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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.0c02721 • Publication Date (Web): 17 Apr 2020

2-Sulfonyl pyridines as tunable, cysteine-reactive electrophiles

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ABSTRACT: The emerging use of covalent ligands as chemical probes and drugs would benefit from an expanded repertoire of cysteine-reactive electrophiles for efficient and diverse targeting of the proteome. Here we use the endogenous electrophile sensor of mammalian cells — the KEAP1-NRF2 pathway — to discover cysteine-reactive electrophilic fragments from a reporter-based screen for NRF2 activation. This strategy identified a series of 2-sulfonyl pyridines that selectively react with biological thiols via nucleophilic aromatic substitution (S_NAr). By tuning the electrophilicity and appended recognition elements, we demonstrate the potential of the 2-sulfonyl pyridine reactive group with the discovery of a selective covalent modifier of adenosine deaminase (ADA). Targeting a cysteine distal to the active site, this molecule attenuates the enzymatic activity of ADA and inhibits proliferation of lymphocytic cells. This study introduces a modular and tunable S_NAr-based reactive group for targeting reactive cysteines in the human proteome and illustrates the pharmacological utility of this electrophilic series.

INTRODUCTION

Owing to the unique reactivity of cysteine among protein coding amino acids, covalent modifiers of this amino acid have emerged as valuable mechanistic probes,[1-4] labeling agents,[5-6] and FDA-approved medicines.[7] Campaigns to develop cysteine-reactive inhibitors of enzyme classes like kinases have typically involved identifying a potent non-covalent inhibitor and then affixing linkers to covalently target an accessible cysteine residue with a tempered electrophile, such as an acrylamide or chloroacetamide.[8] While effective, such strategies require considerable structural information about the druggability of a potential binding pocket and the nature of a given inhibitor's orientation within it. A complementary discovery approach involves the unbiased interrogation of larger systems, like whole cells and whole proteomes, using diverse electrophilic chemical probes and then retroactively deconvoluting the spectrum of protein target engagement using analytical methods such as chemical proteomics.[9-11] These studies have dramatically expanded our view of the covalently druggable content of the cysteine proteome, suggesting that covalent ligands for engaging otherwise undruggable protein targets might be afforded by chemically elaborating upon new cysteine-directed chemotypes with distinct and tunable reactivities.

To expand the scope of available cysteine-targeted electrophilic chemotypes, we envisioned an alternative discovery approach inspired by Nature. The mammalian cell senses and responds to oxidative stress and electrophilic xenobiotics through the activities of the evolved electrophile sensor, Kelch-like ECH-associated protein 1 (KEAP1).[12] KEAP1, a repressor of oxidative stress-responsive transcription factor, Nuclear Factor Erythroid-like 2 (NFE2L2, NRF2 throughout), contains a host of sensor cysteine residues that when oxidized or alkylated by reactive oxygen species or electrophilic chemicals promote the dissociation and nuclear translocation of NRF2.[13] In the nucleus, NRF2 enacts a protective transcriptional program that promotes cellular detoxification and resilience in the context of redox imbalance.[13] Because covalent modification of KEAP1 cysteines results in robust NRF2-driven transcriptional activation at loci containing antioxidant response elements (AREs), we envisioned that a cell-based reporter screen for ARE activation (ARE-LUC, Figure 1A) might be used as a scalable, high-throughput method for identifying new cysteine-reactive chemotypes. Such an approach would, in principle, select for molecules with the requisite physical properties for cellular studies (such as solubility and permeability) and would additionally allow one to rapidly interrogate the largely untapped

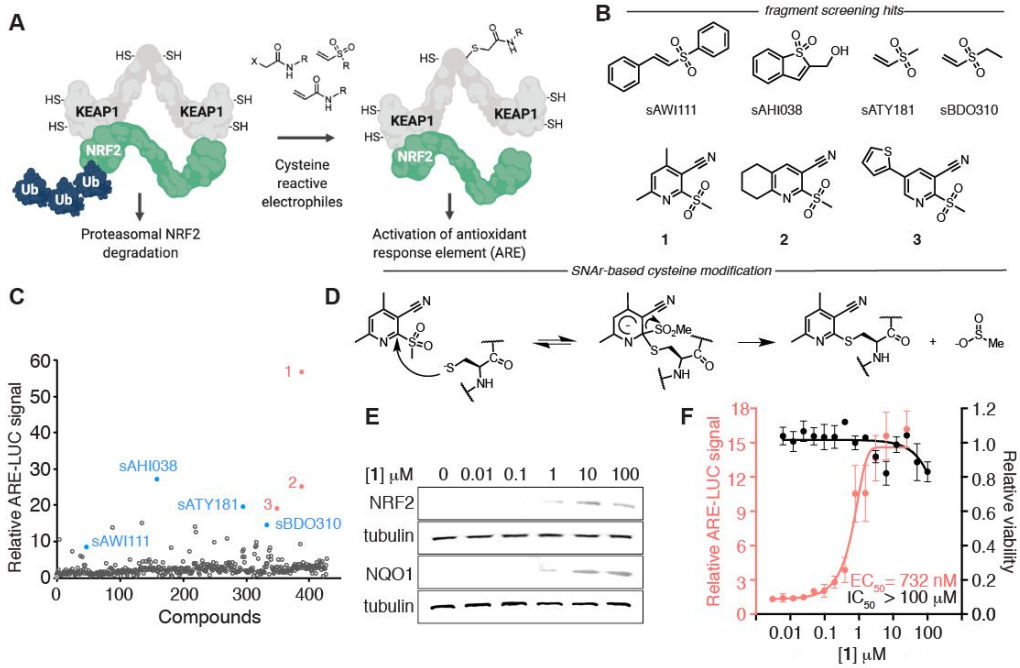

Figure 1. A fragment screen for KEAP1-NRF2 pathway activation identifies S_NAr-based cysteine-reactive groups. (A) Schematic depicting the NRF2-KEAP1 pathway, used in this work to identify cysteine-reactive groups by a reporter-based screen. Structures of top screening hits (B) and scatter plot (C) of relative ARE-LUC luminance values of all compounds delivered to IMR32 cells treated for 24 hours (33 μM). (D) Proposed S_NAr-based mechanism for KEAP1 inhibition by cysteine pyridylation with **1**. (E, F) Representative Western blot analyses for NRF2 and NQO1 protein levels after 24-hour treatment of IMR32 cells with the indicated concentrations of **1**. (F) Relative luminance values of ARE-LUC activity and cytotoxicity measurements from 24-hour treatment of IMR32 cells with indicated concentrations of **1** (n=3, mean and s.e.m.).

reactivity present in the chemical diversity of large chemical libraries.

Here, we describe the execution of a cell-based screening platform that identified the largely unexplored cysteine-reactive electrophilic group, the 2-sulfonyl pyridine. We describe the synthesis and characterization of a library of derivatives, which allowed for the appropriate tuning of this chemotype for diverse targeting of the cysteine proteome. We further demonstrate the utility of this reactive group with the discovery of an allosteric inhibitor of adenosine deaminase.

RESULTS AND DISCUSSION

A reporter-based screen identifies cysteine reactive groups. As proof of concept of this strategy, we assembled a library of 429 commercially available low molecular weight fragments (mean MW = 238 Da, Supplementary Table 1), and, using an optimized, transient transfection-based assay, we then screened this library for ARE-LUC inducing activity in miniaturized format. Four vinyl sulfone-containing fragments (sAWI111, sAHI038, sATY181, and sBDO310) were identified as hits, giving us confidence in our ability to detect low molecular weight, electrophilic activators of NRF2, as vinyl sulfones have been reported both as KEAP1 modifiers[14-15] and as the reactive group in cysteine-targeted covalent inhibitors[16] and probes[17] (Figure 1B, C). Among the highest magnitude hits were also three 2-methylsulfonyl-3-cyano-substituted pyridines (**1**-**3**; Figure 1B, C), which we hypothesized to act via a nucleophilic aromatic substitution-based mechanism (S_NAr)[18-20] to modify reactive sensor cysteines in KEAP1 (Figure 1D).

Treatment of IMR32 cells with resynthesized compound **1** (2-methylsulfony-4,6-diemthyl-nicotinonitrile) was found to efficaciously activate NRF2, dose-dependently promoting the stabilization of NRF2 protein as well as its transcriptional output as monitored by Western blotting for NRF2 target gene NQO1 (NAD(P)H dehydrogenase (quinone) 1; Figure 1E). Further, **1** was found to stimulate ARE-LUC reporter activity with a sub-micromolar half maximal effective concentration (ARE-LUC EC_{50} = 732 nM) while displaying minimal cytotoxicity to IMR32 cells (IC_{50} >100 μM; Figure 1F). Similarly, **1** activated key NRF2-controlled transcripts in IMR32 cells as evaluated by qRT-PCR (Figure S1A). Importantly, robust reporter activation by **1** was dependent on the presence of NRF2 protein (Figure S1B), indicating on-target activation of NRF2 by this chemical series.

To confirm KEAP1's covalent modification by the 2-sulfonyl pyridine group, we synthesized a biotinylated derivative, **4**, which retained NRF2-inducing activity in cellular reporter assays (ARE-LUC EC_{50} = 12.1 μM; Figure S1C, D). We found that treatment with increasing concentrations of **4** could label KEAP1 in HEK293T cells expressing a FLAG-KEAP1 transgene and that labeling with **4** could be competed by pretreatment with a molar excess of **1** (100 μM; Figure S1E). Similarly, we found that biotinylation of KEAP1 by treatment with **4** (10 μM) could

be dose dependently competed away by **1** in a concentration range that coincides with the cellular EC_{50} for NRF2 activation by **1** (EC_{50} labeling = 1.1 μM; Figure S1F, G). Further, we found that previously reported S_NAr-type, cysteine-reactive labeling agents MSBT (Methylsulfonyl benzothiazole)[21] and CNBF (4-Chloro-7-nitrobenzofurazan)[22] are also capable of activating NRF2 in IMR32 cells (ARE-LUC EC_{50} = 901 nM and 409 nM; Figure S1H, I), suggesting that sensor cysteines in KEAP1 are amenable to S_NAr-based modification. Importantly, MSBT and CNBF treatment was found to be considerably more cytotoxic to IMR32 cells (IC_{50} = 9.4 μM and 324 nM respectively; Figure S1J), likely reflecting the heightened electrophilicity of these chemotypes. We additionally found that increasing concentrations of alkyl-biotin probe **4** could label many proteins as evaluated by anti-Biotin Western blotting analyses of whole cell lysates and that labeling with **4** could be competed by pretreatment with cysteine-selective labeling agent iodoacetamide (1mM; Fig S2A, B), reflecting the ability of the 2-sulfonyl pyridine group to selectively label reactive cysteines across the proteome.

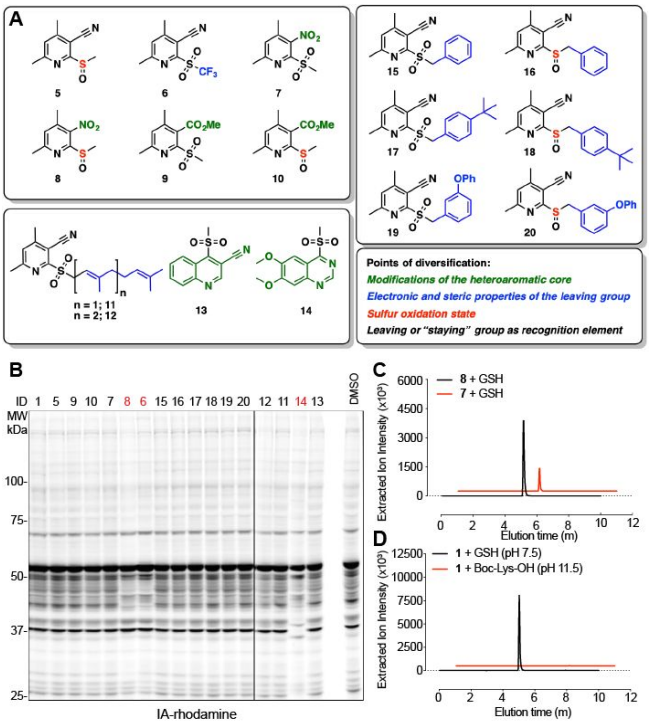

Figure 2. Tuning the reactivity and targeting of 2-sulfonyl pyridines. (A) Structures of compounds and modification strategies used to interrogate the reactivity of the S_NAr-based reactive group with biological thiols. (B) SDS-PAGE analysis of IA-rhodamine labeled proteomes from Ramos cells after 2-hour pretreatment with the indicated compounds (50 μM each). (C) Relative product formation in reactions with GSH and **7** or **8** at pH 7.5. (D) Relative product formation of **1** with GSH or Boc-Lysine-OH at the indicated pH.

Tuning the reactivity and proteome engagement of the reactive group. We next synthesized a library of derivatives of **1** to determine if the reactivity of this series could be predictably tuned for use in covalent ligand discovery (Figure 2A). These modifications included substitution of the nitrile group to other withdrawing groups such as nitro and methyl ester which are potentially capable of stabilizing the negatively charged Meisenheimer intermediate through resonance (e.g., **7**-**10**). Another substitution included changing the sulfur oxidation state from sulfone to sulfoxide (e.g., **5**, **8**, **10**), a modification which has not been explored for similar reagents such as MSBT. We additionally synthesized analogs in which the pyridyl core was changed to bicyclic systems such as quinoline (e.g., **13**) and quinazoline (e.g., **14**). Lastly, we synthesized analogs with altered leaving group appendages for use as a recognition motif (e.g., **11**-**12**, **15**-**20**) or for increasing potential leaving group ability (e.g., **6**). We first evaluated if *in situ* treatment of Ramos cells with the library members might result in global labeling of reactive cysteines as visualized by competitive blockade of the proteomic reactivity profile of an iodoacetamide-conjugated rhodamine probe. Some compounds, such as trifluoromethyl sulfone-substituted analog **6**, nitro-and sulfone-substituted analog **8**, and

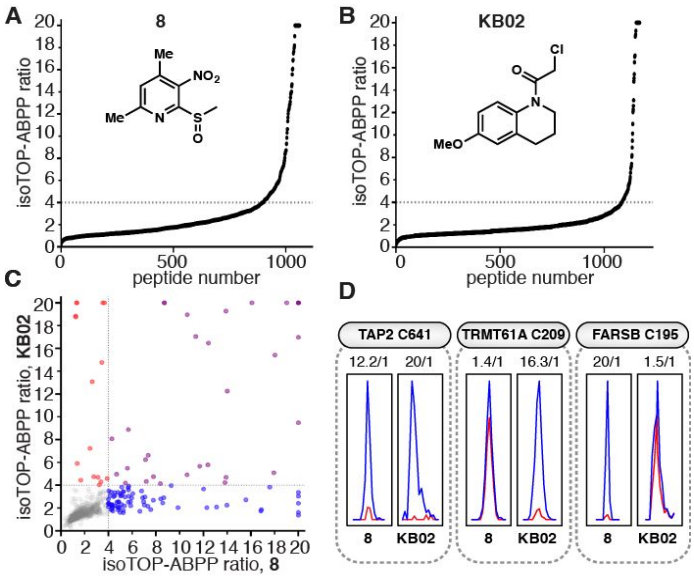

Figure 3. isoTOPP-ABPP defines cysteines liganded by S_NAr-type reactive groups. Structures and isoTOP-ABPP ratios (*R* values) from Ramos cells treated with promiscuous S_NAr probe **8** (A) and chloroacetamide-containing scout fragment KB02 (B). (C) Scatter plot of isoTOPP-ABPP ratios for quantified cysteine-containing peptides targeted by KB02 and **8**, in which red, blue, and purple points represent KB02-selective, **8**-selective and shared targets, respectively. (D) Representative MS1 spectra of liganded cysteines shared by both fragments (C641 of TAP2), specific to KB02 (C209 of TRMT61A), or specific to **8** (C195 of FARSB).

quinazoline **14** displayed heightened proteome reactivity compared to parent compound **1**, which did not visibly compete any IA-labeled bands (Figure 2B), suggesting that the cellular cysteine reactivity of this chemotype can be dramatically enhanced.

We next evaluated a core subset of these analogs (**1**, **5**, **7**-**10**) for *in vitro* reactivity with reduced glutathione (GSH) and for ARE-LUC inducing activity in reporter assays. The majority of these compounds reacted with GSH *in vitro*,

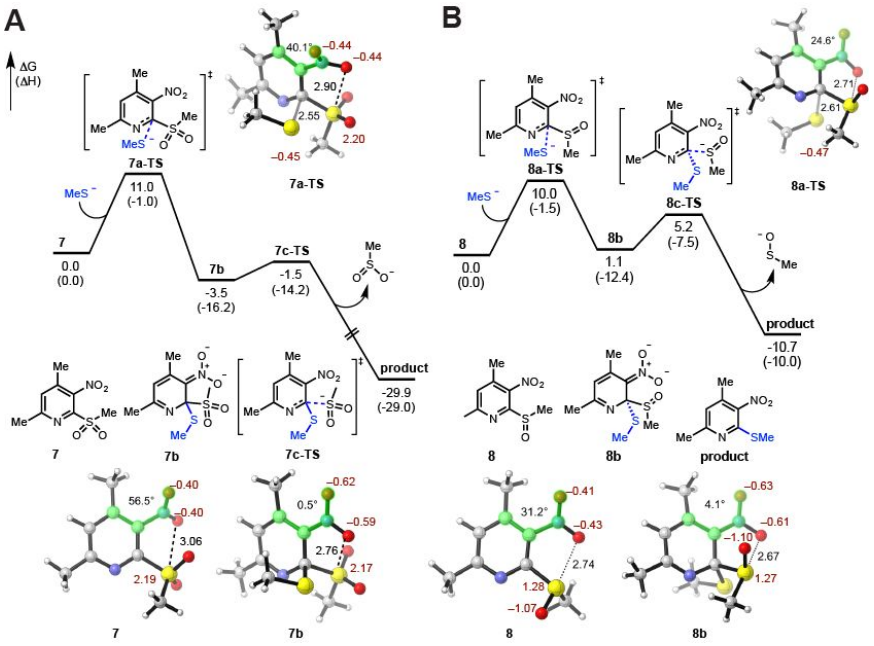

Figure 4. DFT calculations reveal nucleophilic addition as the rate limiting step in S_NAr-based reaction with thiol nucleophiles. Computed energy profiles and transition state structures for product formation via reaction with **7** (A) or **8** (B) with methanethiolate (Gibbs free energies and enthalpies, kcal/mol; distances (in black), angstrom; red numbers, natural population analysis (NPA) charge of indicated atoms).

yielding the appropriate mass for the S_NAr product (Figure S3A). Similarly, the majority of these compounds were additionally active in cellular ARE-LUC assays with minimal cytotoxicity (Figure S3B, C). A notable exception was methyl ester- and sulfone-substituted **9**, which was largely unreactive in these assays (Fig S3). Conversely, we found nitro- and sulfoxide-containing fragment **8** to be the most active of this subset (ARE-LUC EC_{50} = 60 nM; Figure S3B), a result consistent with its promiscuous reactivity profile among iodoacetamide-reactive cysteines and its enhanced cytotoxicity relative to other analogs (IC_{50} = 11.9 μM; Figure 2B, Figure S3C). Interestingly, sulfoxide **8** differs only in its sulfur oxidation state from sulfone-substituted analog **7**, which was considerably less active in reporter assays (ARE-LUC EC_{50} = 1.53 μM, Fig 2C, Figure S3B). Importantly, we found that no library member reacted with lysine (Boc-Lys-OH) at pH 7.5 or 11.5, indicating the specificity of this chemotype for reaction with thiol nucleophiles (Fig 2D, Fig S3A).

We next performed a quantitative chemical proteomics study to assess the global cysteine reactivity trends of individual library members in comparison to established fragment electrophiles, such as chloroacetamide KB02.[10] Using previously established, quantitative mass spectrometry (MS)-based activity-based protein profiling (isoTOP-ABPP) experimental protocols, we found that both fragment **8** and KB02 liganded a similar number of cysteines across the proteome of the Ramos human B cell line (Fig S4, Figure 3A, B), with **8** labeling both a shared set and unique set of cysteines compared to KB02 (*R* value >4; Figure 3C, D). To confirm the proposed S_NAr-based mechanism for the modification of cysteines in proteins, we performed a site of labeling experiment using **8** and recombinant glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Our mass spectrometry results confirmed the ability of **8** to covalently pyridylate C152 and C247 of GAPDH (Fig S5B). Together, these studies suggest that the reported S_NAr probe set represents a modular chemotype amenable to tuning of its reactivity for engaging many cysteines across the proteome.

DFT studies on S_NAr probe reactivity. To gain a deeper mechanistic understanding of the reactivity of the S_NAr probe set with biological thiols and to explain the differences in reactivity between similarly substituted analogs, we performed density functional theory (DFT)-based calculations. Given the enhanced reactivity of nitro- and sulfoxide-substituted **8** relative to analog **7**, we first elected to study the reaction between methanethiolate with **7** (sulfone) and **8** (sulfoxide) This reaction is found to proceed through a stabilized, Meisenheimer-complex intermediate (**7b** and **8b**, Fig 4A, B), which is formed via high-energy transition state **7a-TS** or **8a-TS**. Nucleophilic addition of the thiolate anion is the rate-determining step of the reaction and is thus determining of reactivity of **7** and **8** ($G^{\ddagger}$ = 11.0 and 10.0 kcal/mol for **7a-TS** and **8a-TS**, respectively). Structural analysis suggests that the electrostatic interaction between the nitro-group oxygen and the sulfur leaving group are preferentially stabilizing to **8a-TS** and enhancing of its reactivity, as the O-S distance is shortened to that of **7a-TS** (2.71 Å vs. 2.90 Å, Fig 4A, B). NBO second order perturbation theory analysis precluded the contributions of $LP_O \rightarrow \sigma^*_{S-C}$ interactions in these transition states (Fig S6), indicating the predominance of electrostatic rather than

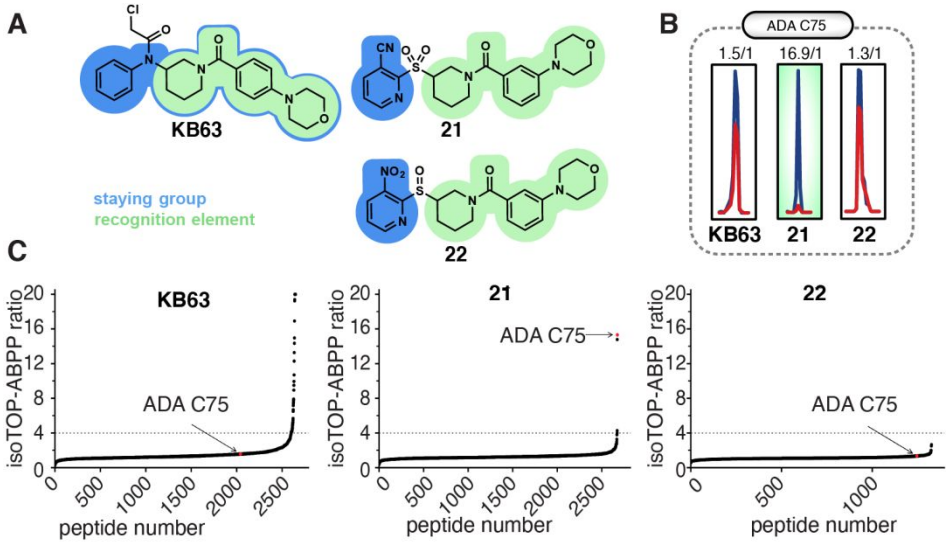

Figure 5. Proteomic profiling of elaborated S_NAr-based ligands identifies a selective modifier of adenosine deaminase. (A) Structures of KB63, **21**, and **22** with recognition elements and staying groups depicted in green and blue respectively. (B) Representative MS1 profiles of ADA C75 labeling and (C) competitive isoTOPP-ABPP ratios (*R* values) from Ramos cells treated *in situ* with the indicated inhibitors (2 hr, 50 μM), with the *R* value of ADA C75 depicted in red.

orbital interactions in stabilizing the nucleophilic addition transition state. Further calculations with **7** and **8** using methylamine as the nucleophile revealed the basis for selective reactivity with thiols, as we found a likely unsurmountable free energy of activation for nucleophilic amine addition to this chemotype ($\Delta G^{\ddagger}$ = 23.3 and 22.5 kcal/mol for **7** and **8**, respectively, Fig S7). Further calculations with nitrile analog **5** revealed a similar trend explaining its tuned electrophilicity. The reaction of **5** and methanethiolate has an increased activation free energy of nucleophilic addition (13.5 kcal/mol) and a weakened nitrile-sulfur interaction in the **5a-TS** (3.66 Å. Figures S8). Likewise, **5** is predicted to be unreactive with amine nucleophiles as this reaction is unlikely to proceed given an insurmountable free energy of addition in reactions with methylamine ($\Delta G^{\ddagger}$ = 26.8 kcal/mol, Figure S8).

Identifying inhibitors based on the 2-sulfonyl pyridine reactive group. Lastly, we sought to demonstrate the utility of this electrophilic series by generating selective covalent inhibitors. We therefore synthesized and evaluated the proteome-wide reactivity of a matched pair of nitro- and cyano-substituted 2-sulfonyl and 2-sulfoxide pyridine-containing compounds by quantitative proteomics. Based on chloroacetamide KB63, a scaffold we previously demonstrated to engage a distinct set of cellular protein targets,[10] we synthesized nitrile- and sulfone-substituted **21** and nitro- and sulfoxide-containing **22** (Figure 5A), designed such that the elaborated leaving group could serve as a recognition element for protein targeting. isoTOP-ABPP experiments with these analogs in Ramos cells revealed that KB63 was more promiscuous than the S_NAr-based electrophiles. Whereas **22** did not significantly modify any quantified cysteines, **21** was found to selectively target two cysteines, C54 of protein calcineurin-like phosphopesterase domain-containing 1 (CPPED1, *R* = 14.78) and C75 of adenosine deaminase (ADA, *R* = 15.32, Figure 5A-D, Supplementary Table 2).

ADA catalyzes the deamination of adenosine to form inosine, an essential step in metabolizing purines and in limiting the buildup of the immunosuppressive molecule adenosine. Additionally, ADA is thought to modulate immune signaling through its extracellular interaction with dipeptidyl peptidase 4 (DPP4, contextually called CD26).[23-24] As such, ADA is the target for a number of active site targeted nucleoside-based inhibitors including Cladribine, an approved immunosuppressive drug for treating multiple sclerosis.[25] We confirmed that **21** labeled C75 of ADA by evaluating competition with iodoacetamide-conjugated biotin (IA-biotin) labeling of immunoprecipitated ADA-FLAG transgene (EC_{50}= 4.4 μM, Figure 6A-C). IA-biotin was confirmed to specifically label the one quantified cysteine by isoTOP-ABPP, as a C75S mutant of ADA resulted in a loss in biotinylation (Figure S9A). Further, we found that treatment with **21** resulted in the proposed pyridylation-based modification of C75 in a mass spectrometry site of labeling experiment (Figures S9B-C and S10).

C75 is not contiguous to the enzymatic active site of ADA, but lies in a distal site between G74 and R76, two residues which have been reported as mutated sites resulting in severe combined immunodeficiency (called ADA-SCID, Figure 6A).[26-28] Given these data, we hypothesized that

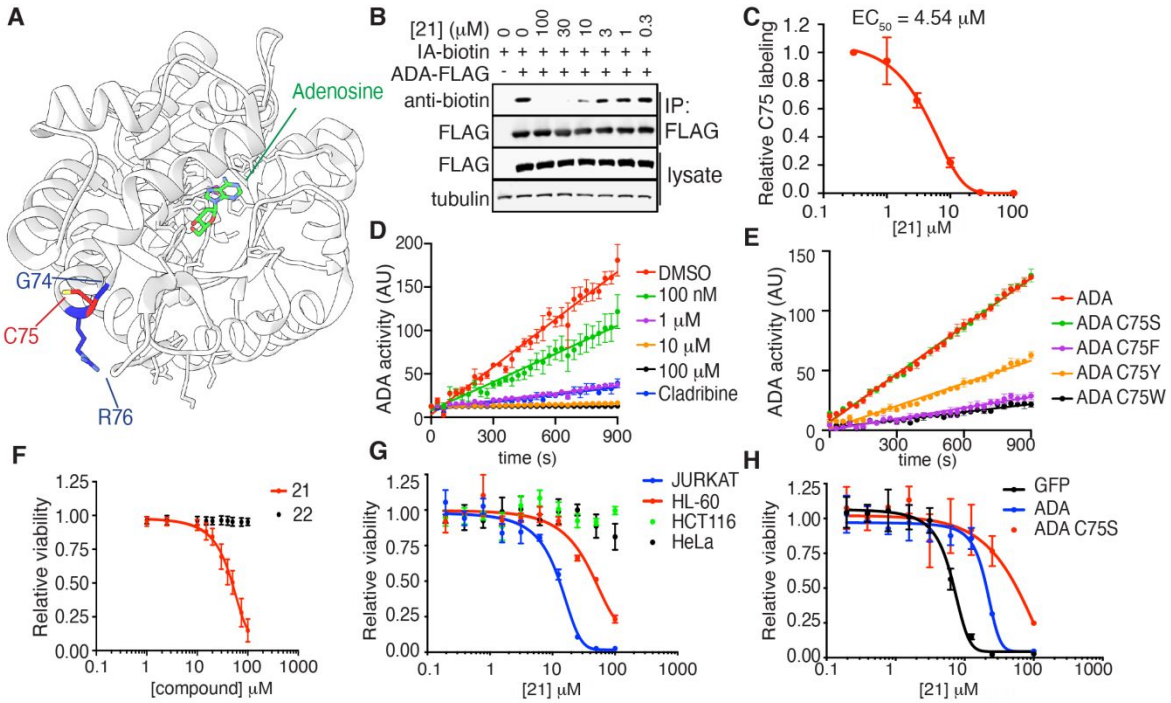

Figure 6. Covalent modification by 21 allosterically inhibits the activity of adenosine deaminase. (A) Ribbon structure of substrate bound, human ADA (adenosine depicted in green; PDB: 3IAR) with sites of SCID-inducing mutations (G74, R76) in blue and site of modification by **21** in red (C75). Western blot analyses (B) and quantification (C) of biotin labeling at C75 of ADA from FLAG-immunoprecipitated material after *in situ* treatment of HEK293T cells transfected with ADA-FLAG and then treated with the indicated concentrations of **21** for 1 hour followed by treatment with 100 μM IA-biotin for an additional hour. (D) Enzymatic activity of ADA (AU = arbitrary fluorescent units) after a 1-hour pre-treatment with the indicated concentrations of **21** or Cladribine (10 μM). (E) Enzymatic activity (AU = arbitrary fluorescent units) of the indicated ADA point mutants recombinantly expressed in mammlian cells (n=3, mean and s.e.m.). Relative viability measurements of primary human T cells (F) and of the indicated ADA-expressing cell lines (G) after 24-hour treatment with the indicated concentrations of **21** and **22.** (H) Relative viability measurements of JURKAT cell lines stably overexpressing GFP, ADA (wildtype), or ADA C75S after 24-hour exposure to the indicated concentration response of **21**.

modification by **21** might antagonize the enzymatic activity of ADA, resulting in a diminished proliferative capacity of lymphocytic cells. We found that pre-treatment of recombinant ADA with **21** lead to the concentration-dependent inactivation of enzymatic activity, a result similarly observed for treatment of the active-site inhibitor Cladribine during the assay (Figure 6D). Unlike the smaller, more polar C75S mutant, mutating C75 to larger aromatic residues Phe, Tyr, and Trp was found to largely inactivate ADA (Figure 6E). We additionally found that **21** was incapable of inhibiting the enzymatic activity of the unmodifiable C75S mutant (Fig S11A), further suggesting that pyridylation by **21** at this site is responsible for diminished enzymatic activity.

We found that **21**, but not **22**, effectively inhibited primary human T cell proliferation (Figure 6F). Similarly, treatment with **21** also inhibited the growth of lymphocyte-derived cancer cell lines expressing detectable amounts of ADA (JURKAT and HL-60; Figure 6G; Fig S11B-C), but not non-immune human cell lines (e.g., HeLa, HCT116),[29] a result not observed for **22**, which does not target ADA (Figure S11C). Additionally, we observed similar diminished proliferative capacity of JURKAT and HL-60 cells when treated with the active site ADA inhibitors Cladribine, Pentostatin, EHNA, and 1-deazaadenosine (Fig S11E-I). Stable overexpression of wildtype ADA in JURKAT cells was found to decrease the anti-proliferative potency of **21**, and overexpression of the modification incompetent C75S ADA mutant further decreased the antiproliferative activity of compound (Figure 6H, Fig S11J). Further, we confirmed that ADA is essential to the viability of JURKAT and HL60 cells, as shRNA-mediated knockdown of ADA transcript decreased the proliferative fitness of these cell types (Fig S11K, L). Together these data suggest that genetic or pharmacological inhibition of ADA antagonizes cellular survival in these cell types, and that allosteric pyridylation at C75 of ADA is responsible, at least in part, for the anti-proliferative activities of **21**.

CONCLUSIONS

Reporter based screens have proven a scalable and effective means to interrogate chemical libraries, yielding new ligandable targets and diverse chemical matter for protein targeting. Here we have used a reporter-based screen to identify a largely underexplored cysteine-reactive chemotype – the 2-sulfonyl pyridine—and demonstrated its utility for the chemoproteomic discovery of a covalent, allosteric inhibitor of the ADA enzyme. We anticipate that these and other high throughput methods for assessing intrinsic compound reactivity will yield additional reactive

chemotypes from the structural diversity lying dormant within chemical libraries. Indeed, recent reports have demonstrated the utility of using cell-based screens at uncovering the latent electrophilic reactivities of small molecules which are dependent on the enzymatic and cellular milieus of their target proteins.[30-31] As such, we are broadening our efforts (screens of ~10^6 compounds) to explore this hypothesis in greater detail.

Chemical optimization of the 2-sulfonyl pyridine scaffold enabled tuning of the electrophilicity of this series. We demonstrated that by replacing the withdrawing group at position 3 and by manipulating the oxidation state or availability of the leaving group that we could increase (e.g., **8**) or decrease (e.g., **9**) the reactivity of this series with biological thiols. Chemical proteomics experiments with nitro- and sulfoxide-substituted probe **8** demonstrated that it modified both a shared and unique set of ligandable cysteines in the proteome when compared to promiscuous chloroacetamide-containing scout fragment KB02, suggesting that this chemotype could be useful in the discovery of selective protein ligands if outfitted with the appropriate chemical recognition motifs. Despite a larger size and polar surface area in comparison to other electrophilic reactive groups (e.g., epoxide, chloroacetamide), the 2-sulfonyl pyridine allows for chemical elaboration upon the leaving group (i.e., extension of the sulfonyl group) and the staying group (i.e., substitutions to positions 4, 5, and 6 of the pyridyl group) for use a recognition element in protein targeting, a feature which has been essential in the design logic in generating selective carbamate-based inhibitors of serine hydrolases.[32-34] In its current iteration, the 2-sulfonyl pyridine promotes pyridylation of target cysteine residues, which may allow for covalent inactivation of key reactive cysteine residues (e.g., in an enzymatic active site) or may endow proteins with neo-functionalities via the non-natural modification of cysteine.

To demonstrate the utility of this chemotype for covalent inhibitor discovery, we performed iterative synthesis of analogs substituted such that an extended leaving group might endow selective protein targeting. These synthetic efforts coupled with proteomics studies ultimately identified **21**, a molecule which selectively pyridylates C75 of ADA. This modification interferes with the enzymatic activity and essential cellular functions of ADA in promoting lymphocytic survival, despite targeting a residue distal to the active site. We anticipate **21** will serve as a unique tool in future genetic and biochemical studies aimed at understanding how modification of residues away from the active site results in an inhibitory effect on ADA activity and T cell function, as observed in ADA-SCID. ADA has additionally emerged as an important target in autoimmunity and oncology indications given its centrality in promoting lymphocyte survival and activation. We anticipate that **21** and future analogs will be of complementary utility to active site inhibitors of ADA in studying the enzymatic role of ADA in these contexts, as nucleotide-based inhibitors (e.g., Figure S11I) have the potential to target other purine-binding active sites in cells. Projecting forward, we anticipate the 2-sulfonyl, and possibly 2-sulfoxide, pyridine chemotypes along with the design logic presented here will provide fertile ground for the future development of diverse cysteine-directed covalent ligands.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website.

Supplementary figures and experimental procedures (PDF)
Supplementary table 1 (.xlsx)
Supplementary table 2 (.xlsx)

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Author Contributions

† These authors contributed equally to this work.

Notes

The authors declare the following competing financial interest: B.F.C is a founder and scientific advisor to Vividion Therapeutics, a biotechnology company interested in developing small-molecule therapeutics.

ACKNOWLEDGMENT

The authors thank Calibr compound management group personnel Emily Roddy and Alonzo Davila for technical support. The authors also thank Kristen Williams for assistance with manuscript preparation and submission. We acknowledge NIH (R35 CA231991) for financial support and NSF XSEDE for supercomputing resources. E.V.V. was supported by the Life Sciences Research Foundation Fellowship.

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