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Imidazoles are Tunable Nucleofuges for Developing Tyrosine-Reactive Electrophiles

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Imidazole-1-sulfonyl and -sulfonate (imidazylate) are widely used in synthetic chemistry as nucleofuges for diazotransfer, nucleophilic substitution, and cross-coupling reactions. The utility of these reagents for protein bioconjugation, in contrast, have not been comprehensively explored and important considering the prevalence of imidazoles in biomolecules and drugs. Here, we synthesized a series of alkyne-modified sulfonyl- and sulfonate-imidazole probes to investigate the utility of this electrophile for protein binding. Alkylation of the distal nitrogen activated the nucleofuge capability of the imidazole to produce sulfonyl-imidazolium electrophiles that were highly reactive but unstable for biological applications. In contrast, arylsulfonyl imidazoles functioned as a tempered electrophile for assessing ligandability of select tyrosine and lysine sites in cell proteomes and when mated to a recognition element could produce targeted covalent inhibitors with reduced off-target activity. In summary, imidazole nucleofuges show balanced stability and tunability to produce sulfone-based electrophiles that bind functional tyrosine and lysine sites in the proteome.

Introduction

Imidazole is a five-membered nitrogen heterocycle that has found widespread use in organic chemistry because of its distinct chemical properties (e.g., aromaticity and basicity). When attached to sulfonyl and sulfonate groups at the N1 position, the resulting sulfonyl- and sulfonate-imidazole (also referred to as imidazylates), and activated analogs (N3 methylated imidazoles),[1] can serve as effective nucleofuges in various functional group transformations[2] including nucleophilic substitution reactions.[3] In addition, imidazole-1-sulfonyl azide is widely adopted in synthetic chemistry as an inexpensive, stable, and effective alternative to triflyl azide as a diazotransfer reagent.[2l,m]

The biological applications of imidazoles are exemplified by the critical role of this heterocycle as a side chain group of histidine residues on proteins.[4] Notably, histidine mediates important acid-base chemistry in the active site of enzymes to enhance the nucleophilicity of catalytic serines in proteases, esterases, lipases, and other members of the serine hydrolase superfamily.[4b,c,5] Imidazoles are also important components of neurotransmitters including histamine.[6] The importance of imidazoles for molecular recognition in biological systems is further illustrated by its prevalence as a component of drugs that modulate a variety of proteins involved in inflammation, infectious disease, and cancer.[7]

To date, the sulfonyl- and sulfonate-imidazole unit serves as a relatively inert nucleofuge but has demonstrated leaving group (LG) capabilities when activated, for example, to the imidazolium ion for amine coupling reactions.[1d,e,8] For biological studies and chemical proteomics specifically, only a handful of applications of imidazole as a LG have been reported.[9] For example, Huang *et al.* explored protein interactions with the clinical candidate but neural toxic drug BIA 10-2474, using a similar structured acyl imidazole probe.[10] The Hamachi group discovered Ligand-Directed Acyl Imidazole (LDAI) chemistry, where target selectivity has been achieved via high ligand specificity and controllable reactivity of the alkyloxyacyl imidazole linker.[11] The Hamachi group recently reported the utility of LDAI for drug and target discovery. For example, ligand-directed labeling of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-activated subtype of the glutamate receptor family (AMPAR) was achieved in neurons both *in situ* and *in vivo*.[12] Tamura *et al.* reported ligand-directed labeling using an *N*-acyl-*N*-alkyl sulfonamide in live cells, exemplified by selective labeling of K58 in Hsp90α.[13] Recent applications of acyl imidazoles utilized copper-dependent bioconjugation of proximal proteins at sites of elevated labile copper in live cells.[14]

Here we developed a series of imidazole-1-sulfonyl and -sulfonate probes to investigate chemical reactivity and application of this compound class for chemoproteomics. We discovered that the imidazolium functioned as a 'supercharged' nucleofuge that broadly reacted with nucleophiles in solution but was not suitable for biological applications because of stability issues. On the opposite end of the reactivity spectrum, sulfonate imidazoles (Imates) were largely inert against nucleophiles in solution and proteomes. Aryl sulfonyl imidazoles (Imyl), however, exhibited a tempered reactivity that could be

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tuned, by substitutions at the 4-position of the imidazole nucleofuge, for covalent binding to lysine and tyrosine sites on proteins in live cells.

Results

Synthesis of Sulfonyl-Azoles

Sulfonyl-azole (SufAz) probes were synthesized by (i) coupling propargylamine to 4-(chlorosulfonyl) benzoyl chloride followed by (ii) nucleophilic substitution by the azole to yield the alkyne-modified SufAz (sulfonyl-tetrazole, -pyrazole, and -imidazole) probes. The alkyne handle enables detection of probe-modified sites in chemoproteomic workflows as previously described.[15] The imidazole sulfonate probe (Imate-01) was synthesized in a similar fashion where 1,1′-sulfonyldiimidazole was coupled with 4-hydroxy-*N*-(prop-2-yn-1-yl) benzamide and purified via silica gel flash chromatography (Figure 1). To determine whether the reactivity of SufAz probes could be modulated, we synthesized analogs bearing electron-withdrawing (EWG) and -donating (EDG) elements on the heterocyclic group. Methylation of the imidazole using methyl trifluoromethanesulfonate could increase the LG ability of this heterocycle. This activated counterpart was designated Imyl-01+.

HPLC Reactivity Analyses of Sufaz Probes

Reactivity of SufAz probes was initially evaluated in solution against nucleophiles using a high-performance liquid chromatography (HPLC) assay as previously described (Figure 2A and B).[15b] We selected a series of nucleophiles for evaluation to mimic side chains of amino acids in proteins: *p*-cresol (tyrosine), *n*-butylamine (lysine), *n*-butanethiol (cysteine), butyric acid (aspartic/glutamic acid) and propionamide (glutamine/asparagine). The reaction was initiated by addition of 1,1,3,3-tetramethylguanidine (TMG) base and progress was monitored at set timepoints by quantifying the area under the curves (AUC) of probe consumption (Figure 2B and S1).

First, we evaluated the LG ability of imidazole for reaction of Imyl-01 with various nucleophiles. Imyl-01 was mostly consumed after 6 hours of reaction time with *p*-cresol, whereas only ~50% and ~15% consumption was observed with butanethiol and *n*-butylamine mimetics, respectively (Figure 2C). A phenyl substitution on the imidazole (Imyl-02) did not have a significant effect on reactivity compared with Imyl-01 ($t_{1/2} \approx 100$ min), and both Imyl-01 and Imyl-02 were overall less reactive than the sulfonyl-triazole (SuTEx[15b]) congener HHS-481 ($t_{1/2} = 1.1$ min, Figure 1B and 2D). We found that Py-02 and Tet-02 were largely inert under the reaction conditions

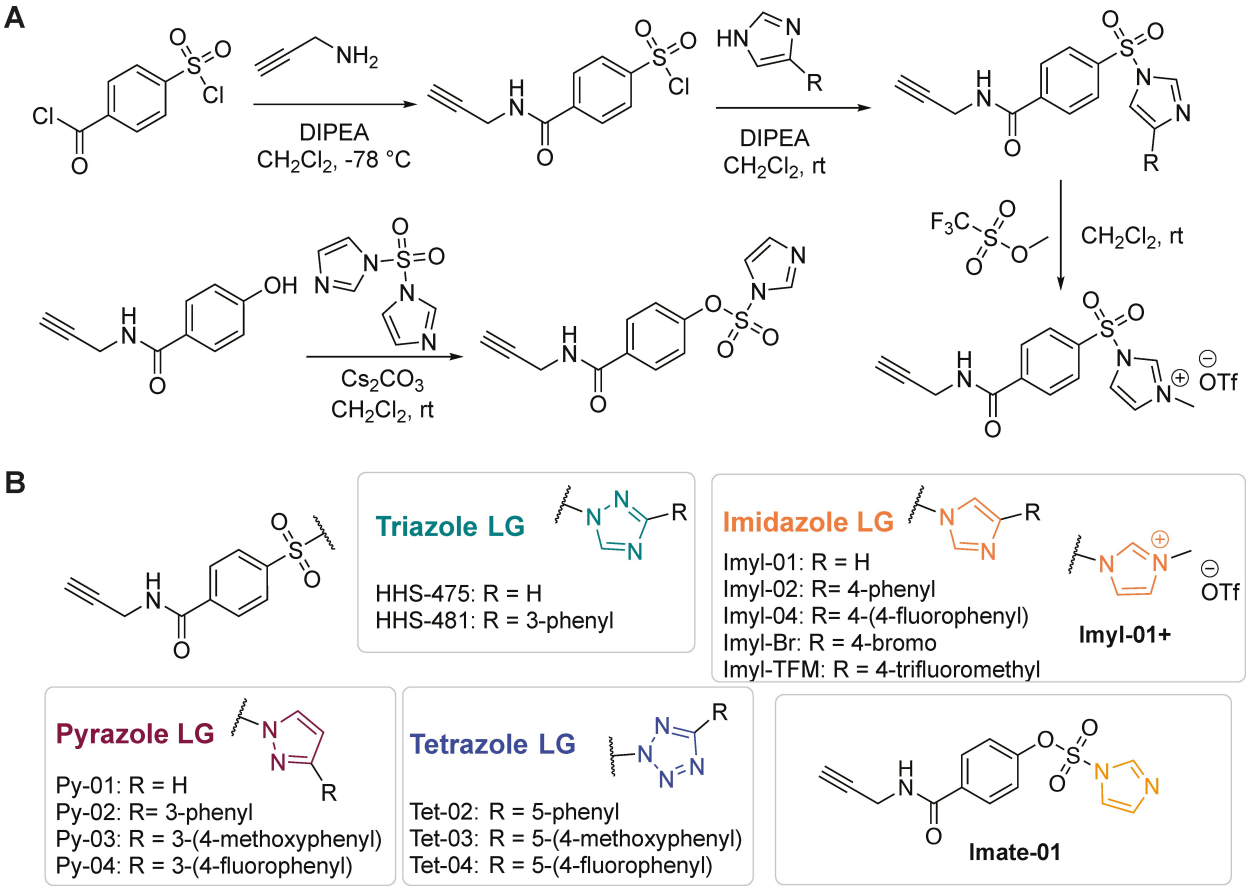

Figure 1. Synthesis of sulfonyl-azole (SufAz) probes. General synthetic scheme (A) for evaluating the effects of various heterocyclic leaving groups (Py = pyrazole; Imyl = imidazole; Tet = tetrazole) on activity of SufAz probes (B). Sulfonyl-triazole (SuTEx) electrophiles are included for comparison.

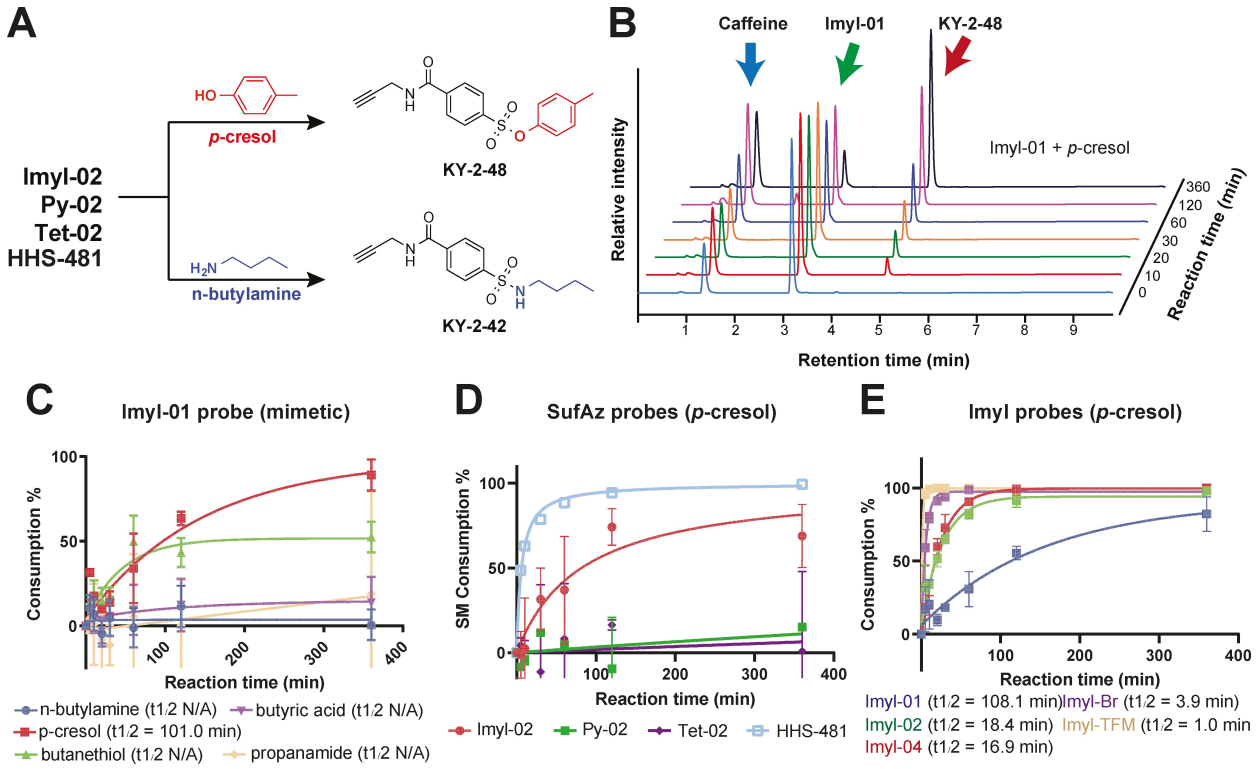

Figure 2. Solution reactivity of SufAz probes as determined by HPLC. A) Representative reactions of SufAz probes with nucleophiles that mimic side chain groups of tyrosine (*p*-cresol) and lysine (*n*-butylamine). B) Overlay of HPLC chromatograms of SufAz reaction as a function of time. The blue arrow denotes the caffeine internal standard. The green arrow denotes starting material (Imyl-01) and the red arrow denotes the probe-nucleophile product, which was confirmed by a stand-alone injection of a synthetic standard (e.g., the Imyl-01-*p*-cresol adduct KY-2-48). Plots of probe consumption as a function of time for (C) Imyl-01 and amino acid mimetics, (D) Imyl-02, Py-02, Tet-02 and HHS-481 reaction with *p*-cresol, and (E) reaction of *p*-cresol with Imyl probes.

against both *p*-cresol and n-butylamine (Figure 2D and Figure S2, respectively).

Next, the imidazole was modified to alter LG ability in order to assess whether reactivity of sulfonyl-imidazoles was tunable. Installing EWGs on the imidazole increased the reactivity of Imyl probes with *p*-cresol. A 4-fluorophenyl substitution (Imyl-04) resulted in a slight increase in reactivity; however, addition of a EWG directly to the imidazole, such as a 4-bromoimidazole (Imyl-Br) or 4-trifluoromethylimidazole (Imyl-TFM), significantly increased reactivity (Figure 2E). We observed rapid reaction of Imyl-01 + with *n*-butylamine and *p*-cresol, which supports the imidazolium functioning as a highly activated nucleofuge. Our findings match previous reports of arylsulfonyl imidazolium triflates as effective sulfonating reagents for preparing sulfonamides and sulfonates.[1b–e,8] Imyl-01 + appeared to react rapidly with all nucleophiles tested and may indicate potential stability issues in solvent due to general hydrolysis ($t_{1/2} < 1$ min, Figure S3A). Next, we compared reactivity of sulfonyl (Imyl-01)- and sulfonate (Imate-01)-imidazole probes by HPLC and found that Imate-01 was largely inert under the reaction conditions tested (Figure S3B).

Chemical Proteomic Evaluation of Sufaz Probes

To evaluate activity of SufAz probes at protein sites, HEK293T cell lysates were treated with compounds (100 μM, 37 °C, 30 min) followed by copper-catalyzed azide-alkyne cycloaddition (CuAAC) with a rhodamine-azide fluorescent tag and SDS-PAGE analysis. Differences in probe reactivity were assessed by comparing intensity of fluorescent protein bands detected.

Results from SDS-PAGE were largely consistent with the HPLC assay results described above. Probes containing a pyrazole or tetrazole LG (Py and Tet analogs) were relatively inert in the proteome. In contrast, Imyl-01 showed enhanced activity compared with Imyl-02 and Imyl-04 (Figure 3A). Compared to HHS-475, Imyl-01 binding activity was dramatically lower in HEK293T, Jurkat, and DM93 lysate (Figure S4). Both Imate-01 and Imyl-01 + showed minor activity and the lack of activity for the latter is likely due to hydrolysis of the sulfonyl-imidazolium electrophile in aqueous conditions (Figure 3B). The imidazole LGs modified with EWGs such as a 4-bromo (Imyl-Br) or 4-trifluoromethyl (Imyl-TFM) showed augmented binding activity with evidence for tunable binding to proteins in lysates (Figure 3B and Figure S5).

To determine if Imyl probes are cell permeable, we treated DM93 cells with Imyl-01 at various time points (25 μM, 10–120 min) and observed time dependent protein labeling with the highest fluorescent probe labeling observed at 2 hours

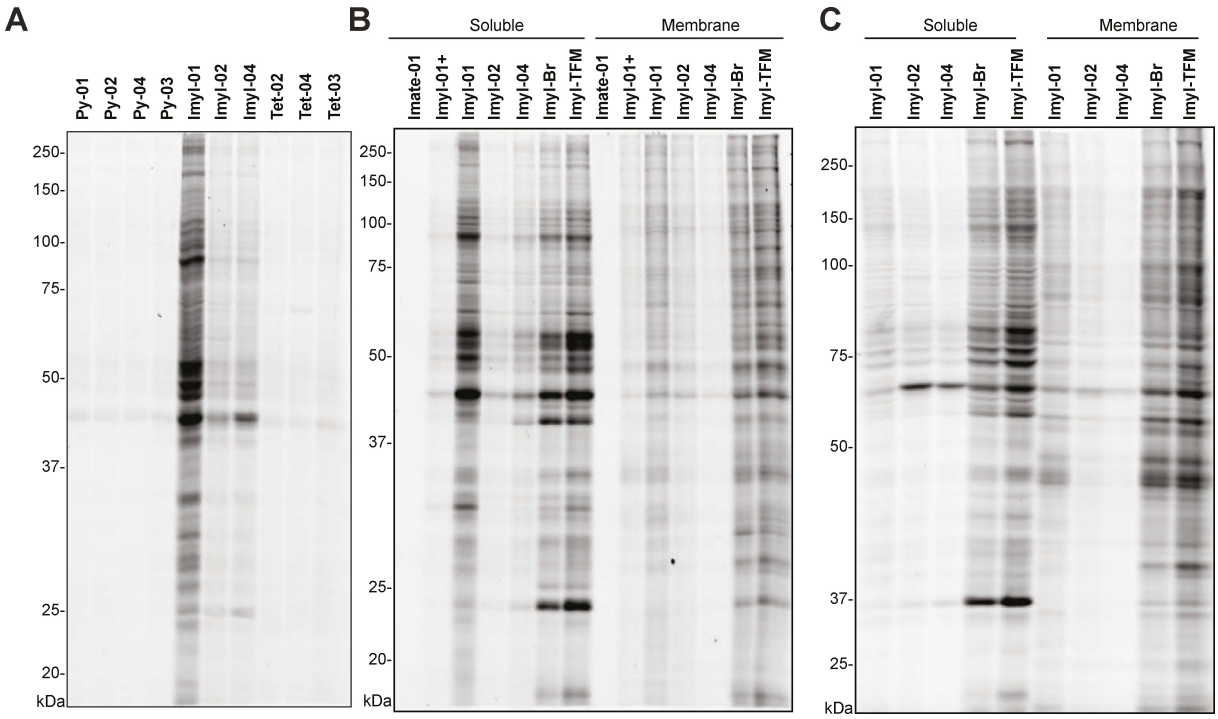

Figure 3. SufAz probe binding activity in lysates and live cells. A) Gel-based chemical proteomic evaluation of SufAz probe labeling of HEK293T soluble proteomes (100 μM, 30 min, 37 °C). B) Comparison of SufAz probe labeling activity in DM93 soluble and membrane lysate (100 μM, 30 min, 37 °C). C) Evaluation of SufAz probe labeling in live HEK293T cells (100 μM probe, 2 hr). Each gel is representative of 3 independent replicates.

using gel-based chemical proteomics (Figure S6). We expanded our cellular treatment studies to additional Imyl analogs (100 μM, 37 °C, 120 min) in HEK293T cells and found the *in situ* labeling profiles were comparable with the lysate evaluations, providing evidence for cell permeability and further corroborated the tunable nature of this electrophile (Figure 3C). Based on the collective data, sulfonyl-imidazoles were determined to be more suitable for chemoproteomic applications because of the tunable nature of this scaffold for covalent binding to proteins in lysates and live cells.

Arylsulfonyl Imidazoles are Tempered Tyrosine- and Lysine-Reactive Probes in Cells

We performed liquid chromatography-mass spectrometry (LC-MS/MS) chemical proteomics to identify the target proteins and binding sites of Imyl-01 and the 4-substituted counterparts. SILAC light and heavy DM93 cells were differentially treated with DMSO or Imyl probes *in situ* (100 μM, 2 hrs, 37 °C). After treatment, cells were harvested, washed, and fractionated to yield the soluble proteome that subsequently underwent CuAAC conjugation with a desthiobiotin-PEG_3 azide tag. The tagged proteins were digested with trypsin and probe-modified peptides were enriched by avidin affinity chromatography, eluted, and analyzed by LC-MS/MS as previously described (Figure 4A and B).[15] High quality probe-modified peptides were identified using quality control criteria that included a 1% protein false discovery rate (FDR), Byonic score $\geq$300, and $\leq$5 ppm mass accuracy .[15]

For the imidazole probes, probe modification occurred principally on tyrosine (Y) and lysine (K) residues, with 583 tyrosine and 289 lysine sites (corresponding to 439 total proteins) reliably quantified (SILAC ratio or SR$\geq$5) across at least 2 replicates (n$\geq$2) of each probe treatment. Proteome coverage by Imyl- probes was variable. The Imyl-TFM probe showed highest apparent reactivity (545 distinct probe-modified sites; 419 sites unique to Imyl-TFM). Imyl-01 was less reactive than Imyl-TFM, resulting in 393 probe-modified sites with >70% of these sites unique to Imyl-01. Imyl-Br showed tempered reactivity with only 82 modified sites that largely overlapped with Imyl-01- and Imyl-TFM-modified sites (Figure 4C). The proteomic reactivity of Imyl-02 and Imyl-04 was drastically reduced with no detectable sites for Imyl-02 and only a single probe-modified site for Imyl-04 that meet our quality control criteria probe (Table S1). A large fraction of Imyl probe-modified sites (>85%) overlapped with sites detected by sulfonyl-triazole probes in treated cells (aggregate sites from HHS-475 and HHS-481 treatments in DM93 cells, 100 μM, 2 hrs; Figure 4D).

The chemoselectivity for tyrosine (Y) versus lysine (K) modification from cellular labeling studies was variable across the Imyl probes tested. Imyl-01 exhibited a modest preference for modification of lysine over tyrosine (Y/K ratio of <1, Figure 4E). Imyl-TFM and Imyl-Br were more akin to chemoselectivity of SuTEx probes and preferentially modified tyrosines (Y/K ratio >4). Imyl-TFM displayed the highest chemoselectivity

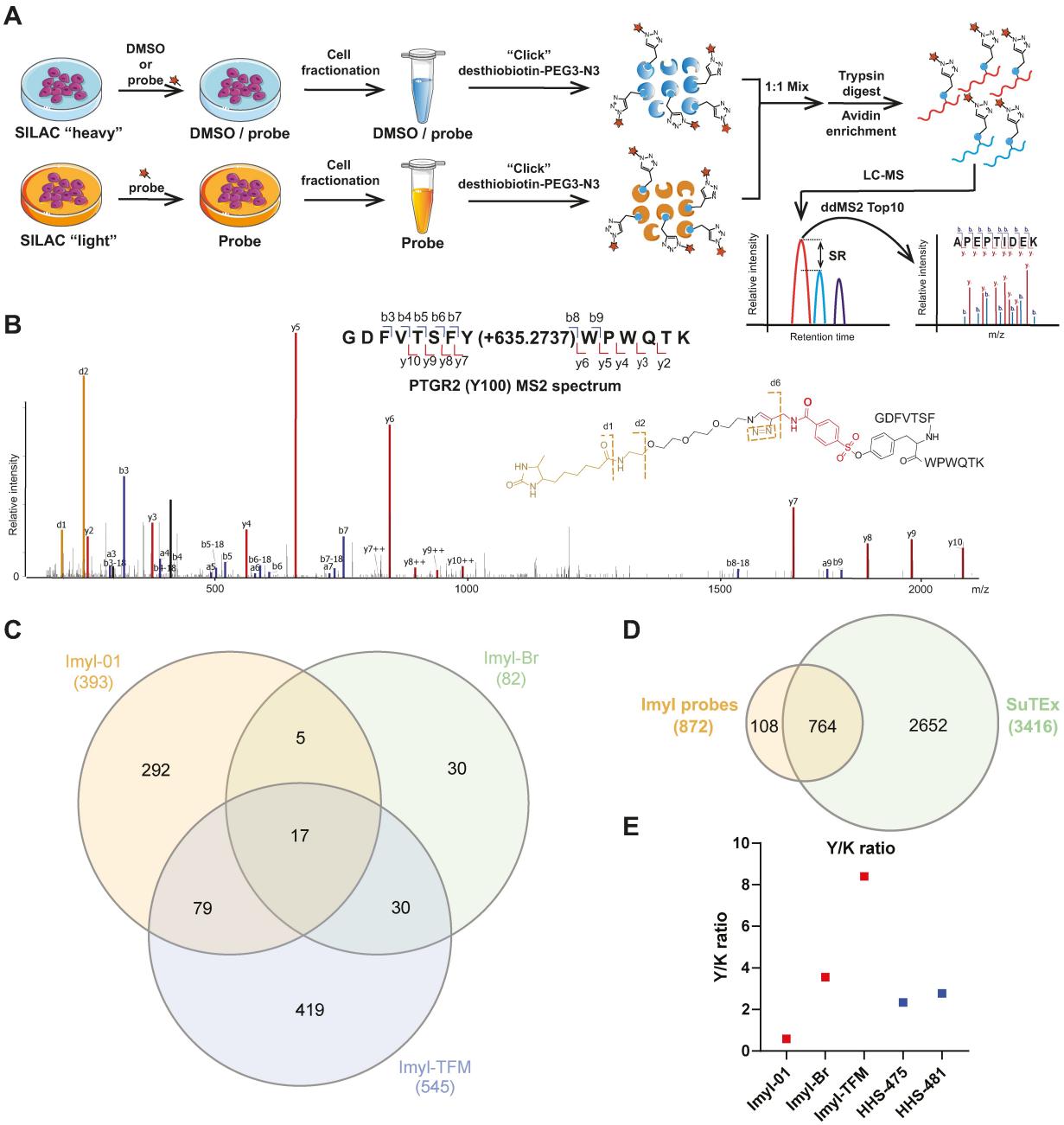

Figure 4. Imyl probes are tempered tyrosine- and lysine-reactive electrophiles. A) SILAC LC-MS/MS workflow for chemoproteomic profiling of Imyl probes. B) Annotated MS2 spectrum of a Imyl-Br modified tyrosine site (Y100) found in PTGR2. The covalent modification adds 635.2737 Da to the modified amino acid. The data was generated from n = 2–4 independent replicates. C) Comparison of overlapping and distinct probe-modified tyrosine and lysine sites detected by Imyl probes evaluated in cellular labeling studies. D) Evaluation of overlapping and distinct probe-modified sites using Imyl compared with SuTEx probes. E) Chemoselectivity (Y/K ratio) of Imyl compared with SuTEx probes. Red squares denote Imyl probes, and the blue squares denote SuTEx probes.

towards tyrosine while maintaining good proteome-wide coverage (Figure 4C and E). A complete list of sites evaluated by SILAC LC-MS/MS chemoproteomics can be found in Table S1.

Proteome-Wide Evaluation of Imyl Fragments

Next, we synthesized a series of Imyl fragment compounds to evaluate whether this sulfone-based electrophile can be used as ligands for targeting protein sites. Our probe binding studies demonstrated high and low reactivity for the TFM- and Br-imidazole LGs, respectively (Figure 4C). We developed Imyl ligands bearing these modified imidazole LGs in conjunction with varying adduct group modifications and tested their ability to ligand sites (Figure 5A). First, we screened the chemical reactivities of the Imyl fragment ligands by HPLC. The cyclopropyl-modified Imyl compound KY-5 did not react appreciably with *p*-cresol or *n*-butylamine. KY-2, modified with the same LG but different AG, displayed moderate activity against *p*-cresol ($t_{1/2}$ = 25 min) and low activity against *n*-butylamine. Both KY-

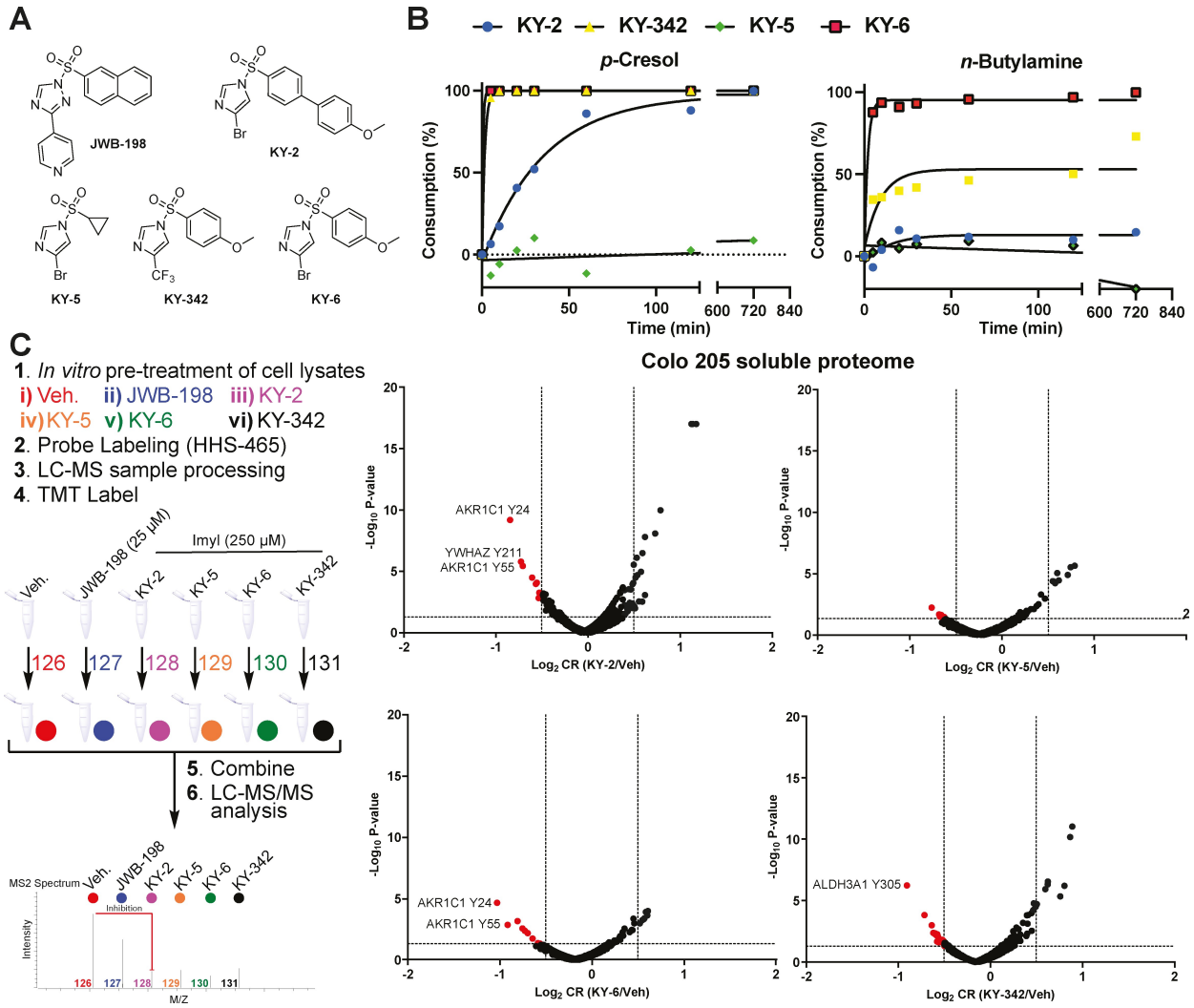

Figure 5. Evaluating Imyl fragment binding activity by TMT-SuTEx. A) Structures of Imyl fragment compounds tested. B) Reactivity of Imyl fragment compounds with tyrosine (*p*-cresol) and lysine (*n*-butylamine) side chain group mimetics were compared by HPLC. C) Experimental workflow for detecting protein sites liganded by Imyl fragment compounds (250 μM, 37 °C, 1 hr) in Colo205 membrane and soluble proteomes as measured by competition of HHS-465 probe labeling. JWB198 (SuTEx fragment) was tested at 25 μM under the same treatment conditions. Additional details on the TMT-SuTEx assay can be found in the Supporting Methods. Peptide isoforms displayed were quantified using Proteome Discoverer to determine site of binding and calculate a competition ratio (CR) for Imyl fragment binding activity. Isoforms denoted in red have a $\log_2$ CR ratio >0.5 with a *p*-value <0.05. Data are representative of $n=4$ biological and technical replicate analyses.

342 and KY-6 showed high activity towards *p*-cresol while only the latter compound also showed a similar reactivity towards *n*-butylamine ($t_{1/2}<5$ min, Figure 5B).

Next, we performed LC-MS/MS chemical proteomics to identify protein sites liganded by the Imyl fragment compounds evaluated in our HPLC studies. We deployed a 6-plex tandem mass tag (TMT) chemical proteomics workflow for quantitative and multiplexed evaluation of probe-modified sites competed by Imyl fragment pretreatment. TMT achieves isotopic labeling at the peptide level using isotopically labeled, amine-reactive reagents that can be combined in a highly multiplexed fashion to yield an isobaric peptide MS1 mass and 6 unique MS2 reporter fragment ions for simultaneous quantitation by LC-MS/MS.[16] Since Imyl and SuTEx probes principally modify Y and K sites, we used a broad spectrum SuTEx probe for evaluating Imyl fragment compound activity. See Figure S7 and Supporting Information for experimental details of the TMT-SuTEx method.

Colo 205 soluble and membrane proteomes were pre-treated with Imyl compounds (250 μM, 1 hr) followed by labeling with the broad-reactive SuTEx probe HHS-465[17] under comparable labeling conditions. Proteomes were subjected to CuAAC with desthiobiotin-azide, proteolytically digested with trypsin protease, and isotopically labeled with amine-reactive TMT reagents (6-plex). Afterwards, desthiobiotinylated peptides were enriched and subjected to LC-MS/MS analysis as previously described.[17] In total, we detected >9,300 probe-modified sites across ~2,400 proteins from aggregate membrane and soluble proteome datasets using TMT-SuTEx. Probe- and TMT-modified peptides used for quantitation were selected based on quality control metrics including Byonic score (>300) and

mass accuracy (≤5 ppm). See Table S2 for a list of probe-modified sites evaluated by TMT-SuTEx.

Proteome-wide coverage using TMT-SuTEx was comparable to previous studies using SILAC[17] with the added benefit of high multiplexing that enabled simultaneous comparison of Imyl fragment binding activity in a single mixed sample. If we considered reproducibly liganded sites (competition ratio or CR < 0.5 for inhibitor/DMSO vehicle comparisons across 2 biological replicates), the Imyl fragment compounds, in general, displayed a restricted binding profile compared with the SuTEx fragment ligand JWB198 tested at a 10-fold lower concentration (Figure 5C and S8). The tempered protein binding activity of Imyl fragment compounds were more akin to the reactivity of aryl fluorosulfates[18] when directly compared to sulfonyl-triazole counterparts.[15b]

Proteins reproducibly and significantly liganded by Imyl fragment compounds included enzymes, adaptor proteins and helicase proteins in soluble proteomes although a small number of binding events were detected in membrane fractions (log_2 competition ratio or CR < 0.5 for Imyl fragment/DMSO vehicle, $p < 0.05$; Figure 5C and S8). Notable targets of KY-2 and KY-6 include aldo-keto reductase family 1 member C1 (AKR1C1 Y24 and Y55) and 14–3-3-zeta (YWHAZ Y211). We observed prominent binding of KY-342 to aldehyde dehydrogenase 3 family member A1 (ALDH3 A1 Y305, Figure 5C). Akin to sulfonyl-triazoles, a cyclopropyl modification on the AG reduced binding activity; KY-5 displayed very minor to negligible binding activity in both soluble and membrane proteomes evaluated (Figure 5C and S8).

Development of a Sulfonyl-Imidazole GST and PTGR2 Inhibitor

The restricted binding profiles of Imyl ligands suggested that the imidazole LG could be leveraged for improving selectivity of covalent inhibitors. We recently disclosed covalent inhibitors of prostaglandin reductase 2 (PTGR2), a lipid enzyme that catalyzes the NADPH-dependent reduction of 15-keto-PGE_2 to produce 13,14-dihydro-15-keto-PGE_2.[15a] From this series, we identified a lead 1,2,4-sulfonyl-triazole inhibitor RJG-2096 that blocked PTGR2 biochemical activity with moderate potency as determined by a LC-MS substrate assay (IC_{50} of ~960 nM, Figure 6A–C; see Supporting Methods for details of assay). Here, we asked whether substitution of the triazole for an imidazole LG counterpart in the RJG-2096 scaffold (i) could retain PTGR2 inhibitory activity, and (ii) improve selectivity against targets commonly bound by SuTEx compounds including glutathione S-transferase (GST) enzymes.[15b,18]

We synthesized RJG-3016 and -3017, which retain the AG binding element but differed from RJG-2096 via incorporation of a substituted imidazole LG (Figure 6A). The PTGR2 inhibitory activity of these Imyl compounds were evaluated by LC-MS substrate assay. We observed significantly increased production of 13,14-dihydro-15-keto-PGE_2 in PTGR2 overexpressed com-

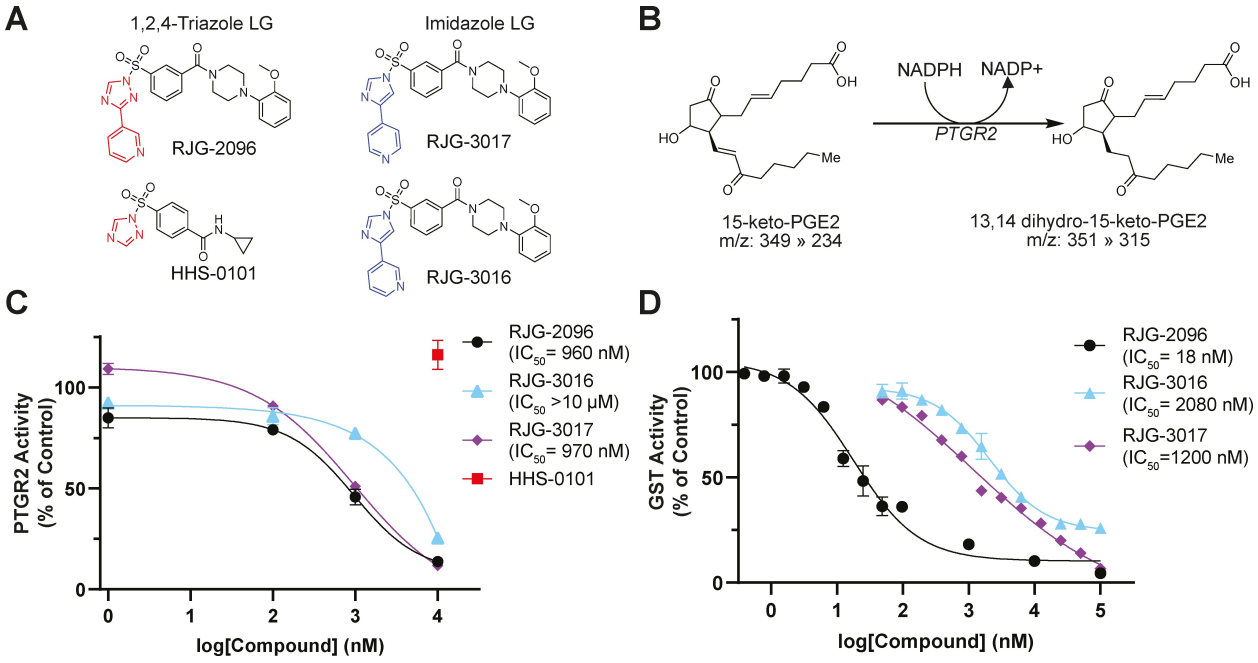

Figure 6. Imidazoles can serve as an effective LG for development of PTGR2 and GST inhibitors. A) Chemical structures of SuTEx and Imyl-based PTGR2 inhibitors. B) PTGR2 catalyzes the NADPH dependent reduction of 15-keto-PGE2 to 13,14-dihydro-15keto-PGE2. C) LC-MS substrate assay for evaluating PTGR2 activity and inhibition by SuTEx and Imyl inhibitors. Recombinant PTGR2-overexpressed HEK293T lysates were pretreated with compound at the indicated concentrations for 30 min followed by addition of 15-keto-PGE2 substrate. Total lipids were extracted and PTGR2-mediated production of 13,14-dihydro-15-keto-PGE2 were quantified by LC-MS as described in Supporting Methods. PTGR2 inhibition was calculated as a %control (inhibitor treatment/vehicle). D) PDAC608T lysates were pretreated with compound then allowed to react with GSH and 1-chloro-3,5-dinitrobenzene (CDNB) to evaluate activity of native GSTs and inhibition by SuTEx and Imyl inhibitors. Inhibition was determined using a %control (inhibitor treatment/vehicle) of the absorbance measured at 340 nm.

pared with mock transfected HEK293T proteomes exposed to 15-keto-PGE_2 substrate (Figure S9). Pretreatment with RJG-3017 resulted in concentration-dependent blockade of recombinant PTGR2 activity with equipotency compared to RJG-2096 (IC_{50} values of ~900 nM for Imyl and SuTEx inhibitors, Figure 6C). Interestingly, the 3-pyridyl substituted Imyl inhibitor (RJG-3016) showed substantially reduced inhibitory activity. Both RJG-3016 and -3017 showed reduced activity against endogenous GST enzymes detected in cell proteomes using a reported biochemical substrate assay[15b] (Figure 6D). These results support imidazole as an effective LG for developing covalent inhibitors with good potency and reduced activity against targets commonly bound by sulfone-based electrophiles.

Discussion

Activity-based protein profiling (ABPP) is a powerful technique used to covalently modify functional sites in the proteome. Our lab disclosed the utility of sulfur-triazole exchange (SuTEx) chemistry to study tyrosine and lysine sites on proteins using sulfonyl-triazole electrophiles.[17] The ability of SuTEx to modify tyrosines enabled the discovery of hyper-reactive tyrosines (i.e. increased nucleophilicity due to the local protein environment) as well as pervanadate sensitive phosphorylation sites.[15b] Altering the electronics of the LG (e.g. by modifying the 1,2,4-triazole group) modulated the number of probe-modified sites and the Y/K ratio detected by LC-MS/MS chemical proteomics.[15b]

We speculated that a change to the heterocyclic nucleofuge itself would produce a proportional alteration to accessible binding sites and amino acid preference due to the various pK_as of the azoles. We explored the innate reactivity of the alternative azole analogs (e.g. pyrazole, imidazole, imidazolium, and tetrazole) and discovered that the imidazole-based LGs provided a tunable scaffold for chemoproteomics (Figure 3). For example, addition of a strong electron-withdrawing trifluoromethyl group to the LG of Imyl compounds facilitated rapid reaction with *p*-cresol whereas the unsubstituted imidazole analog Imyl-01 was significantly less reactive both in solution and proteomes (Figure 2 and 4).

The reactivity of SufAz probes (Py-02, Imyl-02, Tet-02 and HHS-481) with *p*-cresol is in accordance with their respective pK_as except for Tet-02, which, in principle, should be the most reactive. Similar to the imidazolium electrophiles tested herein, we attribute reduced reactivity of sulfonyl-tetrazoles to rapid decomposition upon exposure to aqueous solvent although further studies are needed to fully support this hypothesis. Our findings show that arylsulfonyl imidazolium triflates appear to react readily with all nucleophiles tested by HPLC and may be indicative of potential stability issues in aqueous solvent. In stark contrast, arylsulfonate imidazoles are show minimal activity against amino acid mimetics by HPLC and negligible labeling in the proteome as determined by ABPP. Arylsulfonyl imidazoles show balanced reactivity with nucleophiles that can be tuned by substitutions at the 4-position of the imidazole LG. Akin to sulfonyl-triazoles,[15b] we observed a correlation between probe reactivity and the EWG character of functional group modifications to the azole LG (Figure 2E).

Tuning the LG on Imyl probes provided an opportunity to explore binding specificity in cell proteomes. Probe labeling experiments in DM93 cells revealed that Imyl-01 and -TFM provided broad proteomic coverage with preferential modifications on K and Y, respectively (Figure 4). Despite activity against *p*-cresol in solution, Imyl-02 and −04 were not effective probes in biological systems, which could be due to stability and/or solubility issues. In contrast, Imyl-Br showed binding activity against sites captured by Imyl-01 and -TFM but with reduced reactivity to highlight the tunable nature of the Imyl scaffold for developing protein-targeted agents (Figure 4). Importantly, the unusually high preference of Imyl-TFM for tyrosine binding highlights future opportunities to further improve chemoselectivity through imidazole modifications.

We developed fragment compounds using the Imyl scaffold to ligand a restricted set of protein sites detected in Colo 205 proteomes (Figure 5). These findings contrasted with our HPLC studies that showed larger differences in reactivity and could reflect effects from protein recognition captured by LC-MS/MS chemical proteomics. Although these findings would suggest Imyl compounds function as highly attenuated electrophiles, we demonstrated that substitution of a more reactive triazole with an imidazole LG on an optimized PTGR2 inhibitor scaffold retained inhibitory activity and reduced off-target activity (Figure 6). Instead, we propose that sulfonyl-imidazoles, akin to aryl fluorosulfates,[18] are well positioned for appending to high affinity binding elements to develop targeted covalent inhibitors with enhanced stability and selectivity. In support of this hypothesis, a recent study used imidazole as a LG for developing more stabilized electrophilic thalidomide compounds.[9a]

In conclusion, we synthesized a suite of SufAz probes to explore the ability of various nitrogen-containing 5-membered azoles to undergo covalent reaction with amino acid residues in the proteome. Sulfonyl-imidazoles were identified as tunable electrophiles with tempered reactivity in the proteome compared to established SuTEx compounds.[15b] The ability for reactivity tuning combined with moderate proteome-wide coverage of tyrosine and lysine sites positions sulfonyl-imidazoles as a tempered electrophile for covalent targeting of functional protein sites.

Supporting Information

Supporting figures, experimental methods, chemical synthesis and characterization, and NMR spectra.

Supporting Tables: Table S1: LC-MS data for SILAC studies; Table S2: LC-MS data for TMT-SuTEx.

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Conflict of Interests

K.-L.H. is a founder and scientific advisory board member of Hyku Biosciences.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: SuTEx · SuFEx · activity-based protein profiling · chemoproteomics · covalent ligands · drug discovery

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