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Brief Article

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A Fragment-Based Method to Discover Irreversible Covalent Inhibitors of Cysteine Proteases

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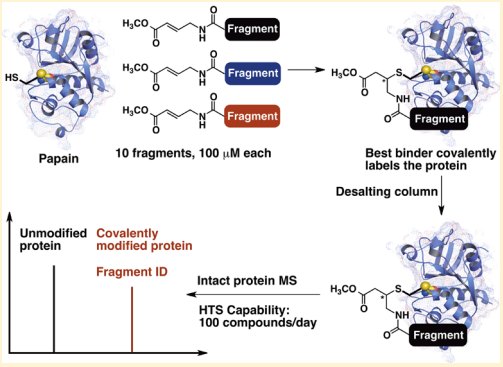
Supporting Information

ABSTRACT: A novel fragment-based drug discovery approach is reported which irreversibly tethers drug-like fragments to catalytic cysteines. We attached an electrophile to 100 fragments without significant alterations in the reactivity of the electrophile. A mass spectrometry assay discovered three nonpeptidic inhibitors of the cysteine protease papain. The identified compounds display the characteristics of irreversible inhibitors. The irreversible tethering system also displays specificity: the three identified papain inhibitors did not covalently react with UbcH7, USP08, or GST-tagged human rhinovirus 3C protease.



INTRODUCTION

Fragment-based drug discovery (FBDD) has emerged as a powerful approach to discover drug leads by exploring greater chemical diversity space with smaller libraries.[1] The major challenge, however, is to detect weak binding interactions between drug-like fragments and their protein targets. Disulfide tethering was developed as one solution to this problem.[2] In this approach, disulfide-containing fragments are covalently trapped on the protein surface via the reversible formation of disulfide bonds. Subsequent MS of the intact protein can identify the covalently bound fragment. The advantages of this method include screening the fragments as mixtures rather than as separate entities. Screening fragments as mixtures increases the throughput capability of the assay and reduces the number of false positives by introducing competition between the fragments. This has proven to be a general and successful approach.[3] Another technique relies on the use of an α-cyanoacrylamide moiety attached to drug-like fragments that react reversibly with noncatalytic cysteines present at the binding site of the protein of interest.[4]

Whether it is possible to design a robust system where the protein can select the best binder from a mixture of electrophilic fragments under irreversible conditions to identify novel leads is not known. Such an approach would be particularly powerful because the identified fragments can subsequently retain their electrophilic tether while being elaborated into a covalent drug. Irreversible tethering would especially benefit the burgeoning field of covalent drug discovery.[5]

However, one concern with such an approach is the danger of selecting the most reactive fragment rather than the fragment with the most specific binding affinity to the protein target.[6] If the electrophilic fragments are too reactive, cysteines or other nucleophilic residues present on the protein surface can undergo nonspecific covalent modifications by the fragments irrespective of their binding affinity.[7] Alternatively, hyper-reactive cysteines or other nucleophilic residues can nonspecifically react with even moderately electrophilic fragments, leading to nonspecific covalent modifications of the protein.[8] In addition, no systematic studies have been done to investigate the kinetic reactivity of cysteine reactive electrophiles attached to a large number (~50) drug-like fragments in order to outline general principles and design rules for irreversible tethering. While this work was in progress, Nonoo, et al. reported the first irreversible tethering method using a small 10-member acrylamide library, which included known reversible thymidylate synthase inhibitor scaffolds.[9] However, a hyper-reactive acrylamide in their library had to be discarded, and no systematic studies have been done further to investigate the reactivity of and outline design rules for drug-like libraries for irreversible tethering. Moreover, there are still no reports of irreversible fragment screening of an unbiased library to identify novel and selective binding fragments. Therefore, whether it is possible to rationally design an electrophilic library of drug-like fragments for irreversible tethering is still a concern.

This report addresses this concern and shows that the proper selection of a cysteine reactive electrophile yields a chemical system that can select weakly bound electrophilic fragments from a mixture and covalently trap the best binders at the highly reactive catalytic cysteine of the model cysteine protease papain. The discovered fragments behave as weak and

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irreversible inhibitors of papain and have novel nonpeptidic structures. The reported method serves as an entry point to discover nonpeptidic inhibitors of other cysteine proteases, which are promising drug targets to treat parasitic infections.[10]

RESULTS

Selecting the Electrophile. To find an electrophile which is suitable for irreversible tethering, we explored the cysteine reactivity profiles of four Michael acceptors: acrylamides **1**, vinylsulfonamides **2**, aminomethyl methyl acrylates **3**, methyl vinylsulfones **4** (Figure 1A,B).

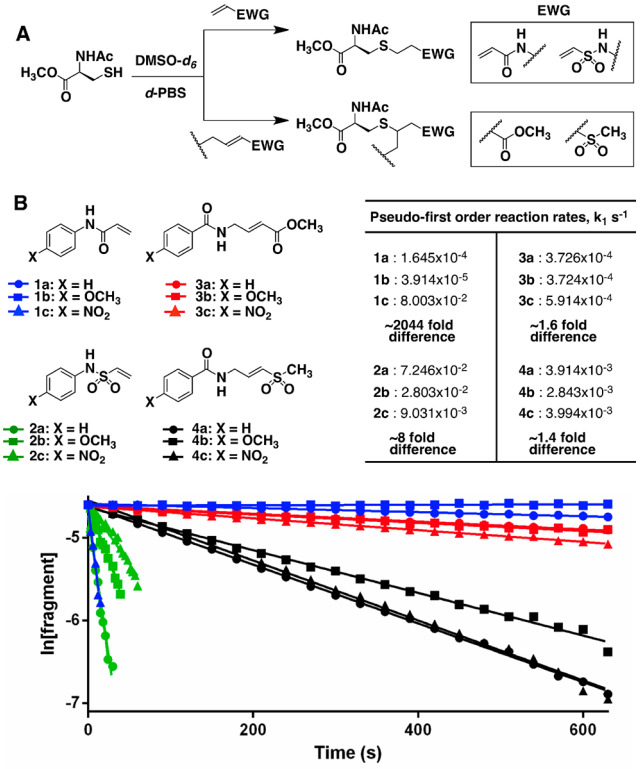

Figure 1. (A) General scheme of NMR rate studies. (B) Chemical structures of the electrophiles **1**–**4** tested for suitability for irreversible tethering and their pseudo-first-order reaction rates with *N*-acetylcysteine methylester at p*D* 8.0 as measured by NMR spectroscopy.

To test how the cysteine reactivity of these electrophiles would be affected by the structure of attached drug-like fragments, we installed acrylamide and vinylsulfonamide electrophiles on aniline, *p*-MeO-aniline, and *p*-NO_2-aniline to yield electrophiles **1a**–**c** and **2a**–**c**. The methyl acrylate and vinylsulfone electrophiles in **3** and **4** were covalently attached to derivatives of benzoic acid, *p*-MeO-benzoic acid, and *p*-NO_2-benzoic acid to yield **3a**–**c** and **4a**–**c**. We envisioned that the different mesomeric and inductive effects of the $-OCH_3$, $-H$, and $-NO_2$ moieties would cause changes in the reactivity of electrophiles **1**–**4** toward cysteine, and these changes would be representative of fluctuations in the reactivity of drug-like fragments toward cysteines. The electrophile that displayed the least fluctuation in reactivity toward cysteine would be the most optimal electrophile to use for irreversible tethering.

We therefore measured the pseudo-first-order reaction rates for each of the compounds **1**–**4** with *N*-acetylcysteine methyl ester using NMR spectroscopy (Figure 1B).[11] Interestingly, we found that acrylamides **1a**–**c** displayed a ∼2044-fold difference in reactivity, with the $-NO_2$ derivative being the most reactive. Because many drug-like fragments contain an amino group attached directly to electron-deficient aromatic rings, we envisioned that similar to compounds **1a**–**c** there could be large fluctuations in the reactivity of such an acrylamide library toward thiols, which would make this library problematic to use. Indeed, as we mentioned previously, in the first publication detailing irreversible tethering method using acrylamides one fragment had to be discarded due to its hyperreactivity.[9]

Vinylsulfonamides **2a**–**c** displayed only an ∼8-fold difference in reactivity toward *N*-acetylcysteine methyl ester. This result was encouraging, yet we sought electrophiles with an even more narrow range of reactivities. To our delight, both the **3a**–**c** and **4a**–**c** series displayed much more balanced reactivity toward cysteine, with only 1.6- and 1.4-fold differences, respectively, in the reactivity between the least reactive and the most reactive electrophiles. We chose acrylates **3** for further studies because they were 10-fold less reactive than vinylsulfones **4** and therefore less prone to nonspecific covalent modifications of nucleophilic amino acid side chains in proteins.[12]

In addition, acrylates are established electrophiles present in irreversible inhibitors of cysteine proteases with activities in vitro biochemical and cell-based assays.[13] Importantly, in vitro k_{inact}/K_i values of acrylate cysteine protease inhibitors vary dramatically (up to 170-fold in the case of falcipain inhibitors) with changes in the structure of the peptide-derived directing group.[13] This indicates that useful levels of kinetic discrimination can be achieved upon structural changes of the directing group despite the high reactivity of the catalytic cysteine in cysteine proteases. Moreover, the acrylate functionality has been shown to have good pharmacokinetic properties and is present in an orally bioavailable inhibitor of human rhinovirus 3C protease.[14] These considerations further confirmed to us that acrylate **3** is a good starting point for validating irreversible tethering. Because known acrylate inhibitors are mostly peptidic in nature, we sought to discover novel nonpeptidic inhibitors with irreversible tethering.

Building and Characterizing the Library. We further validated the utility of electrophile **3** as a thiol-reactive tether by making a library of 100 structurally diverse drug-like fragments **6**–**105** containing this electrophile. The library was constructed with an HBTU amide coupling with commercially available carboxylic acid fragments (Figure 2A). The acids were selected with "rule of three" criteria[15] and a subsequent diversity analysis. We measured the reaction rates for the first 50 fragments to confirm that this library would have balanced cysteine reactivity and could be used for irreversible tethering (Figure 2B). As we expected, these 50 fragments displayed a narrow range of chemical reactivities similar to **3a**–**c**. Overall, we observed only a 2.4-fold difference in the reactivity between the least reactive (k_1 3.327×10^{-4} s^{-1}) and the most reactive (k_1 7.951×10^{-4} s^{-1}) fragment (Figure 2B, Supporting Information (SI) Table S1).

Screening against the Cysteine Protease Papain. Encouraged by these findings, we asked if we could use this library to discover specific covalent enzyme inhibitors with novel structures. As a model protein we chose the cysteine protease papain. We reasoned that the presence of a highly reactive active site cysteine in papain would serve as a stringent specificity test for the proposed irreversible tethering method. We hypothesized that if the designed chemical system displays

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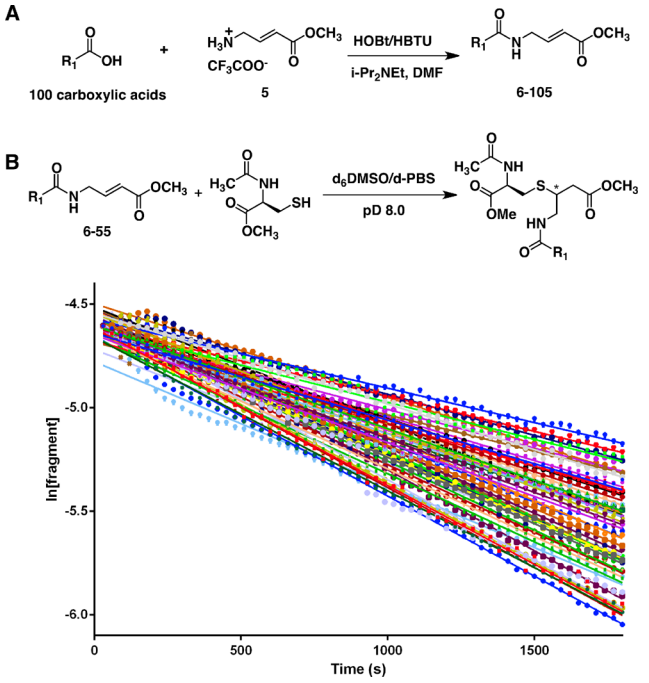

Figure 2. (A) Design and synthesis of the fragment library. (B). Pseudo-first-order NMR rate plots of the reaction of compounds **6**–**55** with *N*-acetyl cysteine methyl ester. Different colors represent different fragments.

specificity in the presence of the highly reactive catalytic cysteine of papain, this system could also be used to discover ligands for less reactive noncatalytic cysteines. In addition, papain is the founding member of a large family of cysteine proteases, so if the developed system produced inhibitors of papain, it could serve as an entry point to discover inhibitors of other medically relevant cysteine proteases.[16] For our initial screening, we used a simple MS assay similar to the original disulfide tethering screening conditions.

Papain (10 μM) was incubated for 1 h with 10 reaction mixtures that each contained 10 electrophilic fragments (100 μM each) (SI Table S2). Each fragment in the reaction mixture had a unique molecular weight (at least 5 Da difference from the closest fragment) to ensure that whole protein ESI-MS could identify candidate hits unambiguously. Hits were defined as any compounds which labeled papain more than 50%. Remarkably, under these reaction conditions, we observed strong monolabeling of papain by three electrophilic fragments in three separate reaction mixtures: **6**, **7**, and **8** (Figure 3). Such selectivity is impressive, given a 9-fold excess of other cysteine reactive electrophiles over compounds **6**, **7**, and **8**. Moreover, we did not detect significant covalent modification of papain with the other seven reaction mixtures (SI Figure S1). This is despite the fact that these reaction mixtures contain a 100-fold excess of cysteine reactive electrophiles relative to the highly reactive catalytic cysteine of papain. Furthermore, compounds **6**, **7**, and **8** labeled papain even though the corresponding reaction mixtures contained fragments that were equally or even more reactive toward *N*-acetylcysteine methyl ester. This observation further suggests that in our system the chemical structure of the drug-like fragment rather than its reactivity determines the covalent labeling of papain.

Additionally, compounds **6**–**8** demonstrated robust labeling of papain in the presence of 10 mM glutathione (1000-fold

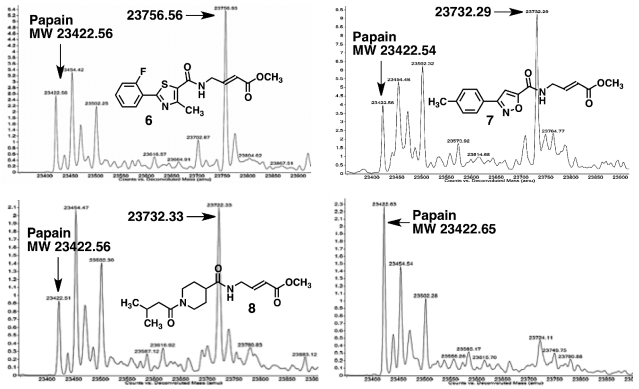

Figure 3. Representative MS spectra of four reaction mixtures containing 10 electrophilic fragments each screened against papain. Papain (10 μM) was incubated with a mixture of 10 electrophilic fragments (100 μM each) for 1 h, followed by gel filtration and ESI-MS of the intact protein.

excess relative to papain), confirming that compounds **6**–**8** covalently label papain due to their specific binding to papain and not simply due to their greater thiol reactivity (SI Figure S2).

We were unable to directly confirm labeling of the catalytic cysteine because the catalytic cysteine peptide was not detectable by ESI-MS or MALDI-TOF upon digestion with trypsin, chymotrypsin, or Glu-C proteases. However, preincubation of papain with compounds **6**–**8**, followed by treatment with **106**, a known papain inhibitor which reacts with its catalytic cysteine,[17] did not cause dilabeling of papain (SI Figure S3A). Additionally, pretreatment of papain with **106** also blocked subsequent labeling by compounds **6**–**8** (SI Figure S3B). These results suggest that compounds **6**–**8** and inhibitor **106** most likely react with the same nucleophilic residue of papain. Compounds **6**–**8** labeled papain in a 1:1 stoichiometry at both 100 μM and 1 mM concentrations, confirming the specificity of these electrophiles for cysteine (SI Figure S4). Moreover, the observed covalent labeling of papain was irreversible because the covalent adducts were stable to dialysis. (SI Figure S5).

Papain Inhibition Assay. We subsequently tested compounds **6**–**8** in an enzymatic assay to confirm that they inhibited papain in the concentration and time dependent manner that is characteristic of irreversible inhibitors.[18] Using assay conditions previously described for papain,[17] we determined k_{inact}/K_i values for compounds **6**–**8** (Figure 4, SI Figure S6). Notably, compound **7** was as potent at inhibiting papain as a known moderate peptidic inhibitor **107**,[17] but compounds **6**–**8** were less potent inhibitors than the known strong peptidic papain inhibitor **106**. This result is expected because irreversible tethering is designed to detect weak binding interactions between the drug-like fragments and the protein target to identify initial hits. Compounds **6**–**8** were all more potent inhibitors than the weak peptidic papain inhibitor **108**.[17] A negative control molecule **19**, which did not label papain in our screen, was ~10-fold less potent at inhibiting papain than the least potent inhibitor **6** and ~33-fold less potent than the most potent inhibitor **7**. Remarkably, compounds **6**–**8** do not have a peptidic character in comparison to traditional cysteine protease inhibitors, including known papain inhibitors (Figure 4).[19] This result is significant because the proposed method can serve as an entry point to

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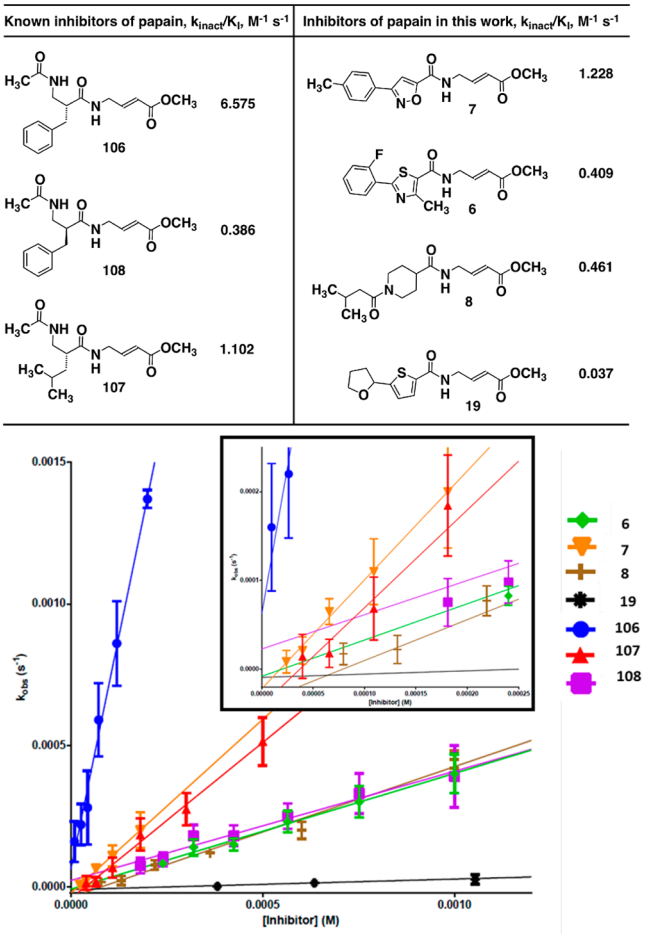

Figure 4. Second-order inhibition plots and k_{inact}/K_i values for papain inhibitor compounds **6**–**8** and known papain inhibitors **106**–**108**. Note: testing of compound **7** at higher concentrations was limited by poor solubility.

discover other types of nonpeptidic inhibitors for medically relevant cysteine proteases, avoiding the known undesirable pharmacological properties of peptidic inhibitors.[20]

Counter-Screen against Human Rhinovirus 3C Protease (HRV3C), USP08, and UbcH7. To further test the specificity of the developed irreversible tethering system, we conducted a counter-screen of the same set of 100 compounds (10 mixtures of 10 compounds each) against three other enzymes: human rhinovirus 3C protease, the deubiquitinase USP08, and the E2 ubiquitin-conjugating enzyme UbcH7. Human rhinovirus 3C protease is a cysteine protease, an antiviral drug target, and there are known orally bioavailable acrylate inhibitors for this protease.[14] Recent reports have indicated that targeting USP08 is a promising approach to overcome gefitinib resistance in lung cancer,[21] while UbcH7 on the other hand regulates the entrance into and progression through the S-phase of the cell cycle.[22] As a source of HRV3C protease for our experiments we used GST-tagged HRV3C protease. We have found that HRV3C protease was labeled by compound **22** (~35% labeling) as well as compounds **32** and **98** (~20% labeling) under the same reaction conditions (SI Figure S7). None of the three papain hits and remaining electrophilic fragments reacted with HRV3C protease under these reaction conditions, indicating that these hits are selective binders. Although the three HRV3C hits did not label their target as strongly as the papain hits did, they could eventually be optimized into potent inhibitors of this clinically important cysteine protease. For UbcH7 and USP08, we found that none of compounds **6**–**105** covalently modified these enzymes (SI Figures S8, S9) under the same reaction conditions. When we increased the incubation time with USP08 to 4 h, we found two compounds that weakly labeled ~30% of USP08. One was compound **6**, while another was a unique compound (**9**) (SI Figure S10). The other two papain inhibitors **7** and **8** did not label USP08 even after 4 h, showing that our system is well behaved and can identify selective binders.

DISCUSSION AND CONCLUSION

In summary, we have rationally designed a chemical system for screening mixtures of electrophilic fragments against the catalytic cysteine of a protein of interest, which eliminates the concern that such an approach would only select the most reactive fragment or otherwise be nonspecific due to the high reactivity of the catalytic cysteine. Using this method, we identified specific, nonpeptidic covalent inhibitors of the cysteine protease papain, which contain novel chemical scaffolds. This is the first example of a successful screen of an unbiased library of electrophilic compounds under irreversible conditions which led to the discovery of specific and novel inhibitor structures for the enzyme of interest.

The key advantage of the reported method is its simplicity. For example, electrophilic fragments **6**–**105** are prepared in one step from commercially available materials using a robust amide bond formation reaction. Moreover, the synthesized electrophilic fragments elicit a predictable and narrow range of chemical reactivities toward thiols and do not react with other nucleophilic residues such as histidine or lysine. The developed screening protocol is simple and is moderately high-throughput. One hundred compounds can be screened in one day without the use of special robotic equipment. Moreover, mixtures of electrophilic fragments can be stored as DMSO stocks, transported, and used to screen fragments against novel protein targets. The developed irreversible tethering method displays a high hit rate (3% for papain and HRV3C protease), and the discovered papain inhibitors have weak potency in enzymatic assays. These are typical characteristics of fragment-based drug discovery methods. Our failure to discover strong inhibitors of USP08 and UbcH7 is most likely not due to the limitations of the method but rather due to the limited sampling of chemical space because only 100 fragments were prepared and tested. Because USP08 and UbcH7 do not have classical hydrophobic binding pockets like the P2 substrate pocket of papain, it is likely that a larger library will be required to find adequate binders.

While the developed approach can be used to tether weakly bound fragments to the highly reactive catalytic cysteine of papain, it remains to be seen whether the same approach can be used to tether weakly bound fragments to noncatalytic cysteines on protein surfaces. We are currently exploring that particular aspect of this technology. Further investigations and applications of the developed method to discover enzyme and protein–protein interaction inhibitors by targeting catalytic and noncatalytic cysteines will be reported in the near future.

EXPERIMENTAL SECTION

Fragment Library Design. Using the Discovery Studio Package with Pipeline Pilot from Accelrys, 94275 commercially available carboxylic acids were identified from the ChemBridge, ChemDiv, MayBridge, NCI, and Sigma-Aldrich libraries using SMARTS query

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strings. Of these, 62000 were removed because they contained reactive functional groups (e.g., acyl halides) or were unsuitable leads (e.g., nitro compounds). Compounds were then filtered based on "rule of three" criteria which were modified to increase the number of resulting compounds: molecular weight (MW) ≤ 350 Da, AlogP ≤ 3, hydrogen-bond acceptors ≤3, hydrogen-bond donors ≤3, rotatable bonds ≤3, and polar surface area ≤80. A principal component analysis and neighborhood algorithm was applied to the 1522 remaining compounds to produce 281 fragments with a 0.75 diversity index. Then 100 of these compounds were initially selected based on affordability and the ease of future analogue synthesis

Synthesis of 6−108. The carboxylic acid fragment (0.2 mmol) was dissolved in dimethylformamide (0.2 M, 1 mL), then **5** (46 mg, 0.2 mmol), HBTU (73.8 mg, 0.16 mmol), and HOBt (29.8 mg, 0.22 mmol) were added, followed by $EtN(i\text{-}Pr)_2$ (100.7 μL, 0.6 mmol). The reaction was stirred at 23 °C for 16 h. TLC at 16 h showed conversion to product. The reaction was quenched with H_2O (5 mL) and extracted three times with CH_2Cl_2 (5 mL). The combined organic layers were washed with 1 M HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and saturated aqueous NaCl (10 mL). The organic layer was dried over $MgSO_4$, filtered, and evaporated. Purified by flash column chromatography with a CH_3OH/CH_2Cl_2, CH_3OH gradient 0−5% to yield compounds **6−108**. Yields ranged from 11% to 100%, with an average yield of 60%. Chemical structures of compounds **6−108** are shown in SI. For initial library creation, compounds were characterized by ^1H NMR and low resolution MS. All compounds tested in enzymatic assays were also characterized by ^{13}C NMR and ≥95% purity was confirmed by HPLC.

NMR Rate Studies. *N*-Acetyl cysteine methyl ester was dissolved in 2:1 deuterated PBS:DMSO-d_6 (78 mM) with 10 mM CH_2Cl_2 as an internal standard. The electrophile (10 mM) was then added immediately prior to acquiring NMR spectra. H^1 spectra were taken every 30 s for 30 min (or every 4 s for 5 min for highly reactive compounds **1c** and **2a−c**). The integrals of the vinyl peaks were used to determine the concentration of the electrophile over time. The natural logarithm of the concentration of the electrophile vs time was then plotted using GraphPad Prism software. The linear slope of this plot was used to determine the pseudo-first-order rate constant. Deuterated PBS recipe: 20 mM Na_3PO_4, 50 mM NaCl in D_2O was adjusted to p*D* 8 with DCl solution.

Irreversible Tethering Screening Assay. Papain (Sigma P4762, 10 μM), UbcH7 (recombinantly expressed, 10 μM), GST-264 HRV3C protease (recombinantly expressed, 10 μM), or USP08 (recombinantly expressed, 10 μM) in 50 mM HEPES, 150 mM NaCl, and 0.1 mM EDTA pH 7.5 was treated with a mixture of 10 fragments (SI Table S2) (10 mM DMSO stock solutions, final concentrations: 100 μM of each fragment, and 1% DMSO). The reaction mixture was incubated for 1 h or 4 h at 23 °C before being passed through Zeba gel filtration columns (Thermo, 7K MWCO) to remove unreacted fragments. The protein solution was then immediately analyzed by whole protein LC/ESI-MS.

LC/ESI-MS Protocol. Accurate-mass data were obtained on an Agilent 6210A LC-TOF mass spectrometer in positive ion mode using electrospray ionization. Samples were chromatographed on the LC-TOF instrument using a Poroshell 120 EC-C18 HPLC column (2.1 mm × 50 mm, 2.7 μm), an Agilent Series 1200 HPLC binary pump, and an Agilent Series 1200 autoinjector. The HPLC column was held at 45 °C, and the autosampler was held at 8 °C. Mobile phase A was a solution of 0.1% formic acid in water:acetonitrile (19:1). Mobile phase B was a solution of 0.1% formic acid in acetonitrile. The flow rate was set to 250 μL/min. The gradient used was 0% B for 2 min, ramping linearly to 90% B from 2 to 5 min, holding at 90% B from 5 to 7 min, and then returning to 0% B at 7.1 min. The column was allowed to equilibrate for 2.7 min before the next injection was initiated. The eluent from the column was diverted to waste for the first 2 min. The spectra were acquired from 301 to 3200 Da using a gas temperature of 340 °C, a gas flow of 7 L/min, and the nebulizer gas at 35 psi. The following voltages were used: capillary 4200 V, fragmentor 230 V, skimmer 64 V, and octapole RF peak 250 V. Spectra were acquired at a rate of 1 spectra/s. The data was processed using MassHunter software version B.02.00. Maximum entropy deconvolutions were performed with a mass step of 1, S/N threshold of 30, average mass at 90% of peak height, and 5 charge states minimum.

Papain Activity Assays. Papain (4.8 μM) in 50 mM Na_3PO_4 and 2 mM EDTA was preactivated with 1 mM DTT for 30 min. Activated papain (3.84 μM) in 4:1 mixture of 50 mM Na_3PO_4 and 2 mM EDTA at pH 6.2 and acetronitrile was then preincubated for 1 h with varying concentrations of the electrophilic fragment. Every 10 min, 10 μL of the reaction mixture was added to a well of 96-well plate containing 100 μL of 4:1 mixture of 50 mM Na_3PO_4/2 mM EDTA/pH 6.2:acetronitrile with 400 μM Cbz-Gly-ONp. *p*-Nitrophenol product formation was monitored by absorbance at 340 nM (ε: 6800 M^{-1} cm^{-1}) with a Biotek Synergy 4 plate reader. All reactions were performed in duplicate. Product concentration vs time was plotted with GraphPad Prism software, and the initial slope was calculated to determine enzymatic activity (E). The values of k_{inact}/K_i for each inhibitor were then determined according to the method of Kitz and Wilson.[23] Briefly, the slopes of the plots of $\ln(100 \times E_{inhibited}/E_{uninhibited})$ vs time were used to determine the pseudo-first-order inhibition constant k_{obs} for a given concentration of a given inhibitor. The slope of the plot of k_{obs} vs [Inhibitor] was then used to determine the second-order inhibition constant k_{inact}/K_I (because $[I] \ll K_i$, the plots were linear at the concentrations tested).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures, characterization of the synthesized chemical compounds, and supplementary figures. 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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