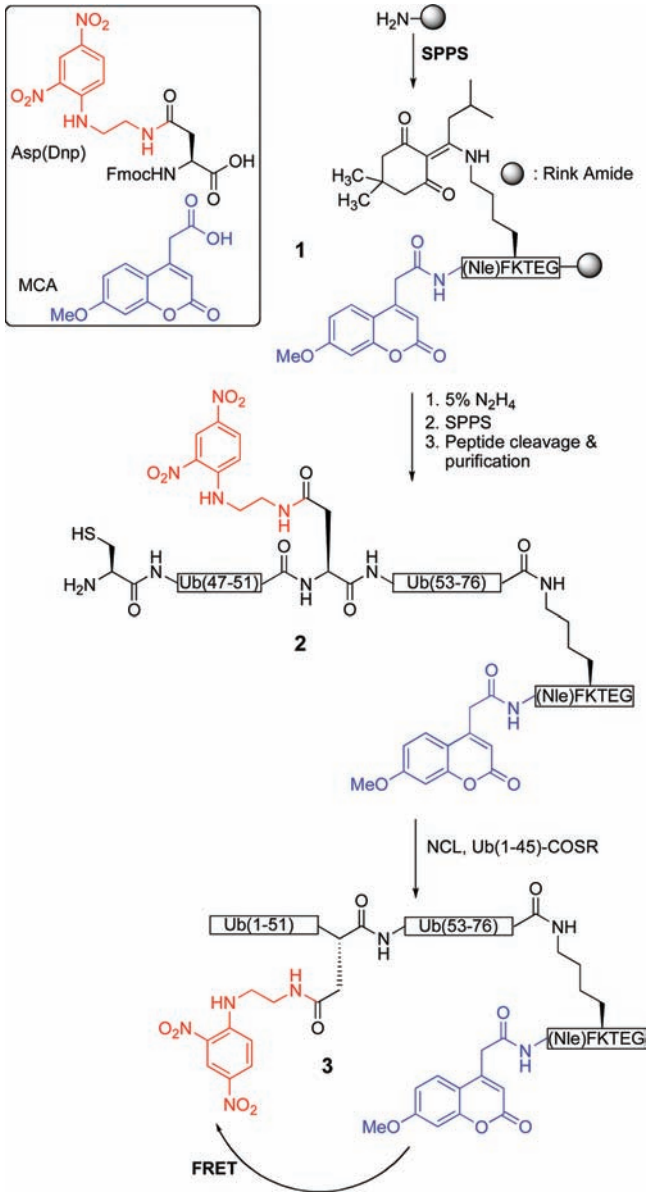
Scheme 3. Synthesis of Ubiquitinated Peptide Bearing the Dnp and MCA Donor−Quencher Pair on Ub Asp52 and the N-Terminus of the p53 Peptide, Respectively[a]



[a]Norleucine (Nle) was used instead of Met to avoid oxidation during synthesis and handling (R = $-CH_2CH_2-COOMe$).

cleavage (Figure 3), further supporting the correct design of our FRET system.

These results were also supported by LC−MS analyses in which substrate **3** showed the expected cleavage products, {(MCA)-p53(384−389)} and {Ub(Asp52-Dnp)} (Supporting Information), consistent with the fluorescence data (Figure 3). Our study indicated that the ability of UCH-L3 to cleave the isopeptide bond is largely affected by the position of the Dnp moiety. Circular dichrosim (CD) spectral analysis of both systems produced comparable secondary structures, indicating that the introduction of the donor−quencher pair does not induce major conformational changes (Supporting Information). To further evaluate the effect of Dnp in {(MCA)-p53(384−389)}-{Ub(Asp52-Dnp)}, we used LC−MS to compare the hydrolysis efficiency of UCH-L3 in this system with an unmodified substrate, {(MCA)-p53(384−389)}-Ub, bearing only the MCA probe and not Dnp (Supporting Information). Notably, a similar percentage of cleavage was observed for both substrates under similar cleavage conditions, demonstrating that the Dnp moiety in substrate **3** does not interfere with the enzymatic activity of UCH-L3. In light of these results, we chose to continue with substrate **3** as our preferred UCH-L3 substrate in the HTS assay.

To maximize the amount of material obtained from our synthetic protein strategy, we decided to test UCH-L3 efficiency in cleaving the nondesulfurized analogue of {(MCA)-p53(384−389)}-{Ub(Asp52-Dnp)}. Notably, the cleavage percentage of this substrate with UCH-L3 was nearly identical to that realized with the unmodified and desulfurized analogue. These findings encouraged us to use the FRET system involving the undesulfurized version of {(MCA)-p53(384−389)}-{Ub(Asp52-Dnp)}, thereby reducing the number of steps required for substrate synthesis. Using the synthetic scheme described above (Scheme 3), the synthesis of the building block **2**, and Ub(1−45)-thioester at the 0.1 mmol scale, followed by performing the NCL step on purified material, afforded ∼60 mg of highly pure substrate **3**, ready for the kinetic characterization and the HTS step. This amount of substrate is also sufficient to perform approximately 10 000 measurements based on the HTS conditions we developed, further supporting the practicality of our screening system.

3. Kinetic Characterization of the FRET System. To kinetically characterize UCH-L3 activity with our substrate **3**, we assayed different substrate concentrations (1−10 μM) with the optimal enzyme concentration (20 nM) and followed the fluorescence emission of the hydrolysis product, {(MCA)-p53(384−389)}. The rates of initial velocity were calculated and fitted to the Michaelis−Menten equation to determine the affinity constant, K_M, the maximal velocity, V_{max}, and the catalytic turnover rate constant, k_{cat} (Figure 4). The fitted data correspond to a K_M of 3.8 μM and a k_{cat} of 0.92 s^{-1}. These kinetics parameters are different from those reported for Ub-AMC (K_M = 0.05 μM, and k_{cat} = 9.1 s^{-1}),[15] Lys(TAMRA)-linked Ub (K_M = 0.86 μM, and k_{cat} = 4.5 s^{-1}),[17] and the more recently chemically prepared Gly-Lys(TAMRA)-linked Ub (K_M = 0.07 μM, and k_{cat} = 27 s^{-1}).[44,45] The lower catalytic efficiency determined with our FRET assay could be the result of changes in the size of the attached peptide (composed of seven residues) to the C-terminus of Ub that UCH-L3 targets. Indeed, previous structural and biochemical analysis showed that UCH-L3 is highly sensitive to the size of the peptide linked to the C-terminus of Ub.[23,32−35]

4. HTS Screening of a NCI Library. As a proof of concept for the ability of our system to screen a chemical library with the aim of identifying novel inhibitors of UCH-L3, we screened a relatively small library consisting of 1000 compounds. The NCI library contains relatively rigid scaffolds with a tendency to be planar and contain not more than a single chiral center with pharmacologically desirable features. We initially screened the library of NCI compounds at a 25 μM concentration, with UCH-L3 at 20 nM. On the basis of the differences in rate obtained in the absence or presence of the NCI compounds, we found several inhibitors for UCH-L3 that exhibited ≥50% inhibition, and notably, all featured a quinone motif in their structure. When these inhibitors were tested at lower concentrations (Figure 5), the LS1 compound showed complete inhibition at 5 μM. Notably, the removal of the

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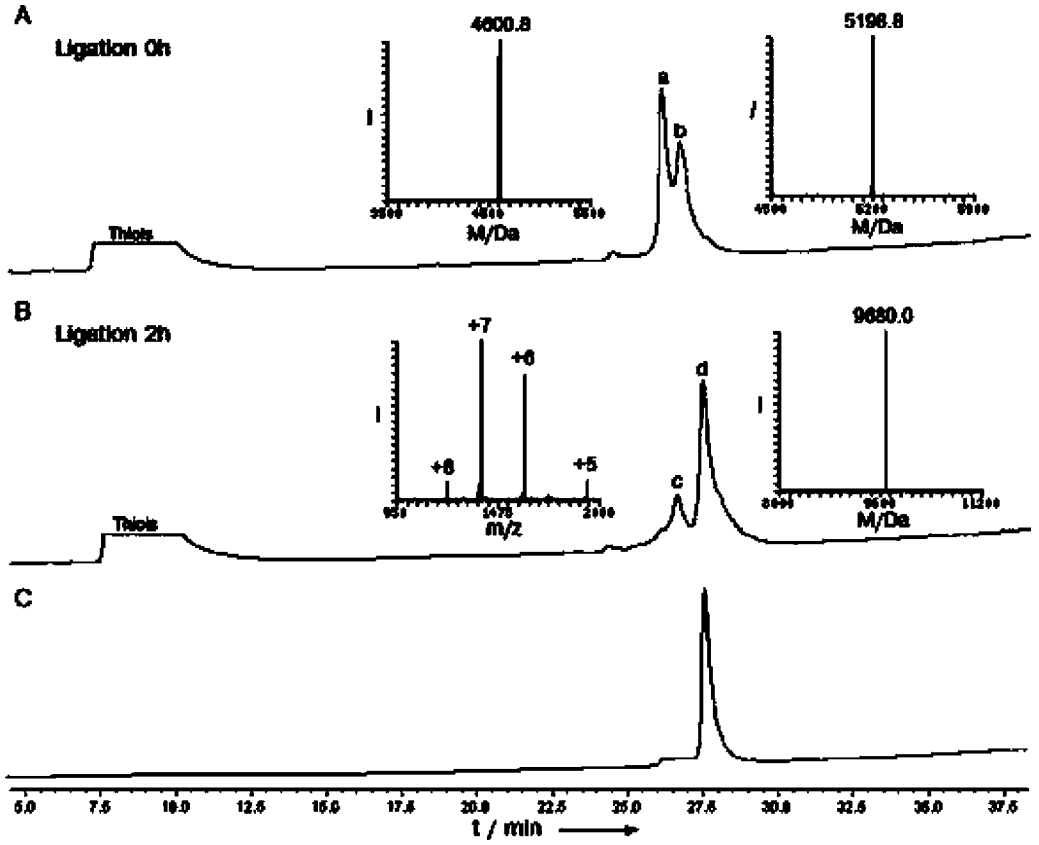

Figure 2. Analytical HPLC traces of the ligation reaction and mass spectrometry analysis (ESI−MS) of {(MCA)-p53(384−389)}-{Ub(Asp52-Dnp)}, **3**. (A) Reaction at time 0 h; peak a corresponds to peptide **2**, with the observed mass of 4600.8 Da (calculated mass, 4598.8 Da); peak b corresponds to Ub(1−45)-thioester with the observed mass of 5198.8 Da (calculated mass, 5198.9 Da). (B) Ligation after 2 h; peak c corresponds to the hydrolyzed byproduct of Ub(1−45)-thioester with the observed mass of 5096.1 Da (calculated mass, 5095.9 Da); peak d corresponds to the desired ligation product **3** with the observed mass of 9680.0 Da (calculated mass, 9678.7 Da). (C) HPLC analysis of purified ligation product **3**.

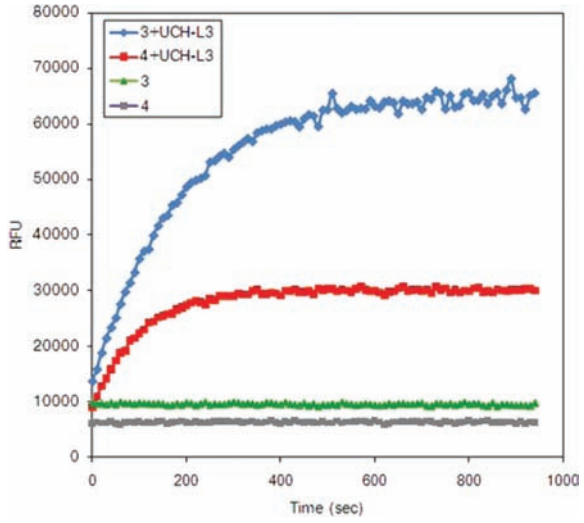

Figure 3. Characterization of the FRET systems based on **3** and **4** using a fluorescence-based assay in the presence (blue and red for substrate **3** and **4**, respectively) or absence (green and gray for substrate **3** and **4**, respectively) of UCH-L3.

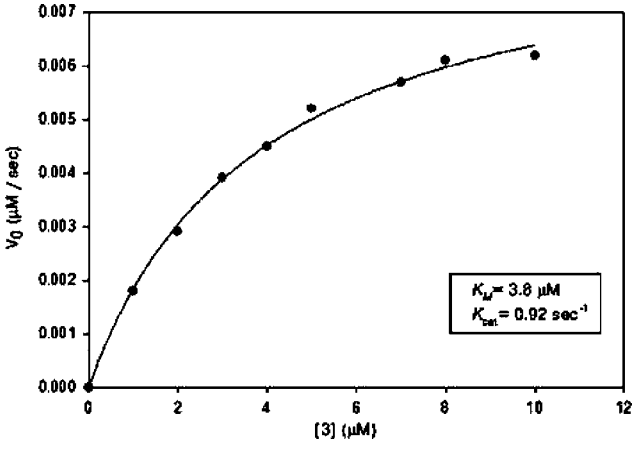

Figure 4. Kinetic characterization of UCH-L3 activity toward substrate **3**. Each value represents the mean ± SE of three independent experiments.

methyl group of LS1 to give the acid form, LS2, led to a ∼10-fold decrease in the potency of inhibition against UCH-L3.

Interestingly, LS2 also known as streptonigrin, is an antitumor agent isolated from cultures of *Streptomyces flocculus*, which belongs to a group of antitumor agents that possess an aminoquinone moiety, such as mitomycin C, rifamycin, and geldanamycin.[46] Streptonigrin shows antitumor activity against a broad range of tumors and cancers (e.g., breast, lung, head and neck cancer, lymphoma, and melanoma).[47] Hence, streptonigrin has served as a lead drug molecule for chemical modifications and synthesis so as to correlate specific structure features with the biological activity of the derived molecule.[48−50] To the best of our knowledge, neither streptonigrin

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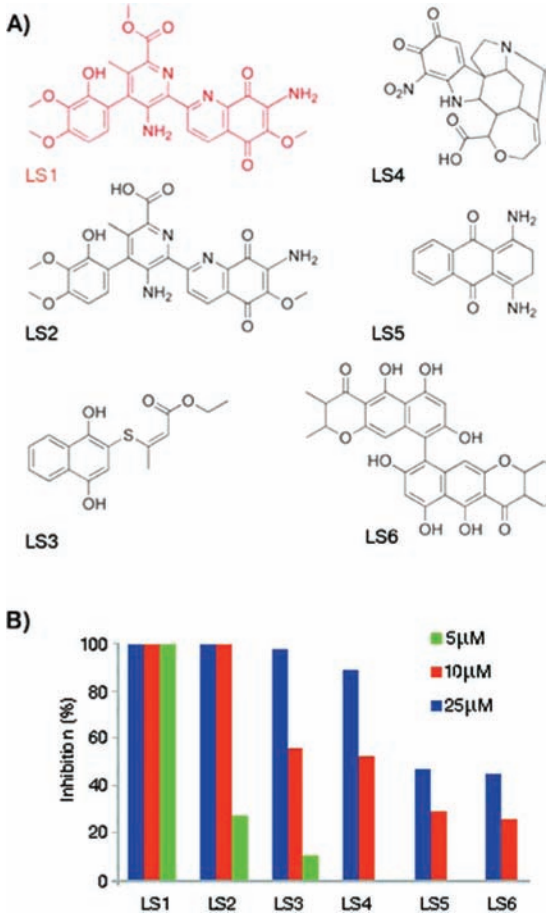

Figure 5. Structures of the selected inhibitors (A) showing their percent of inhibition of UCH-L3 at various concentrations (B). Each value represents the mean ± SE of three independent experiments.

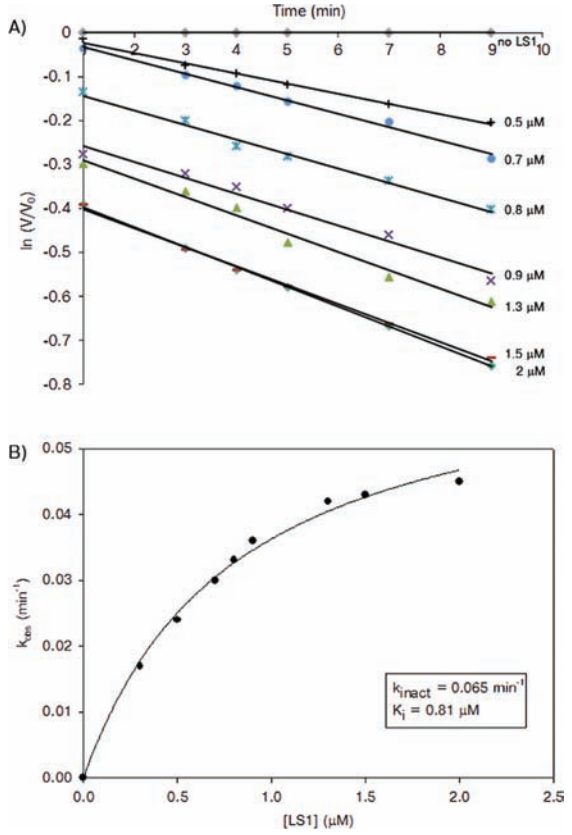

Figure 6. Time-dependent assay of LS1-mediated inhibition. (A) Plot of $\ln(V/V_0)$ versus time to obtain inactivation rate constants (k_{obs}) at each concentration. (B) Plot of the inactivation rate constants (k_{obs}) versus LS1 concentrations to obtain the maximal rate of enzyme inactivation (k_{inact}) and the LS1 concentration required to reach the half-maximal rate of inactivation (K_i). Each value represents the mean ± SE of two independent experiments.

nor any of its analogues have been reported to show inhibitory activity against Cys proteases, in general, or against any of the known DUBs.

Since UCH-L3 is a Cys protease and some quinone derivatives had been suggested to inhibit Cys proteases irreversibly, we asked whether LS1 could act as an irreversible inhibitor. To learn more about the inhibitory mechanism of LS1, several additional experiments were carried out. LS1 was tested after different preincubation times with UCH-L3 and showed a clear time-dependent inhibition with k_{inact} = 0.065 min^{-1} and K_i = 0.8 μM (Figure 6). However, when examining whether UCH-L3 undergoes covalent modification by LS1, presumably through alkylation of the Cys residue, we found no change in the molecular weight of UCH-L3 (Supporting Information). Together, the mass spectrometry data and the time-dependent inhibition suggest that LS1 is a reversible, slow-binding inhibitor of UCH-L3. Further studies on the exact inhibitory mechanism of LS1 is required and will be reported upon in due course.

5. Examining the Activity of UCH-L1 with the FRET Assay. Next, we tested whether our FRET assay could be applied to UCH-L1, a scenario that would allow the use of the assay in future studies seeking novel inhibitors. UCH-L1 has high sequence homology to UCH-L3 in terms of conserved catalytic residues (i.e., Cys, His and Asp) involved in the removal of small molecules and amide-conjugates of Ub.[51,52] Yet, UCH-L1 is 200-fold less active than UCH-L3 toward a model Ub amide substrate.[12,16] UCH-L1 is considered as one of the most abundant proteins in the brain[51] and has implicated in Parkinson's disease and several forms of cancer. Not surprisingly, several groups have been searching for potent inhibitors of this enzyme.[16,53] To support these efforts, we tested our HTS with UCH-L1 and found that this enzyme can also cleave our substrate, albeit at a lower efficiency than UCH-L3, consistent with previous data on the slow activity of UCH-L1 with different substrates.[12,16] Nevertheless, we were able to optimize cleavage conditions to obtain a linear increase in cleavage velocity over a 25-min period such that the decrease in fluorescence correlates to enzyme inhibition. To validate this, LS1 was screened at various concentrations and was found to completely inhibit UCH-L1 (0.16 μM) at 2.5 μM after 20 min incubation without noticeable selectivity over UCH-L3 (Figure 7). These results, thus, confirm that this assay could also be used to search for new inhibitors of UCH-L1.

■ SUMMARY

We have developed a highly efficient FRET-based HTS for DUBs by applying our recent advances in the expeditious synthesis of ubiquitinated peptides. The system was synthesized in large quantities that enabled HTS of 1000 compounds from which LS1 was identified as a slow-binding UCH-L3 inhibitor with k_{inact} = 0.065 min^{-1} and K_i = 0.8 μM. This system, which bears the isopeptide bond linkage, was also found to be suitable

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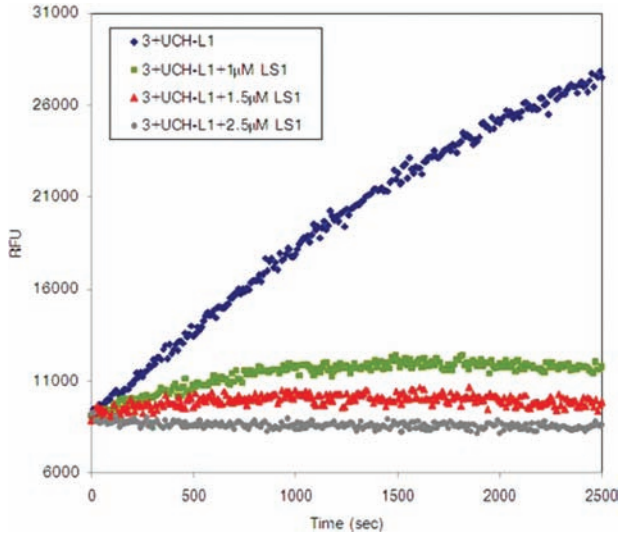

Figure 7. Characterization of substrate **3** with UCH-L1 and its inhibition by various concentrations of LS1.

against UCH-L1 and should enable HTS of libraries and optimization for the discovery of novel inhibitors of DUBs from the UCH family. Moreover, by adjusting this system and taking advantage of our ability to prepare all Lys-linked di-Ub chains, we should be able to extend our strategy to prepare a HTS assay for the specific DUBs that act on these chains. Together, this study provides the first proof of concept of using advanced protein synthesis strategies to assist ongoing efforts targeting different components of the Ub system,[54] including those focusing on the role of DUBs in health and disease, and ultimately, for drug development.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HPLC analysis of UCH-L3 cleavage, circular dichrosim of the different substrates, ^{1}H NMR and mass spectrometry analysis of LS1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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