ABSTRACT: Covalent ligands are a versatile class of chemical probes and drugs that can target noncanonical sites on proteins and display differentiated pharmacodynamic properties. Chemical proteomic methods have been introduced that leverage electrophilic fragments to globally profile the covalent ligandability of nucleophilic residues, such as cysteine and lysine, in native biological systems. Further optimization of these initial ligandability events without resorting to the time-consuming process of individualized protein purification and functional assay development, however, presents a persistent technical challenge. Here, we show that broadly reactive electrophilic fragments, or “scouts”, can be converted into site-specific target engagement probes for screening small molecules against a wide array of proteins in convenient gel- and ELISA-based assay formats. We use these assays to expediently optimize a weak potency fragment hit into a sub-μM inhibitor that selectively engages an active-site cysteine in the retinaldehyde reductase AKR1B10. Our findings provide a road map to optimize covalent fragments into more advanced chemical probes without requiring protein purification or structural analysis.

Functionalized Scout Fragments for Site-Specific Covalent Ligand Discovery and Optimization

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we show that this approach is suitable for assaying recombinantly expressed targets of interest directly in human cell proteomes without requiring protein purification.

We initially considered using iodoacetamide (IA) probes\textsuperscript{11} for the development of a ligand optimization platform, but the broad reactivity of such IA probes, while an attribute for the global profiling of cysteine ligandability by MS-based proteomics, renders them less suitable for gel- and ELISA-ABPP assays of individual cysteine residues in targets of interest, as most proteins possess several IA-reactive cysteines. We instead pursued the adaptation of recently described electrophilic “scout” fragments\textsuperscript{24,26} as potential site-selective probes of ligandable cysteines on proteins. Scout fragments have been found to capture a large fraction of the total quantity of cysteines liganded by larger electrophilic compound libraries,\textsuperscript{24,26,27} thus potentially providing privileged structures for evaluating ligandable cysteines in diverse assay formats.

We installed alkyne handles into scout fragments, KB02 and KB05, to furnish KB02yne and KB05yne, respectively (Figure 1a), and compared the reactivity of these compounds to IA-alkyne (Figure 1b, Supplementary Figure S1). Interestingly, however, at lower test concentrations (0.5−10 μM), the scout and IA probes exhibited similar overall proteomic reactivity and displayed markedly distinct patterns of protein engagement (Figure 1b, Supplementary Figure S1). We interpret these results to indicate that the scout fragment recognition groups (KB02: 6-methoxy-1,2,3,4-tetrahydroisoquinoline; KB05: N-(4-bromophenyl)aniline) promote binding to specific sites in the proteome, thereby enabling preferential reactivity of proximal cysteines that matches or exceeds the intrinsic reactivity of these residues with IA probes. Such site-specific interactions are likely most evident at lower probe concentrations in gel-ABPP experiments because they are not obscured by additional cysteine reactivity events on the same protein or comigrating proteins that occur at higher probe concentrations.

Encouraged by evidence of preferred scout fragment-cysteine reactions in the proteome, we next set out to identify the proteins harboring these cysteines by MS-based proteomics. Here, we adapted a previously described ABPP platform to quantify the intrinsic reactivity of cysteines with electrophilic compounds that involves comparing the proteome-wide reactivity of these compounds tested at high and low concentrations, where a [high]/[low] reactivity ratio of...
~1.0 for a given cysteine would reflect “hyperreactivity” with the electrophilic compound.\textsuperscript{1,27} Because we intended to convert our findings into whole protein assays for ligand optimization, we adapted the ABPP platform to quantify the reactivity of KB02yne, KB05yne, and IA-alkyne with proteins rather than individual cysteines. In brief, cell lysates were treated with 10 μM ([low]) or 100 μM ([high]) of each alkyne probe followed by CuAAC with biotin-PEG4-azide and enrichment with streptavidin beads. Enriched proteins were digested with trypsin on-bead, and the eluted peptides were
quantiﬁed by LC-MS-based proteomics using either 1) isotopic labeling by reductive demethylation (ReDiMe) with heavy ([low]) or light ([high]) formaldehyde in pairwise comparisons35,36 or 2) isobaric tandem mass tagging (TMT-10plex) for multiplexed comparisons (Figure 2a).27 An \( R \) value ([high]/[low]) approaching 1.0 in either quantiﬁcation format was interpreted as evidence of robust, site-speciﬁc reactivity between an alkyne probe and an individual cysteine on the protein, whereas \( R \) values much larger than 1.0 were interpreted as reﬂecting incomplete alkyne probe reactions at the low probe concentration at one or more cysteine residues on the protein. We considered \( R \) values < 2 for KB02yne or KB05yne as marking scout fragment-hyperreactive proteins

Table 1. Representative Proteins Showing Hyperreactivity (\( R < 2.0 \)) with Scout Fragment Alkyne Probes KB02yne or KB05yne

| gene          | protein                                       | scout fragment alkyne ratio | IA-alkyne ratio | liganded cysteine | scout   |
|---------------|-----------------------------------------------|----------------------------|-----------------|------------------|---------|
| AKR1B10       | aldo-keto reductase family 1 member B10       | 0.6 (KB02yne)              | 3.2             | C299             | KB02    |
| COG8          | conserved oligomeric Golgi complex subunit 8 | 1.6 (KB02yne)              | 3.8             | C96              | KB02    |
| DUS2L         | dihydrouridine synthase 2                     | 1.0 (KB02yne)              | 3.6             | C116             | KB02    |
| HELS          | lymphoid-speciﬁc helicase                    | 1.5 (KB02yne)              | 4.9             | C836             | KB02    |
| WDR45B        | WD repeat domain phosphoinositide-interacting protein 3 | 1.1 (KB02yne) | 4.3             | C63              | KB02    |
| ALG3          | Del-P-Man:Man(5)GlcNac(2)-PP-Dol alpha-1,3-mannosyltransferase | 1.1 (KB05yne) | 3.3             | C21              | KB05    |
| CDKN2AIP      | CDKN2A-interacting protein                    | 1.5 (KB05yne)              | 6.5             | C516             | KB05    |
| FAM40A        | protein FAM40A                                | 1.4 (KB05yne)              | 3.4             | C769             | KB05    |
| SLC4A11       | sodium bicarbonate transporter-like protein 11 | 1.2 (KB05yne) | 4.3             | C170             | KB05    |
| SMARC1D1      | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1 | 1.3 (KB05yne) | 4.3             | C460             | KB05    |

“Also shown are cysteine residues in these proteins found in previous chemical proteomic studies24,26,27,37,38 to be liganded by the corresponding scout fragments (KB02 or KB05, respectively).

Figure 3. Recombinantly expressed protein targets show site-speciﬁc reactivity with scout alkyne probes. (a, b) Gel-ABPP of proteins showing hyperreactivity with KB02yne (a) or KB05yne (b), verifying the scout fragment-sensitive cysteine in each protein target. Wild-type (WT) and cysteine-to-alanine (C-to-A) mutant forms of proteins were recombinantly expressed as FLAG epitope-tagged fusions in HEK293T cells (transient transfection), and cell lysates were treated with scout alkyne probes (1 \( \mu \)M, 1 h), subject to CuAAC conjugation to an azide-rhodamine reporter tag, and analyzed by SDS-PAGE followed by in-gel ﬂuorescence scanning. Anti-FLAG immunoblotting was used to conﬁrm a similar expression for WT and mutant forms of proteins. Gel-ABPP data are from a single experiment representative of at least two biological replicates. (c) Workflow for the ELISA-ABPP assay to screen for more advanced ligands of scout fragment-hyperreactive proteins, where biotinylated scout probes are used for site-speciﬁcally labeled cysteines in recombinantly expressed proteins evaluated in transfected cell lysates. Active and inactive denote competitor ligands that do or do not engage a scout fragment-sensitive cysteine, respectively. (d) Quantiﬁcation of scout probe (alkyne or biotin) reactivity with WT and C-to-A mutant forms of hyperreactive proteins via gel- and ELISA-ABPP, respectively. Data represent average values ± standard deviation for at least two independent biological replicates.
potentially suitable for site-specific assay development and screening.

In total, our MS-ABPP studies identified 667 and 447 proteins with $R$ values < 2 for KB02yne and KB05yne, respectively, out of a total of $\sim$5500 quantified proteins (Supplementary Data set 1). Notably, several of these proteins showed much higher $R$ values with IA-alkyne (Figure 2b–d), supporting preferential reactivity with scout fragments. We next cross-referenced the KB02yne/KB05yne hyperreactive proteins with previously published MS-ABPP data mapping cysteines that are liganded by KB02 and KB05, 24,26,27,37,38 which revealed that 47% and 42% of these proteins possessed at least one cysteine residue that had been identified as liganded by KB02 or KB05, respectively (Supplementary Data set 1). Examples of proteins showing preferential reactivity with KB02yne and/or KB05yne that also harbor a KB02 and/or KB05-liganded cysteine are shown in Figure 2d and Table 1. These data, taken together, support generally consistent cysteine reactivity profiles for the scout fragments and their corresponding alkyne probes. We next sought to determine if the scout alkyne probes could label recombinantly expressed protein targets of interest with site specificity for liganded cysteine residues.

Proteins from diverse functional classes (e.g., enzymes (AKR1B10, CDK2, IDH1, NAGK), transcription factors (IRF4), adaptors (HPCAL1, MOB4, CDKN2AIP), RNA-binding proteins (WDR43)), and subcellular localizations (e.g., membrane (ERLIN1)) were selected for follow-up studies to evaluate the performance of scout probes in gel- and ELISA-ABPP assays. Proteomic lysates from HEK293T cells transiently expressing wild-type (WT) and cysteine-to-alanine (C-to-A) mutants of each protein were treated with a scout probe (1 μM, 1 h) followed by CuAAC conjugation to a rhodamine azide tag. Samples were then subjected to SDS-PAGE and in-gel fluorescence. Anti-FLAG immunoblotting was used to confirm a similar expression between WT and mutant forms of proteins. Gel-ABPP data are from a single experiment representative of at least two biological replicates. (b) ELISA-ABPP comparison of probe reactivity. Lysates were treated with KB02-biotin (1 μM, 1 h) or IA-biotin (1 or 10 μM, 1 h). Data represent average values ± standard deviation for at least two biological replicates.

Figure 4. Comparison of site-specific cysteine reactivity by scout and IA-based probes. (a) Gel-ABPP comparison of probe reactivity. WT and C-to-A mutants were recombinantly expressed in transiently transfected HEK293T cells and cell lysates treated with alkyne probes (1 or 10 μM, 1 h) followed by CuAAC conjugation to a rhodamine azide tag. Samples were then subjected to SDS-PAGE and in-gel fluorescence. Anti-FLAG immunoblotting was used to confirm a similar expression between WT and mutant forms of proteins. Gel-ABPP data are from a single experiment representative of at least two biological replicates. (b) ELISA-ABPP comparison of probe reactivity. Lysates were treated with KB02-biotin (1 μM, 1 h) or IA-biotin (1 or 10 μM, 1 h). Data represent average values ± standard deviation for at least two biological replicates.
KB05yne (Figure 3b), and mutation of the principal scout fragment-sensitive cysteine in these proteins ablated the majority of the labeling (Figure 3a,b). Mutation of other cysteines in representative proteins did not substantially alter scout fragment reactivity (Figure 3a,b). Cysteines in representative proteins did not substantially alter scout fragment reactivity (Figure 3a,b). Mutation of other cysteines in these proteins ablated the majority of the labeling (Figure 3a,b). Having determined that site-specific reactions between scout probes and protein targets could be readout by gel-ABPP, we next sought to develop a more convenient assay format for competitively screening large numbers of electrophilic small molecules that, at the same time, did not require purification of the protein targets. Toward this end, we established a 96-well ELISA plate-based assay where lysates of HEK293T cells expressing FLAG epitope-tagged protein targets were pretreated with electrophilic compounds (5 μM, 1 h) or DMSO as a control followed by incubation with biotinylated scout probes (KB02-biotin or KB05-biotin, 1 μM each, 1 h; Supplementary Figure 2a) and transfer to a streptavidin-coated microplate to enrich biotinylated scout probe-labeled proteins (Figure 3c). After washing, the plate was then incubated with an anti-FLAG-HRP antibody, washed again, and treated with the HRP substrate tetramethylbenzidine (TMB) to provide a colorimetric measurement of protein target enrichment in DMSO- versus electrophilic compound-treated samples (Figure 3c). We first verified the accuracy of the ELISA-ABPP assay by comparing biotinylated scout probe reactivity with WT vs C-to-A mutants of protein targets, which revealed consistently superior enrichment of the WT proteins with good Z’ scores and data quality that generally matched the gel-ABPP results (Figure 3d).59

We next compared the site-specific cysteine reactivity of scout probes to more broadly reactive IA probes, which revealed that, in either gel-ABPP (Figure 4a) or ELISA-ABPP (Figure 4b) formats, the scout fragment probes consistently showed superior performance. When tested at equivalent concentrations (1 μM), the scout probes generally exhibited greater intensity of site-specific labeling of cysteines in proteins of interest compared to IA probes (e.g., AKR1B10, IDH1, NAGK, CDKN2AIP, HCPAL1). Attempts to increase the intensity of signals generated by IA probes by, for instance, using higher probe concentrations (10 vs 1 μM) were confounded by greater background labeling of additional cysteines on either the proteins of interest or other proteins in the cell lysate (e.g., AKR1B10, CDKN2AIP, IRF4, HCPAL1). This issue even led to a paradoxical reduction in signal intensity for AKR1B10 in ELISA-ABPP experiments performed with 10 versus 1 μM IA-biotin (Figure 4b), possibly reflecting suppression of AKR1B10 enrichment on the streptavidin plate due to binding of many other IA-biotin-labeled proteins in the cell lysate. These results indicate that the greater potency and specificity displayed by scout probes are critical features to enable screening of individual cysteines in proteins by gel- and ELISA-ABPP.

As a case study for electrophilic compound screening by ELISA-ABPP, we selected the NADPH-dependent reductase AKR1B10, which metabolizes aliphatic carbonyl-containing compounds, including all-trans retinol, and plays a protumorigenic role through detoxification of reactive oxygen species and regulation of fatty acid synthesis and lipid metabolism.40,41 We have also found that AKR1B10 is a NRF2-regulated protein in KEAP1 mutant nonsmall cell lung cancers (NSCLCs).26 The scout fragment-sensitive cysteine in AKR1B10−C299− is located in the active site of this enzyme (Figure 5a), and natural electrophilic compounds that engage this cysteine, such as prostaglandin A1, inhibit AKR1B10 activity.72

We performed ELISA-ABPP on FLAG-AKR1B10-transfected HEK293T cell lysates treated with a set of electrophilic compounds (138 total, tested at 5 μM; 1 h) representing elaborated structural analogues of KB02 that preserved the α-chloroacetamide reactive group and were derivatized through modification of the methoxy group of the 1,2,3,4-tetrahydroisoquinoline core or members of our in-house electrophile library (Supplementary Data set 1).26,27 This collection of compounds provided diverse recognition and reactive groups, including ~25% acrylamides. A number of hit compounds were identified, the most potent of which were two α-
Figure 6. Characterization of KB02-derived ligands showing improved potency and selectivity for AKR1B10. (a) Concentration-dependent profiles for the blockade of KB02-biotin engagement of AKR1B10_C299 by KB02, VC59, and VC63, as measured by ELISA-ABPP. Data represent average values \( \pm \) standard deviation for at least two independent biological replicates. (b) VC59 blocks KB02-biotin engagement of endogenous AKR1B10 in H460 cell lysates (in vitro) (left) but not in intact H460 cells (in situ) (right). (c, d) MS-ABPP ratio plots showing cysteines liganded by VC59 in H460 lysates (in vitro; c) or H460 cells (in situ; d). Active site (C299) and other (C187) cysteine residues in AKR1B10 are highlighted in red. R values correspond to cysteine reactivity ratios in DMSO-treated samples/VC59-treated samples. (e) Heat map of cysteines liganded by VC59 (R values > 4.0) in vitro (left) and their corresponding R values from in situ (right) experiments, demonstrating that AKR1B10_C299 is unusual in showing a lack of engagement by VC59 in situ. The in vitro and in situ R values for each cysteine shown in the heat map are internally normalized to 100% for ease of visualization.

chloroacetamides—VC59 and VC63—that suppressed KB02-biotin enrichment of AKR1B10 by >80% (Figure 5c). Additional hits included EV97, a tryptoline acrylamide that stereoselectively blocked KB02-biotin reactivity with AKR1B10 (61%; Figure 5c).

We then used ELISA-ABPP to calculate IC\(_{50}\) values for VC59 and VC63, both of which showed low-\( \mu \)M activity (0.9 \( \pm \) 0.3 \( \mu \)M and 1.6 \( \pm \) 0.7 \( \mu \)M \( \text{IC}_{50}\) values, respectively), representing an \(~30\)-fold improvement over KB02 (IC\(_{50}\) of 36 \( \pm \) 1 \( \mu \)M) (Figure 6a). VC59 was also evaluated by gel-ABPP, which determined a similar IC\(_{50}\) value of 0.7 \( \pm \) 0.3 \( \mu \)M (Supplementary Figure S3a), and confirmed to engage endogenous AKR1B10 by pretreating H460 cell lysates with VC59 (0.5–20 \( \mu \)M, 1 h) followed by KB02-biotin (1 \( \mu \)M, 1 h), streptavidin enrichment, and immunoblotting for AKR1B10 (Figure 6b). We also used gel-ABPP to confirm a stereoselective blockade of KB02-alkyne reactivity with AKR1B10 by the acrylamide EV97, albeit at lower potency (\(~5\) \( \mu \)M; Supplementary Figure S3b) than \( \alpha \)-chloroacetamides VC59 and VC63. We confirmed that VC59 engagement of AKR1B10_C299 inhibited the reductase activity of this enzyme using a previously reported NADPH absorbance assay (Supplementary Figure S3c). In contrast, the AKR1B10_C299A mutant was not inhibited by VC59 but maintained sensitivity to previously described noncovalent AKR1B10 inhibitors (tolrestat and isolithocholic acid, Supplementary Figure S3c,d). Notably, the binding of these reversible inhibitors to AKR1B10 was assayable by ELISA-ABPP (Supplementary Figure S3e) suggesting that this method could also be used to screen reversible small-molecule libraries against proteins of interest.

We next determined the proteomic selectivity of VC59 (5 \( \mu \)M, 1 h) in H460 cell lysate by competitive isoTOP-ABPP, which confirmed complete engagement of C299 of AKR1B10 and identified only a handful of additional cross-reactivity events (e.g., ACAT1_C196, ALDH3A2_C231, CKAP4_C100) among >3000 quantified cysteines (Figure 6c and Supplementary Data set 1). Surprisingly, we found that VC59 did not engage C299 of AKR1B10 in living H460 cells, even when tested at 20 \( \mu \)M (Figure 6d,e, 3 h), despite maintaining cross-reactivity with other targets in situ (Figure 6d,e and Supplementary Data set 1). We suspected that decreased in situ reactivity of VC59 with AKR1B10 reflected a change in the state of this protein in cells. Additional experiments revealed that KB02yne reactivity of C299 of AKR1B10 was blocked by increasing concentrations of NADPH (IC\(_{50}\) = 2.6 \( \pm \) 0.7 \( \mu \)M, Supplementary Figure S3f), suggesting that AKR1B10 may be fully bound to this cofactor in cells, which could then slow or prevent electrophilic compound reactivity with C299 in cells.

Here, we have leveraged the attenuated reactivity of two broadly reactive scout fragment electrophiles to generate probes that can be used for cysteine-specific, HTS-compatible ELISA-ABPP assays on a wide range of proteins directly in transfected cell lysates. We show how this approach can be used to identify more advanced ligands for the cancer-related enzyme AKR1B10, a protein with an active-site cysteine (C299) that displays hyperreactivity with the scout fragment.
KB02. More generally, we believe that ELISA-ABPP should provide a swift and near-universal assay format to discover hit compounds for structurally and functionally diverse proteins; however, its implementation does not negate the need for functional assays to confirm modulation of protein activity and facilitate optimization of both the binding ($K_a$) and reactivity ($k_{\text{on}}$) components of covalent compound-cysteine interactions. Projecting forward, it is noteworthy that several of the other site-specific scout fragment reactions mapped herein also occur with cysteines that reside within or in close proximity to functional regions of proteins. For instance, KB02yne site-specifically reacted with C269 of isocitrate dehydrogenase-1 (IDH1), a residue that is located on a dynamic loop neighboring the NADP(H)-binding pocket of the enzyme and in the same binding region as reported IDH1 inhibitors. (Supplementary Figure S4a). We confirmed that KB02yne also site-specifically reacted with C269 of the oncogenic R132H mutant of IDH1 (Figure 3a), suggesting that covalent ligands targeting this cysteine could provide a way to inhibit IDH1-dependent cancers. Consistent with this premise, Eli Lilly has recently reported a mutant IDH1-selective inhibitor that covalently reacts with C269. The KB02yne-sensitive cysteine in CDK2 (C177) is a surface exposed residue (Supplementary Figure S4b) that resides in an allosteric inactivator site and may provide a way to selectively block this enzyme over other CDKs, which do not share this enzyme. C310 of ERLIN1, C134 of MOB4, and C516 of CDKN1AIP2 all reside at predicated protein–protein interaction sites, and covalent ligands engaging C516 of CDKN1AIP2 have been shown to disrupt interactions with the Wnt ligand Dishevelled, resulting in reduced Wnt signaling. Finally, some of the scout fragment reactivity events occurred at uncharacterized cysteines on oncogenic proteins such as C194 of the multiple myeloma transcription factor IRF4 (Figure 3a) and may provide a path to developing chemical probes for these difficult to target proteins. For such targets, larger compound libraries may need to be screened to find hit compounds. While ELISA-ABPP should be amenable to such larger screens, a current bottleneck is the limited size and structural diversity of covalent chemistry libraries, a topic that is of considerable emerging interest. Our further discovery that ELISA-ABPP identified acrylamide hit ligands for AKR1B10 (e.g., EV97), as well as verified the activity of previously described reversible inhibitors of this enzyme, points to the potential for the platform to discover both reversible and irreversible ligands for proteins that extend beyond the chemotype of the scout fragment probe itself. In considering some of the potential current limitations of using scout probes, we note that several proteins showing hyperreactivity with these probes did not show evidence of possessing a cysteine that was strongly engaged by KB02 or KB05 in our legacy chemical proteomic studies (Figure 2b,c, blue signals in right graphs). While some of these cases may represent cysteines that show superior reactivity with the alkynylated versions of scout fragments, others may reflect proteins where the relevant scout fragment-sensitive cysteine has not yet been identified. In this regard, we found that KB02yne/KB05yne-hyperreactive proteins lacking defined scout fragment-sensitive cysteines were enriched in membrane proteins that contain greater numbers of undetected cysteines in previous chemical proteomic experiments (Supplementary Figure S5). We speculate that the use of alternative protease digests more optimally suited for mapping membrane proteins may facilitate the identification of scout fragment-sensitive cysteines on such membrane proteins, thereby opening up an even broader swath of the proteome for covalent ligand discovery and optimization. Additionally, in considering ways to more effectively identify cell-active electrophilic compounds, we note that, in other studies, electrophilic compound-cysteine interactions have been assessed in situ by MS-ABPP and we anticipate that ELISA-ABPP should also be amenable to future screens performed in cells versus cell lysates.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.0c01336. Additional figures and biological and chemistry methods (PDF) Supporting Information Data set S1: MS-ABPP data for 1) hyperreactivity experiments, 2) isoTOP-ABPP data for VC59 treatment of H460 cells/lysates, and 3) chemical structures and AKR1B10 screen results for covalent library screened (XLSX).

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#### Notes

The authors declare the following competing financial interest(s): Dr. Cravatt is a cofounder and scientific advisor to Vividion Therapeutics, a biotechnology company interested in developing small-molecule therapeutics to treat human disease.

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### REFERENCES

1. Zhang, T.; Hatcher, J. M.; Teng, M.; Gray, N. S.; Kostic, M. Recent Advances in Selective and Irreversible Covalent Ligand Development and Validation. *Cell Chem. Biol.* 2019, 26 (11), 1486–1500.

2. Arrowsmith, C. H.; Audia, J. E.; Austin, C.; Baell, J.; Bennett, J.; Blagg, J.; Bountra, C.; Brennan, P. E.; Brown, P. J.; Bunnage, M. E.; Buser-Doepner, C.; Campbell, R. M.; Carter, A. J.; Cohen, P.;
(29) Chung, C. Y.; Shin, H. R.; Berdan, C. A.; Ford, B.; Ward, C. C.; Olzmann, J. A.; Zoncu, R.; Nomura, D. K. Covalent targeting of the vacuolar H(+)ATPase activates autophagy via mTORC1 inhibition. Nat. Chem. Biol. 2019, 15 (8), 776–785.

(30) Roberts, A. M.; Miyamoto, D. K.; Huffman, T. R.; Bateman, L. A.; Ives, A. N.; Akopian, D.; Heslin, M. J.; Contreras, C. M.; Raffe, M.; Skibola, C. F.; Nomura, D. K. Chemoproteomic Screening of Covalent Ligands Reveals UBAS As A Novel Pancreatic Cancer Target. ACS Chem. Biol. 2017, 12 (4), 899–904.

(31) Pinch, B. J.; Doctor, Z. M.; Nabet, B.; Browne, C. M.; See, H. S.; Mohardt, M. L.; Kozono, S.; Lian, X.; Manz, T. D.; Chun, Y.; Kibe, S.; Zaidman, D.; Daitchman, D.; Yeoh, Z. C.; Vangos, N. E.; Geffken, E. A.; Tan, L.; Ficarro, S. B.; London, N.; Marto, J. A.; Buratowski, S.; Dhe-Paganon, S.; Zhou, X. Z.; Lu, K. P.; Gray, N. S. Identification of a potent and selective covalent Pin1 inhibitor. Nat. Chem. Biol. 2020, 16 (9), 979–987.

(32) Boike, L.; Cioffi, A. G.; Majewski, F. C.; Co; J.; Henning, N. J.; Jones, M. D.; Liu, G.; McKenna, J. M.; Tallarico, J. A.; Schirle, M.; Kitade, Y.; Tajima, K.; Zhao, H. T.; El-Kabbani, O.; Hara, A. Kinetic studies of AKR1B10, human aldose reductase-like protein: endogenous substrates and inhibition by steroids. Arch. Biochem. Biophys. 2009, 487 (1), 1–9.

(33) Endo, S.; Matsunaga, T.; Mamiya, H.; Ohta, C.; Soda, M.; Kitade, Y.; Tajima, K.; Zhao, H. T.; El-Kabbani, O.; Hara, A. Kinetic studies of AKR1B10, human aldose reductase-like protein: endogenous substrates and inhibition by steroids. Arch. Biochem. Biophys. 2009, 487 (1), 1–9.

(34) Gallego, O.; Ruiz, F. X.; Ardevol, A.; Dominguez, M.; Alvarez, R.; de Lara, A. R.; Rovira, C.; Farres, J.; Fita, I.; Pares, X. Structural basis for the high all-trans-retinaldehyde reductase activity of the tumor marker AKR1B10. Proc. Natl. Acad. Sci. U. S. A. 2007, 104 (52), 20764–20769.

(35) Okoye-Okafor, U. C.; Bartholdy, B.; Carter, J.; Gao, E. N.; Pietrak, B.; Rendina, A. R.; Rominger, C.; Quinn, C.; Smallwood, A.; Wiggall, K. J.; Reif, A. J.; Schmidt, S. J.; Qh; S.; Zhao, H.; Joberty, G.; Faeth-Savitski, M.; Bantscheff, M.; Drewes, G.; Duraiswami, C.; Brady, P.; Groy, A.; Narayanagari, S. R.; Antony-Debre, L.; Mitchell, K.; Wang, H. R.; Kao, Y. R.; Christopeit, M.; Carvajal, L.; Barreyro, L.; Paitset; E.; Makishima, H.; Will, B.; Concha, N.; Adams, N. D.; Schwartz, B.; McCabe, M. T.; Maciejewski, J.; Verma, A.; Steid U. New IDH1 mutant inhibitors for treatment of acute myeloid leukemia. Nat. Chem. Biol. 2015, 11 (11), 878–886.

(36) Cho, Y. S.; Levell, J. R.; Liu, G.; Caffer, T.; Sutton, J.; Shafer, C. M.; Costales, A.; Manning, J. R.; Zhao, Q.; Sendzik, M.; Shultz, M.; Chenail, G.; Dooley, J.; Villabla, B.; Farsijani, A.; Chen, J.; Kulatil, R.; Xie; X.; Dodd, S.; Gould; T.; Liang, G.; Heimbach, T.; Slocom, K.; Firestone, B.; Pu, M.; Pagliarini, R.; Gownrey, J. D. Discovery and Evaluation of Clinical Candidate IDH305, A Brain Penetrant Mutant IDH1 Inhibitor. ACS Med. Chem. Lett. 2017, 8 (10), 1116–1121.

(37) Hai, Ma; R.; Yun, C. H. Crystal structures of pan-IDH inhibitor AG-881 in complex with human IDH1 and IDH2. Biochem. Biophys. Res. Commun. 2018, 503 (4), 2912–2917.

(38) Yen, K.; Travins, J.; Wang, F.; David, M. D.; Artin, E.; Staley, K.; Padyana, A.; Gross, S.; DeLaBarre, B.; Tobin, E.; Chen, Y.; Nagaraja, R.; Che; S.; Jin, L.; Konteatis, Z.; Cianchetta, G.; Saunders, J. O.; Salturto, F. G.; Quivoron, C.; Opolon, P.; Bawa, O.; Saada, V.; Paci; A.; Broutin, S.; Bernard, O. A.; de Botton, S.; Marteyn, B. S.; Pilchowska, M.; Xu, Y.; Fang, C.; Jiang, F.; Wei, W.; Jin, S.; Silverman, L.; Liu; Y.; Yang, H.; Dang, L.; Dorsch, M.; Penard-Lacomrine, V.; Biller, S. A.; Su, S. M. AG-221, A First-in-Class Therapy Targeting Acute Myeloid Leukemia Harboring Oncogenic IDH2 Mutations. Cancer Discovery 2017, 7 (5), 478–493.

(39) Brooks, N.; DeWalt, R.; Boulet, S.; Lu, Z.; Kays, L.; Cavitt, R.; Gomez, S.; Sdelrov, J.; Milligan, P.; Roth, K.; Bauers, R.; Antonsamy, S.; Hahn, P.; Rankovic, Z.; Mccann, D.; Mo, G.; Tiu, R.; Burkholder, T.; Geegman, S.; Gilmour, R. Identification and characterization of LY3410738, a novel covalent inhibitor of cancer-associated mutant Isocitrateg Dehydrogenase 1 (IDH1). In Proceedings of the American Association for Cancer Research Annual Meeting 2019, Atlanta, GA, Philadelphia (PA): AACR: Atlanta, GA, Philadelphia, PA, 2019.

(40) Camarillo, J. M.; Rose, K. L.; Galligan, J. J.; Xu, S.; Marnett, L. J. Covalent Modification of CDK2 by 4-Hydroxynonenal as a Mechanism of Inhibition of Cell Cycle Progression. Chem. Res. Toxicol. 2016, 29 (3), 3233–32.

(41) Pednekar, D.; Wang, Y.; Fedotova, T. V.; Wouciwickiz, R. J. Clustered hydrophobic amino acids in amphphilic helices mediate erlin1/2 complex assembly. Biochem. Biophys. Res. Commun. 2011, 415 (1), 135–140.

(42) Chen, M.; Zhang, H.; Shi, Z.; Li, Y.; Zhang, X.; Gao, Z.; Zhou, L.; Ma; J.; Xu; Q.; Guan, J.; Cheng, Y.; Jiao, S.; Zhou, Z. The MST4-MOB4 complex disrupts the MST1-MOB1 complex in the Hippo-YAP pathway and plays a pro-oncogenic role in pancreatic cancer. J. Biol. Chem. 2018, 293 (37), 14455–14469.

(43) He, X.; Zhang, W.; Yan, C.; Nie, F.; Li, C.; Liu, X.; Fei, C.; Li, S.; Song, X.; Jia, Y.; Zeng, R.; Wu, D.; Pan, W.; Hao, X.; Li, L. Chemical biology reveals CARF as a positive regulator of canonical Wnt signaling by promoting TCF/beta-catenin transcriptional activity. Cell Discov 2017, 3, 17003.

(44) Agnarelli, A.; Chevassut, T.; Mancini, E. J. IRF4 in multiple myeloma-Biology, disease and therapeutic target. Leuk. Res. 2018, 72, S8–S5.

(45) Keeley, A.; Petri, L.; Abranyi-Balogh, P.; Keseru, G. M. Covalent fragment libraries in drug discovery. Drug Discovery Today 2020, 25 (S6), 983–996.

(46) Resnick, E.; Bradley, A.; Gan, J.; Douangamath, A.; Krojer, T.; Sethi, R.; Geurink, P. P.; Aimon, A.; Amatia, G.; Bellini, D.; Bennett, J.; Fairhead, M.; Fedorov, O.; Gabizon, R.; Gan, J.; Guo, J.; Plotnikov, A.; Resnik, N.; Ruda, G. F.; Diaz-Salez, L.; Straub, V. M.; Szommer, T.; Velupillai, S.; Zaidman, D.; Zhang, Y.; Coker, A. R.; Dowson, C.
G.; Barr, H. M.; Wang, C.; Huber, K. V. M.; Brennan, P. E.; Ovaa, H.; von Delft, F.; London, N. Rapid Covalent-Probe Discovery by Electrophile-Fragment Screening. *J. Am. Chem. Soc.* 2019, 141 (22), 8951−8968.

(57) Spradlin, J. N.; Hu, X.; Ward, C. C.; Brittain, S. M.; Jones, M. D.; Ou, L.; To, M.; Proudfoot, A.; Ornelas, E.; Woldegiorgis, M.; Olzmann, J. A.; Bussiere, D. E.; Thomas, J. R.; Tallarico, J. A.; McKenna, J. M.; Schiele, M.; Maimone, T. J.; Nomura, D. K. Harnessing the anti-cancer natural product nimbolide for targeted protein degradation. *Nat. Chem. Biol.* 2019, 15 (7), 747−755.

(58) Zhang, X.; Crowley, V. M.; Wucherpfennig, T. G.; Dix, M. M.; Cravatt, B. F. Electrophilic PROTACs that degrade nuclear proteins by engaging DCAF16. *Nat. Chem. Biol.* 2019, 15 (7), 737−746.