JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Mapping the Protein Interaction Landscape for Fully Functionalized Small-Molecule Probes in Human Cells

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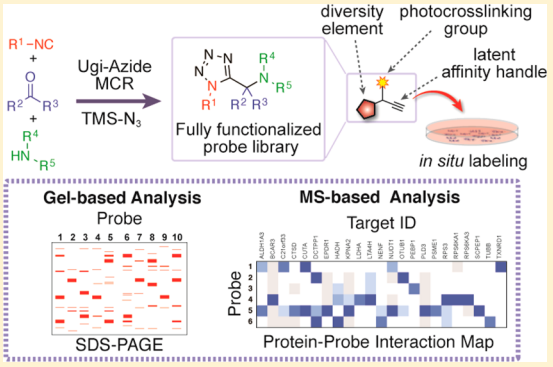
Supporting Information

ABSTRACT: Phenotypic screening provides a means to discover small molecules that perturb cell biological processes. Discerning the proteins and biochemical pathways targeted by screening hits, however, remains technically challenging. We recently described the use of small molecules bearing photoreactive groups and latent affinity handles as fully functionalized probes for integrated phenotypic screening and target identification. The general utility of such probes, or, for that matter, any small-molecule screening library, depends on the scope of their protein interactions in cells, a parameter that remains largely unexplored. Here, we describe the synthesis of an ∼60-member fully functionalized probe library, prepared from Ugi-azide condensation reactions to impart structural diversity and introduce diazirine and alkyne functionalities for target capture and enrichment, respectively. In-depth mass spectrometry-based analysis revealed a diverse array of probe targets in human cells, including enzymes, channels, adaptor and scaffolding proteins, and proteins of uncharacterized function. For many of these proteins, ligands have not yet been described. Most of the probe−protein interactions showed well-defined structure−activity relationships across the probe library and were blocked by small-molecule competitors in cells. These findings indicate that fully functionalized small molecules canvas diverse segments of the human proteome and hold promise as pharmacological probes of cell biology.



INTRODUCTION

The phenotypic screening of small-molecule libraries is a central component of academic and industrial programs aimed at pharmacologically perturbing and understanding biochemical pathways in living systems.[1,2] The output of phenotypic screening can be new chemical probes for basic research and drugs for treating human disorders. Identifying the targets of phenotypic screening hits, however, remains a major technical hurdle and a critical goal for elucidating the mechanism(s) of action of bioactive compounds.[3,4] Several strategies have been introduced to facilitate target identification,[4−6] and each approach has a distinct set of advantages and limitations: some identify targets through direct binding to small molecules, but at the cost of requiring the prior lysis of cells and attachment of compounds to a solid support; others facilitate target identification *in situ* but rely on indirect (e.g., genetic or metabolic) evidence to forge functional connections between compounds and proteins. We,[7] and others,[8] have introduced complementary approaches where the small-molecule library itself possesses design features intended to facilitate target identification. These features can include small-molecule libraries with embedded linker elements so that the point of contact to solid support for biochemical target enrichment is already defined prior to phenotypic assay.[8] We have alternatively described small-molecule libraries that are fully functionalized, in that they contain "variable" binding elements to promote interactions with different proteins in cells and "constant" photoreactive and alkyne groups for covalent modification and detection/identification of protein targets, respectively.[7] These probes have the advantage of forging covalent bonds with protein targets directly in living cells under the same assay conditions used for phenotypic screening. Subsequent target enrichment and identification are then achieved by copper-catalyzed azide−alkyne cycloaddition (CuAAC or "click"[9,10]) chemistry-enabled biotin−(strept)avidin chromatography and mass spectrometry (MS) analysis. Nonetheless, the incorporation of photoreactive and alkyne groups presents synthetic challenges that may limit the structural diversity of fully functionalized probes and impinge on the scope of their protein interactions in cells. Here, we set out to address these challenges by first devising an efficient synthetic route to create a structurally diverse library of fully functionalized probes and then applying these probes in combination with quantitative MS-based proteomic methods to globally assess probe-target interactions in human cancer cells. These studies uncovered a broad protein interaction landscape for fully functionalized probes, including enzymes, channels, adaptor and scaffolding proteins, and proteins of uncharacterized function. Many of these proteins lack known ligands, indicating

Received: June 2, 2014
Published: July 21, 2014

   dx.doi.org/10.1021/ja505517t | J. Am. Chem. Soc. 2014, 136, 10777−10782

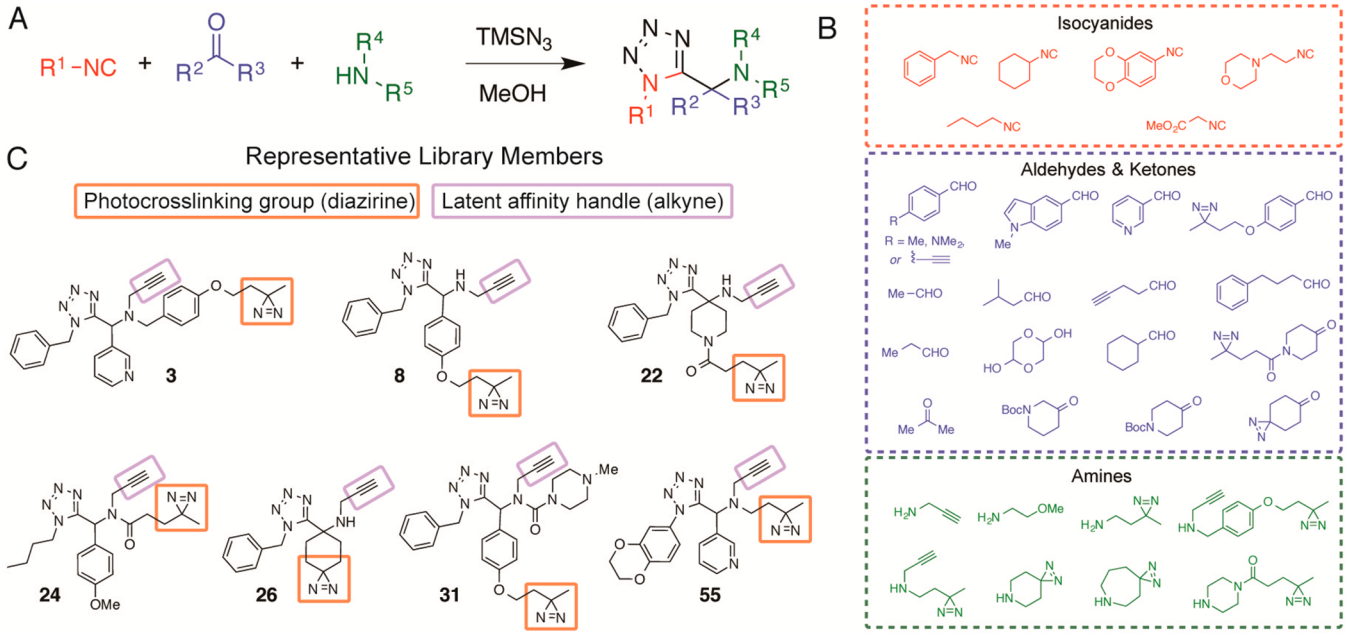


Figure 1. Synthesis and representative members of a fully functionalized small-molecule probe library. (A) Ugi-azide multicomponent condensation reaction used to synthesize a library of 1,5-disubstituted tetrazoles. (B) Components used in Ugi-azide reaction for library synthesis. (C) Structures of representative library members highlighting diazirine and alkyne groups for protein photocrosslinking and ligation of reporter tags to probe-bound targets, respectively. Also see Figure S1 for a complete list of probe structures.

that fully functionalized probes can provide pharmacological access to underexplored portions of the human proteome.

RESULTS

Library Design and Synthesis. We previously reported a first-generation fully functionalized library constructed using a benzophenone photocrosslinking element.[7] While this library proved effective for coupled phenotypic screening and target identification, its structural diversity and, by extension, protein-interaction potential were limited by the size of the benzophenone group and synthetic chemistry challenges facing its derivatization. Toward the goal of developing an efficient synthetic strategy to produce a more structurally varied library, we applied the Ugi-azide multicomponent condensation reaction[11] to afford rapid assembly of 1,5-disubstituted tetrazoles from amine, isocyanide, and aldehyde/ketone building blocks (Figure 1A,B). With the goal of minimizing the steric footprint of the photocrosslinking element, we chose the diazirine group to forge the covalent bond between probes and protein targets. Upon UV irradiation, diazirines extrude nitrogen to form a highly reactive carbene species that is capable of inserting into C−H, N−H, and O−H bonds on a protein, forming a covalent adduct.[12] This mechanism differs from that of benzophenones, which react via a triplet diradical.[13] Diazirine and benzophenone probes may therefore exhibit distinct crosslinking efficiencies for individual amino acids, although how such differences ultimately affect labeling at the protein level is unclear. We also incorporated a terminal alkyne into each library member to allow visualization and enrichment of probe-labeled proteins following CuAAC with azide-bearing reporter groups.[14] We first prepared a collection of diazirines that possess either an amine or a carbonyl, either to serve as one component of the Ugi-azide condensation reaction or to allow immediate functionalization of the corresponding Ugi-azide product. Using these, along with a larger collection of commercially available reactants (Figure 1B), we prepared a structurally diverse, ~60-member "fully functionalized" probe library (Figure 1C and Figure S1). We then set out to globally map the proteomic interactions of this probe library in human cells.

Target Profiling in Cells. We initially evaluated protein targets of the probe library by treating the human PC-3 prostate cancer cell line with each library member (10 μM, 30 min, 37 °C), followed by exposure to UV light for 10 min at 4 °C, cell lysis, and separation of soluble and membrane proteomes by centrifugation. Probe-labeled proteins in these proteomic fractions were coupled to a rhodamine−azide (Rh−N_3) reporter tag[14] using CuAAC chemistry,[9,10] and separated and detected by SDS-PAGE and in-gel fluorescence scanning, respectively. The resulting profiles revealed a striking diversity of *in situ* protein labeling events, with most probes showing distinct labeling patterns across the cancer cell proteome, and, conversely, the protein targets exhibited different preferences for individual probes (Figure 2 and Figure S2). Virtually all protein labeling was UV-light-dependent, with the exception of a 55 and 60 kDa proteins that were labeled by a subset of probes in no-UV-light control experiments (e.g., probes **10** and **20**, Figure 2A). The aggregate structure−activity relationship (SAR) for *in situ* probe−protein interactions enabled selection of a subset of probes—**3**, **8**, **22**, **24**, **26**, **31**, and **55** (Figure 2B)—for in-depth MS studies based on their complementary profiles.

In our initial MS studies, we measured the relative protein enrichments for each test probe versus a common comparison probe **3**, which showed a protein-interaction profile that included most of the proteins that interacted broadly with the probe library, but limited evidence of unique protein-labeling events. Quantification of relative protein enrichments was achieved by SILAC (Stable isotope labeling by amino acids in cell culture[15]) methods, following previously described protocols.[7,16] In brief, we treated heavy-isotope-labeled ("heavy") and light-isotope-labeled ("light") PC3 cells with the test probe (**8**, **22**, **24**, **26**, **31**, or **55**) and probe **3** (10 μM of each probe), respectively, following the general protocol described for gel-based profiling

dx.doi.org/10.1021/ja505517t | J. Am. Chem. Soc. 2014, 136, 10777−10782

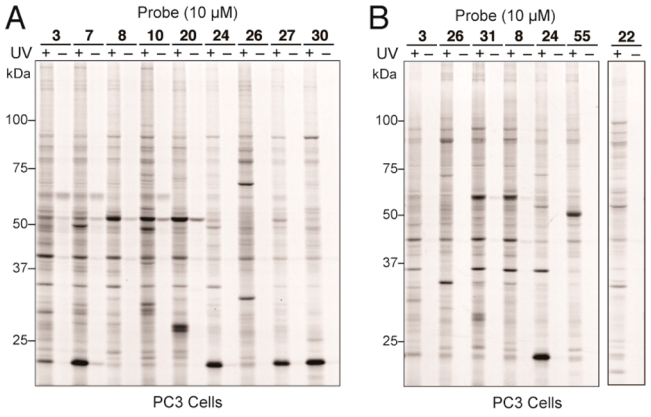


Figure 2. Gel-based profiles of *in situ* protein labeling events of PC3 cells treated with fully functionalized probes. (A) Gel profiles for representative probes (10 μM) incubated with PC3 cells for 30 min prior to UV light exposure (10 min), lysis, conjugation to an Rh–N_3 reporter tag by CuAAC, and analysis by SDS-PAGE and fluorescence scanning. Shown are soluble PC3 proteomes; see Figure S2 for gel profiles of additional probes and membrane proteomes of PC3 cells. (B) PC3 cell gel-based profiles of probes selected for in-depth MS studies.

except that the heavy and light proteomic samples were mixed prior to CuAAC conjugation to an azide-biotin tag. Biotinylated proteins were enriched using streptavidin chromatography, digested on-bead with trypsin, and the resulting tryptic peptide mixture analyzed by liquid chromatography–mass spectrometry (LC-MS) using an LTQ-Orbitrap mass spectrometer. Proteins that exhibited heavy:light SILAC ratios ≥3 were designated as preferred targets of the test probe. These SILAC experiments identified a distinct set of preferred targets for each test probe, with some probes showing a restricted interaction profile (e.g., **24**, which selectively enriched a single protein PEBP1 relative to probe **3**) and others exhibiting a larger number of targets (e.g., **31**, which enriched nine protein targets relative to probe **3**) (Figure 3A). Interestingly, many of the protein targets could be matched with confidence to the gel-based profiles on the basis of their predicted molecular weights and selective interactions across the test probes (Figure S3). Control experiments with representative probes confirmed that targets showed approximately 1:1 ratios in comparisons of heavy and light cells treated with the same probe and that target enrichment was, in general, dependent on UV-light exposure (Figure 3B and Table S1).

Analysis of Protein Targets. In total, 24 preferred protein targets were identified for the six test probes and included diverse classes of enzymes (kinases, peptidases, metabolic enzymes), adaptor proteins, scaffolding proteins, and proteins of uncharacterized function (Table 1). A search of the literature revealed that the targets near-equally distributed into proteins with and without known ligands. Among the proteins with known ligands were NUDT1, a metabolic enzyme that has recently been shown to repair damaged bases in cancer cells[17,18] and found here to interact preferentially with **31** relative to other probes; PEBP1, a protein binding partner and negative regulator of Raf kinase[19] and found here to interact preferentially with **24**; and tubulin, a classical target of many anti-tumorigenic drugs[20] and found here to interact preferentially with **55**. Among the proteins that lack known ligands, it is interesting to note that several could be viewed as traditionally difficult to "drug", including adaptor and scaffolding proteins (e.g., BCAR3,[21] CUTA[22]). We also performed a more limited analysis of membrane proteomes from probe-treated cells and identified a distinct set of proteins that showed differential probe labeling in comparisons of **3** and

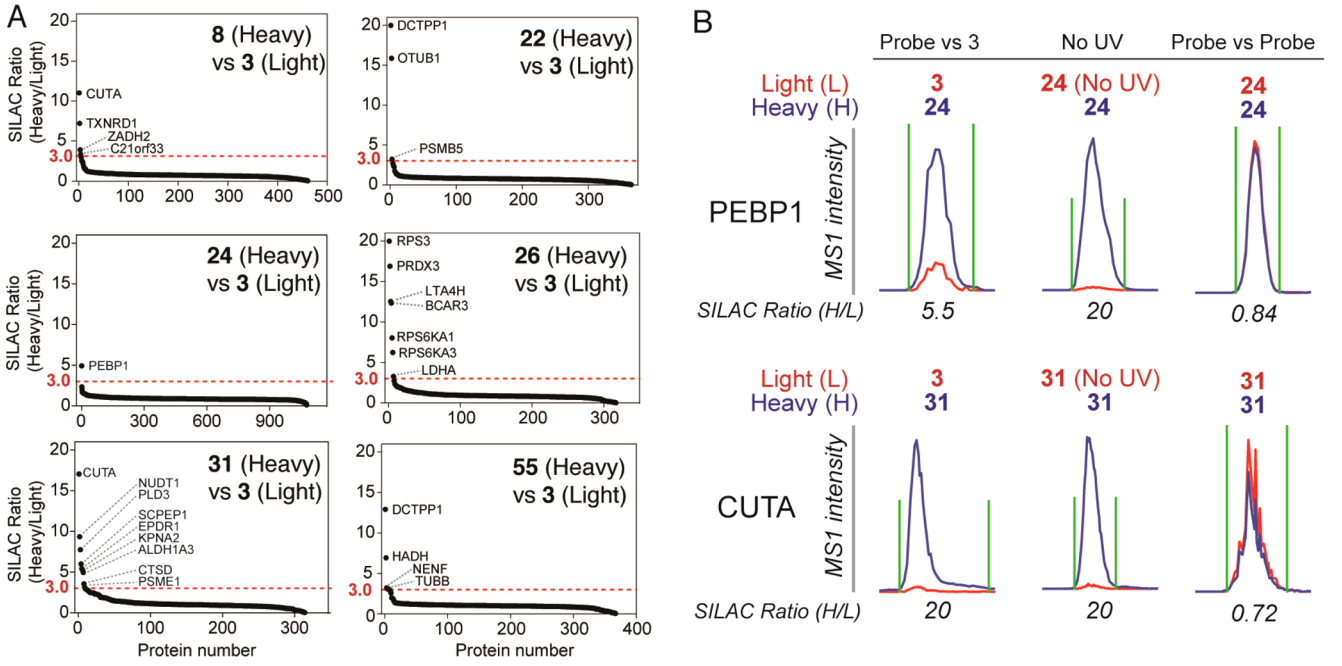


Figure 3. Quantitative proteomic data for representative targets of the probe library. (A) SILAC plots for total proteins identified in experiments comparing cells treated with test probe (**8**, **22**, **24**, **26**, **31**, and **55**) versus **3** (10 μM probes, 30 min). Proteins with median SILAC ratios ≥3 (test probe/**3**) are designated as preferred targets of the test probe (red dashed line marks 3-fold enrichment). Results are a combination of duplicate proteomic experiments performed in PC3 cells. See Table S1 for full proteomic data sets. (B) Representative MS1 peptide traces for protein targets of probes **24** (PEBP1) and **31** (CUTA) in experiments comparing the test probes to probe **3** ("probe vs **3**"), to no-UV-light controls ("no UV"), and to themselves ("probe vs probe"). SILAC ratios ≥20 are reported as 20. SILAC ratios are shown as heavy:light.

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Table 1. Probe Targets, Defined as Proteins That Showed 3-fold or Greater Enrichment in Quantitative Proteomic Comparisons of Test Probes versus Probe 3[a]

protein name	preferred probe(s)	competition	protein type	known ligands
ALDH1A3	**31**	partial	enzyme (dehydrogenase)	yes
BCAR3	**26**	yes	adaptor protein	no
C21orf33	**8**	yes	putative enzyme (amidotransferase)	no
CTSD	**31**	yes	enzyme (peptidase)	yes
CUTA	**8, 31**	yes	scaffolding protein	no
DCTPP1	**22, 55**	yes	enzyme	yes
EPDR1	**31**	partial	uncharacterized	no
HADH	**55**	ND	enzyme (metabolic)	no
KPNA2	**31**	yes	importin	no
LDHA	**26**	no	enzyme (dehydrogenase)	yes
LTA4H	**26**	partial	enzyme (hydrolase)	yes
NENF	**55**	ND	neurotrophic factor	no
NUDT1	**31**	yes	enzyme (phosphatase)	yes
OTUB1	**22**	yes	enzyme (peptidase)	no
PEBP1	**24**	yes	kinase/lipid-binding protein	yes
PLD3	**31**	partial	enzyme (lipase)	no
PRDX3	**26**	no	enzyme (peroxidase)	no
PSME1	**31**	no	proteasome activator	no
RPS3	**26**	yes	ribosomal protein	no
RPS6KA1	**26**	partial	enzyme (kinase)	yes
RPS6KA3	**26**	yes	enzyme (kinase)	yes
SCPEP1	**31**	yes	enzyme (peptidase)	no
TUBB[b]	**55**	ND	tubulin	yes
TXNRD1	**8**	partial	enzyme (reductase)	yes

[a]For competition data, designations of "yes" and "partial" correspond to competition ratios of less than or equal to 0.5 and 0.5–0.67, respectively; ND, not determined. References for known ligands (defined as compounds that have been shown to affect the biochemical or cellular activity of the target): ALDH1A3,[23] CTSD,[24] DCTPP1,[25] LDHA,[26] LTA4H,[27] NUDT1,[17,18] PEBP1,[28] RPS6KA1/3,[29] TUBB,[20] and TXNRD1.[30] [b]Multiple tubulin variants were enriched by **55** and these proteins shared overlapping and distinct peptides. For the sake of clarity, we have listed these variants as a single entry, but the data for all variants can be found in Table S1.

26 (Table S1). These proteins included transmembrane channels, enzymes, and proteins of uncharacterized function.

Protein targets of the probe library not only exhibited diversity in their structure and function, but also in their relative abundance in cells, as estimated by searches of the public CRAPome (Contaminant Repository for Affinity Purification) database,[31] which inventories the detection frequency of proteins across ~400 negative-control affinity chromatography experiments. We found that the most of the protein targets showed very low frequencies of detection in the CRAPome repository (<5%) (Figure 4), suggesting that they are lower abundance proteins. A handful of protein targets were much more commonly detected in CRAPome searches (e.g., TUBB, >50% frequency of detection) (Figure 4), but, even for such abundant proteins, selective probe labeling could be readily discerned over background signals in our comparisons of test probes to probe **3** (Figure S4).

Evidence That Protein Targets Are Substantially Engaged by Probes in Cells. Based on their different interaction profiles with the test probes (Figure 5A), each

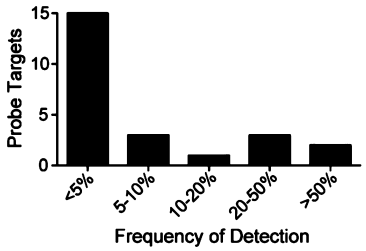

Figure 4. Frequency of detection of probe targets in the CRAPome database (http://www.crapome.org/).[31] Targets show a broad distribution of detection frequencies, indicating that they span a wide range of abundances in human cells.

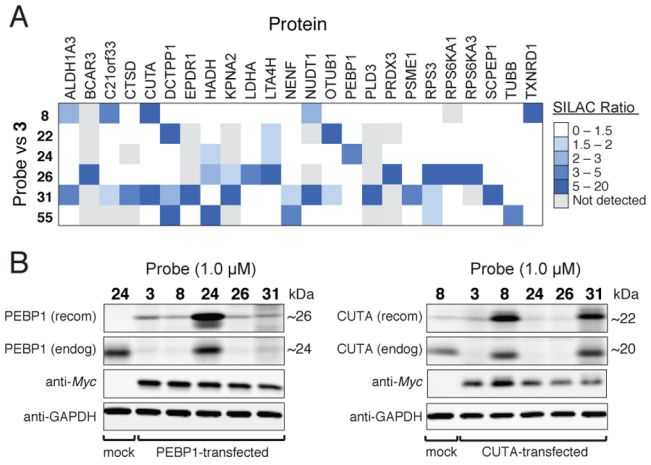

Figure 5. Probe–protein interaction profiles. (A) Heat map showing enrichment ratios for various probe–protein interactions as determined by quantitative proteomic experiments in PC3 cells comparing test probes to probe **3**. (B) Recombinantly expressed (recom) and endogenous (endog) forms of CUTA and PEBP1 show similar probe-interaction profiles in cells. Recombinant, Myc-tagged proteins were assayed for probe labeling in transiently transfected HEK293T cells and the profiles compared to those of mock-transfected cells.

protein could be assigned a preferred probe (Table 1). We confirmed these preferential probe interactions for representative protein targets by recombinantly expressing these proteins in HEK293T cells by transient transfection and testing the transfected cells against the test probe set (Figure 5B). We then generated "non-clickable" alkane (or alkene) versions of each probe for use in competitive profiling experiments to estimate target engagement in cells (Figure 6A and Figure S5). Initial competition experiments were performed by treating PC3 cells with probes (10 μM) and 2× competitors (20 μM) or DMSO for 30 min, followed by UV-light exposure (10 min at 4 °C), cell lysis, coupling of probe-labeled proteins to Rh–N_3 by CuAAC, and analysis by gel-based profiling. Several probe-labeled proteins showed reduced signals in competitor-treated versus DMSO-treated cell preparations (Figure S5).

We next treated isotopically heavy and light cells with probe (10 μM) and either 2× competitor (20 μM; heavy cells) or DMSO (light cells) as described above, coupled probe-labeled proteins to biotin-N_3 by CuAAC, and analyzed the samples by streptavidin chromatography combined with quantitative LC-MS-based proteomics. Proteins that showed heavy:light ratios of 0.5 or less were considered substantially engaged by the competitor, while those showing light:heavy ratios between 0.5 and 0.67 were considered partially engaged by the competitor.

dx.doi.org/10.1021/ja505517t | J. Am. Chem. Soc. 2014, 136, 10777–10782

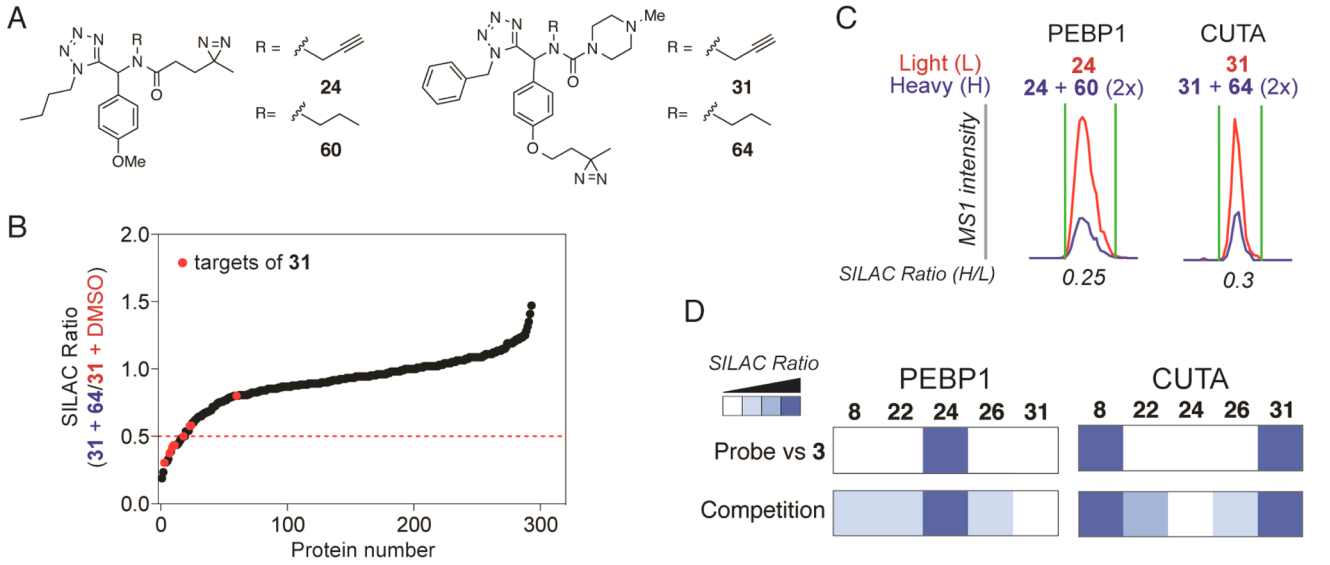

Figure 6. Blockade of probe–protein interactions in cells by nonclickable competitor analogues. (A) Structure of probes **24** and **31** and their nonclickable competitor agents **60** and **64**, respectively. (B) SILAC plots for total proteins identified in experiments comparing PC3 cells treated with test probe **31** (10 μM) and either 2× competitor **64** (20 μM, heavy cells), or DMSO (light). Red dashed line marks a light:heavy ratio of 0.5; protein ratios at or below this line indicate substantial competition. See Figure S5 and Table S2 for competition data for additional probes. (C) Representative MS1 peptide traces for protein targets of probes **24** (PEBP1) and **31** (CUTA) in competition experiments with 2× competitor (**60** and **64**, respectively). (D) Comparison of enrichment ratios for representative targets in test probe-versus-**3** and test probe-versus-competitor experiments. A good correlation is observed between the test probe showing the highest target enrichment and the corresponding nonclickable analogue showing the highest competition (depicted using the inverse of the competition ratio shown in panel B and Figure S5) of target–probe interactions.

Most of the 24 protein targets exhibited clear evidence of partial or substantial engagement by the competitor analogue of its preferred interacting probe in cells (Figure 6B,C and Table 1; also see Figure S5 and Table S2). In some cases, the competitor showed greater than the anticipated 2-fold competition, which could reflect superior binding of the target to the non-clickable analogue compared to the parent alkyne probe. Importantly, for protein targets that were detected across the majority of probe-versus-**3** data sets, we confirmed that the extent of probe enrichment correlated with the extent of competition by the corresponding competitor agent (Figure 6D). This finding indicates that the distinct probe–protein interaction profiles reflect authentic differences in binding as opposed to variations in photocrosslinking efficiency among the probes.

A second set of proteins emerged from our quantitative proteomic experiments that exhibited an initially curious profile of competition without preferential probe interactions (Table S3). These proteins also showed strong enrichment in probe-versus-no UV light control experiments (Tables S1 and S3), confirming that they are direct targets of the probe library. We believe that these proteins represent avid and promiscuous ligand-binders, which would explain their lack of preferential interactions in probe-versus-**3** experiments and pervasive competition in probe-versus-competitor experiments.

CONCLUSIONS

It seems logical to surmise that the general utility of a compound library for phenotypic screening would be coupled to the scope of its target interactions in cells. Despite this expectation, few if any compound libraries, to our knowledge, have been systemically examined for protein interactions in cells. We, and others, have assessed the targets of small-molecule libraries bearing electrophilic functionalities that impart covalent reactivity with proteins in cells,[32−34] but the target landscape of reversibly acting small-molecule probes remains poorly understood. Fully functionalized libraries provide a technically straightforward means to achieve this objective, since probe–protein interactions can be trapped *in situ* by exposing probe-treated cells to UV light. With these considerations in mind, we set forth two primary goals for this study: (1) to synthesize a library of fully functionalized probes that displays substantial structural diversity, while also preserving features for protein capture and enrichment, and (2) to map the protein interactions for this library in human cells.

The first objective was accomplished using Ugi-azide condensation chemistry to install variable recognition elements into the probe library in conjunction with sterically minimized photoreactive (diazirine) and affinity (alkyne) groups. Combining these probes with quantitative proteomics uncovered a striking diversity of protein targets in human cancer cells. Most of these proteins showed unique SARs across the probe library, and their interactions were, in general, blocked by treatment with excess non-clickable analogues of the probes. We are encouraged by these features of specific binding events, inasmuch as they forecast the potential for the ligands to affect protein activity in cells. However, it is important to qualify that we do not yet know the functional consequences of the numerous specific probe–protein interactions characterized in this study and whether they reflect binding to active, allosteric, or ineffectual sites on proteins. This question should be addressable in future studies where the binding sites and biochemical effects of ligands are characterized for individual proteins.

We believe that our results support the future implementation of fully functionalized libraries in phenotypic screens, where the respective pharmacological effects and protein interaction profiles of library members can be integrated to facilitate the target identification process. Such efforts often benefit from the discovery of structurally related active and inactive control probes, the selection of which can be guided by chemical proteomic methods.[7,35] A preliminary survey of the probe–protein interactions mapped so far also indicates that probes with

dx.doi.org/10.1021/ja505517t | J. Am. Chem. Soc. 2014, 136, 10777−10782

similar structural features (e.g., **8** and **31**) share considerable overlap in their targets. It should thus be possible to cross-correlate target profiles (assessed by gel and MS analyses) with the structures of probes to generate preliminary SARs that guide probe optimization. Finally, it is noteworthy that the probe targets discovered herein include both well-established "druggable" proteins, such as enzymes and channels, and proteins that are much less commonly accessed by pharmacology, including adaptor and scaffolding proteins. While more chemical proteomic studies will be needed to determine the generality of our findings, they do suggest that the protein landscape accessed by small molecules in human cells extends far beyond traditionally defined druggable targets. Projecting forward, we wonder whether a broader and more agnostic exploration of the protein interactions of structurally distinct small-molecule libraries is warranted and could yield a tailored set of compounds that maximize coverage of the human proteome for phenotypic screening.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures and tables, materials, detailed experimental methods, and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the NIH (CA132630, DA032541) and Ono Pharmaceuticals.

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