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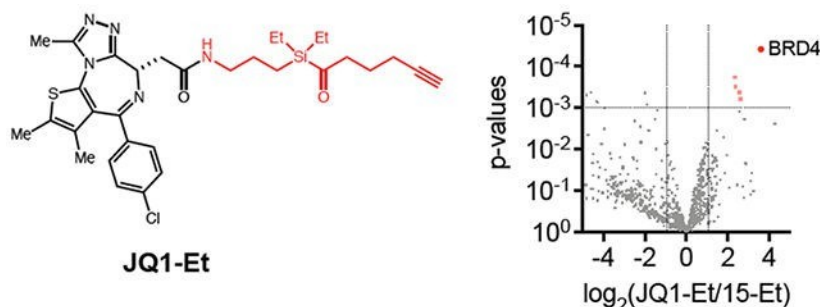
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Abstract

Deconvolution of the protein targets of hit compounds from phenotypic screens, often conducted in live cells, is critical for understanding mechanism of action and identifying potentially hazardous off-target interactions. While photoaffinity labeling and chemoproteomics are long-established approaches for discovering small-molecule-protein interactions in live cells, there are a relatively small number of photoaffinity labeling strategies that can be applied for chemoproteomic target identification studies. Recently, we reported a novel chemical framework for photoaffinity labeling based on the photo-Brook rearrangement of acyl silanes and demonstrated its ability, when appended to protein-targeting ligands, to label recombinant proteins. Here, we report the application of these probes to live cell photoaffinity workflows, demonstrate their complementarity to current state-of-the-art minimalist diazirine-based photoaffinity probes, and introduce a modular synthetic route to access acyl silane scaffolds with improved labeling properties.

Graphical Abstract



Keywords

chemoproteomics; photoaffinity labeling; JQ1; BRD4; acyl silanes

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Competing Financial Interests Statement

DKN is a co-founder, shareholder, and scientific advisory board member for Frontier Medicines and Zenith. DKN is also on the scientific advisory board of The Mark Foundation for Cancer Research, Photys Therapeutics, Oerth Bio, Apertor Pharmaceuticals, Ten30 Biosciences, and Deciphera. DKN is also an Investment Advisory Partner for a16z Bio, an Advisory Board member for Droia Ventures, and an iPartner for The Column Group.

Introduction

Chemoproteomics, particularly photoaffinity labeling (PAL), is a powerful approach for mapping small-molecule-protein interactions within the native cellular context¹⁻⁸. Identifying the protein targets of bioactive small molecules, especially those emerging from phenotypic screens, is critical for elucidating mechanisms of action and anticipating potential off-target effects¹⁻⁸. Current photoaffinity labeling methods predominantly employ reactive moieties such as benzophenones, aryl azides, and diazirines, each offering distinct advantages and limitations. Benzophenones, while highly stable and easily activated by UV light, often require prolonged irradiation and exhibit limited specificity due to nonspecific labeling. Aryl azides feature relatively simple synthesis and efficient crosslinking; however, their short-lived and highly reactive nitrene intermediates frequently lead to nonspecific protein labeling and lower yields. Diazirines have gained popularity due to their compact size, efficient photolysis to highly reactive carbenes, and enhanced labeling selectivity¹⁻⁸ (Figure 1A). In particular, linear aliphatic diazirines have been employed as so-called “minimalist” probes due to their small size and have experienced widespread use in the community. Nonetheless, additional PAL probes that can be used for intracellular chemoproteomic studies would expand the available toolkit for target identification campaigns⁹.

We recently reported a novel chemical framework for PAL based on the photo-Brook rearrangement of acyl silanes¹⁰. In this method, photoactivation of the acyl silane species with long-wavelength UV light enables the formation of a reactive α -siloxy carbene species, which can react with amino acid residues in an unbiased manner (Figure 1B). We reported a small series of acyl silane probes differentiated by substitution around the photoactivatable moiety and observed improved labeling profiles as the steric bulk increased. In this initial report, we validated labeling with established small-molecule protein binders in pure proteins and cell lysates. Here, we further optimized our acyl silane reagents and workflow for using these PAL probes for live cell chemoproteomic target identification. We demonstrate that our acyl silane PAL probes can be used for intracellular chemoproteomic target identification, showing comparable or superior results compared to traditional diazirine handles. We also developed a modular synthetic route to enable access to diversified acyl silanes, which can enhance the properties of probes. Taken together, these findings establish acyl silanes as another alternative to traditional diazirine-based PAL probes for live-cell workflows and highlight the importance of modularity in a PAL probe scaffold.

Results

Synthesis of Second-Generation Acyl Silanes

Our initial report on acyl silane photoaffinity scaffolds aimed to develop a new scaffold for PAL, while also introducing a modular handle through which photo- and physico-chemical properties could be tuned. Through testing several probes, we determined that the labeling efficiency of these probes correlated with the steric bulk of the silane. Additionally, we observed that the bulkier probes were less prone to thermal background reactions, likely

resulting from an attack by lysine followed by a thermal Brook rearrangement¹⁰. Our initial study examined these labeling events in the context of whole proteins and proteomes by gel-based assessment of labeling. In this study, we aimed to determine whether these probes could be utilized in live cells to facilitate target identification in complex biological settings using chemoproteomic approaches and how our acyl silane PAL probes compared with traditional diazirine probes.

While previous efforts have investigated varying the linker of the PAL probe to improve its physicochemical properties, we aimed to take advantage of the substitution handle provided by the silane to modulate probe properties, enabling a better comparison to current probes while minimizing the distance between the targeting ligand and reactive species to further reduce non-specific labeling. Additionally, a small library of PAL probes that differed

only by substitution on the photoactivatable warhead could enable higher-confidence target identification.

As we have established the importance of steric bulk around the acyl silane for labeling efficiency, we hypothesized that a diethyl- or silacyclopentyl-substituted probe could reduce hydrophilicity while preserving labeling efficiency. As such, we selected such dichlorosilanes for our initial reaction screens (Figure S1). We began our efforts by examining our previously reported synthetic route to acyl silane probes. In our initial studies, the silane moiety was installed through initial hydrosilylation of an alkene with a disubstituted chlorosilane (HR_2SiCl). While this route was successful for our initial series of substituents, the relative dearth of these chlorosilane reagents hampered further probe diversification. We therefore turned to the much more available dichlorosilane species for our diversification attempts. The installation of the silane was achieved by reacting the respective dichlorosilane with a Grignard species, followed by the capture of the silyl chloride intermediate by a lithiated dithiane. We found that bromoethyl dioxolane was the most compatible synthon for the Grignard sequence (Figure S2). The installation of the alkyne moiety via deprotonation and substitution yielded compound **2**. The tetramethylsilane (TMS) functionality on the alkyne was deprotected by addition of potassium carbonate in methanol to give terminal alkynes **3**. Sequential acid-promoted deprotection of the acetal and reduction of the corresponding aldehyde with sodium borohydride afforded alcohol intermediates **4**. Following installation of the phenyl carbamate moiety, deprotection of dithianes **5** by PIFA revealed substituted probes **15-Et** and **15-cyclo** (Scheme 1).

Evaluation of Second-Generation Acyl Silane Probes

With two new substituted acyl silane probes in hand, we sought to evaluate their labeling compared to the best-performing acyl silane from our original report, the isopropyl-substituted probe **15-iPr**. We began by evaluating their labeling performance in cell lysate. Upon photoaffinity labeling in cell lysates and CuAAC-mediated appendage of an azide-functionalized rhodamine handle, labeled proteins were subsequently visualized by in-gel fluorescence. Probe **15-Et** displayed light-dependent labeling of proteins to a

similar extent as probe **15-*i*Pr**, while preserving the minimal thermal background labeling characteristic of its bulkier congener (Figure 2). However, samples treated with the silacyclopentane-substituted probe **15-cyclo** showed significantly greater fluorescence in

the samples incubated in the absence of light, suggesting that this species is susceptible to thermal background reactivity (Figure 2). From these studies, we determined that the ethyl- and isopropyl-substituted probes were better suited than the cyclopentane-substituted probe for further design of chemoproteomic probes.

Synthesis and Testing of Fully-Functionalized First and Second-Generation Acyl Silane Probes

Next, to assess the utility of ethyl and isopropyl-substituted acyl silane PAL handles for chemoproteomic applications, we generated PAL probe based on the BET inhibitor JQ1, bearing isopropyl- and ethyl-substituted acyl silane handles and an alkyne handle, **JQ1-*i*Pr** and **JQ1-Et**, for subsequent cellular treatment, photoaffinity-mediated capture of protein targets, copper-catalyzed azide-alkyne cycloaddition (CuAAC) appendage of a biotin-azide enrichment handle, and avidin-enrichment and chemoproteomic identification of targets. While we previously described the synthesis of **JQ1-*i*Pr**, we synthesized **JQ1-Et** for this study. Conversion of the alcohol **4-Et** to amine **6-Et** under Mitsunobu conditions enabled access to versatile amine handle **7-Et** after removal of the Boc protecting group. An amide coupling of the carboxylic acid derived from (+)-JQ1 to this amine allowed access to the fully-functionalized diethyl acyl silane **JQ1-Et** (Scheme 2, Figure 3). We also generated a probe based on the established diazirine PAL handle, **JQ1-DA**, as well as a negative control diazirine PAL handle analogous to **15-Et/*i*Pr** to compare the performance of **JQ1-*i*Pr** and **JQ1-Et** with that of an established chemoproteomic probe (Figure 3A-3D)¹.

To establish a benchmarking standard to which we could compare our acyl silane probes, we first tested the previously reported **JQ1-DA**, which had not yielded the successful identification of its known targets, BRD2, BRD3, and BRD4, in K562 leukemia cancer cells (Figure 4A; Table S1)¹¹. While we observed significant enrichment of BRD4 compared to dimethylsulfoxide (DMSO)-treated controls, the enrichment observed was less than two-fold, with 43 other off-target proteins identified. When compared to a nonselective control diazirine probe (**DA control**, Figure 4B), we observed over two-fold enrichment of both BRD3 and BRD4, as well as 69 other off-target proteins (Figure 4B; Table S2). BRD3 and BRD4 enrichment by **JQ-DA** was also outcompeted by pre-incubation with an excess of JQ1 (Figure 4C; Table S2).

We next sought to compare the performance of our **JQ1-*i*Pr** and **JQ1-ET** probes to these data. **JQ1-*i*Pr** showed more than 2-fold significant enrichment of BRD4 compared to DMSO-treated controls, but also enriched 172 other off-target proteins—more than what we had observed with **JQ1-DA** (Figure 4D; Table S3). In contrast, **JQ1-*i*Pr** excitingly showed remarkably selective and robust (>50-fold) enrichment of BRD4 compared to the negative control **15-*i*Pr** probe with only one off-target protein identified (Figure 4E; Table S4). Importantly, we also observed that BRD4 enrichment by **JQ1-*i*Pr** was significantly outcompeted by pre-treating cells with excess JQ1, indicating specific interactions of the probe and not a non-specific pulldown (Figure 4F; Table S5).

Seeking to minimize the number of off-targets enriched by our probes, we hypothesized that

reduced hydrophobicity of the **JQ1-Et** probe compared to **JQ1-*i*Pr** could lead to improved

labeling specificity. Cellular chemoproteomic profiling of **JQ1-Et** not only demonstrated significant enrichment of BRD4 compared to DMSO control, but also less non-specific labeling and enrichment with 57 other off-target proteins enriched compared to 172 off-target proteins with **JQ1-*i*Pr** (Figure 4D, 4G; Table S3, Table S6). Similar to **JQ1-*i*Pr**, we also observed highly selective and robust enrichment of BRD4 with **JQ1-Et** treatment

compared to its corresponding negative control **15-Et** probe (Figure 4E; Table S7). We also observed that BRD2 and BRD4 enrichment by **JQ1-Et** was significantly outcompeted by pre-treating cells with excess JQ1, once again suggesting specific interactions of the probe (Figure 4I; Table S8). Overall, our results strongly demonstrated the utility of acylsilane PAL probes in cellular chemoproteomic target identification applications.

In testing our new generation of acyl silane probes in live cells, we aimed to facilitate peptide mapping to observe the direct modification of BRD4 with our probes and explore potential residue selectivity, as has been achieved by previous groups with diazirine handles⁵. However, we were unable to identify any mass adducts corresponding to our probes in proteomics experiments. We surmised that the siloxy product of the acyl silane PAL reaction could be acid-labile, as our LC-MS/MS workflows require 0.1% formic acid in the mobile phase. To evaluate this, we performed PAL labeling in lysate using a modified workflow that included titration of formic acid after labeling and the click reaction, but before adding the reducing reagent and running SDS/PAGE. As expected, the amount of labeling product observed decreased with increased acid (Figure S3). The acid-lability of these probes is under further investigation in our labs for use as a traceless probe for protein labeling. In total, our work demonstrates the utility of acyl silane PAL probes in cellular chemoproteomic workflows.

Assessing Rapamycin Targets with Acyl Silane Probes

We next sought to determine whether the acyl silane handle could be used to assess target identification with another small-molecule. We chose rapamycin as our second proof-of-concept, generating **rapamycin-*i*Pr** (Figure 5A). While we did not observe one of the primary targets of rapamycin, FKBP12¹², our cellular chemoproteomic assessment of **rapamycin-*i*Pr** identified a razor peptide found in IFITM1, IFITM2, or IFITM3 as the primary target enriched by the probe (Figure 5B; Table S9). Interestingly, IFITM proteins were identified by Shokat as the primary mechanism of cellular uptake for rapamycin-linked compounds¹³, and rapamycin's immunosuppressive effects are in part through promoting endolysosomal degradation of IFITM3¹⁴. Thus, we did identify a known target of linker-containing rapamycin analogs with our probe. We conjectured that **rapamycin-*i*Pr** may not have entered cells to engage FKBP12, so we performed chemoproteomic profiling with this probe in cell lysates. While we still did not observe FKBP12 enriched by the probe in cell lysate, capturing FKBP12 in bottom-up proteomics may be challenging due to its extremely small size. Excitingly, we did observe outcompetition of FKBP3 and IFITM1/2/3 with excess rapamycin pre-treatment (Figure 5C-5D; Table S10-S11). FKBP3 (also known as FKBP25) has previously been shown to be a target of rapamycin¹⁵. While we did not detect FKBP12 as a target of our probe, we did identify other known direct targets of rapamycin, thus further showcasing the utility of the acyl silane handle in chemoproteomic

applications.

Discussion

Photoaffinity labeling, coupled with chemoproteomic profiling, is a powerful tool for deconvoluting small molecule-protein interactions; however, more photoaffinity tools are needed to increase the chances of successful target identification in chemoproteomic experiments. We recently reported acyl silanes as a novel scaffold for photoaffinity labeling, based on the 1,2-photo-Brook rearrangement of acyl silanes to form an α -siloxy carbene intermediate. In this study, we optimize and demonstrate the utility of acylsilanes for PAL and cellular chemoproteomic and target identification applications as exemplified with JQ1 and rapamycin probes. To improve probe labeling properties, we developed a modular synthetic route to enable access to acyl silanes with diverse substituents at silicon and demonstrated that reducing the hydrophobicity of the photoaffinity probe improves specificity of probe labeling in living cells. We do, however, still observe some non-specific labeling and enrichment of proteins that are unlikely targets of JQ1 or rapamycin. Specificity of labeling in our study could perhaps be further increased by comparison of additional linker types functionalized with either acyl silane. We also showed that site of labeling was not possible with our current scaffolds due to their acid liability under mass spectrometry conditions, so future work may include optimization of adduct stability under acidic conditions. Alternatively, these acid-labile adducts could allow for temporal control of covalent labeling, which can be released in a scarless manner via acidification of the samples. Overall, our results establish acyl silanes as a new tool for photoaffinity labeling for target identification studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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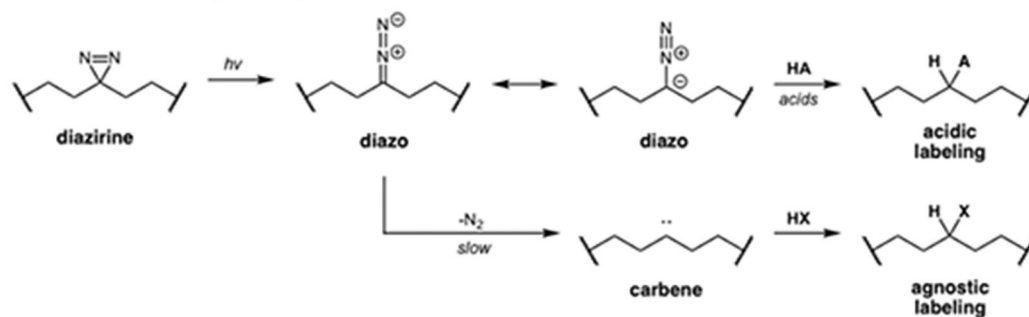
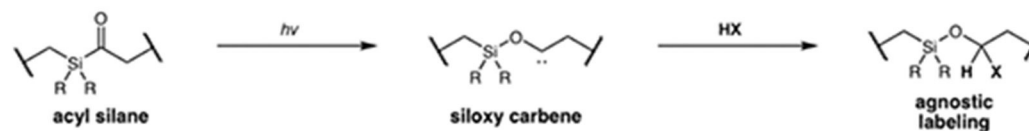
A. "Minimalist" diazirine (Yao 2013)**B. "Minimalist" acyl silane (Toste 2022, *this work*)**

Figure 1. Photoaffinity labeling with diazirines versus acyl silanes.

(A) Pathways for photoaffinity labeling by aliphatic "minimalist" diazirines. (B) Pathway for photoaffinity labeling by acyl silanes.

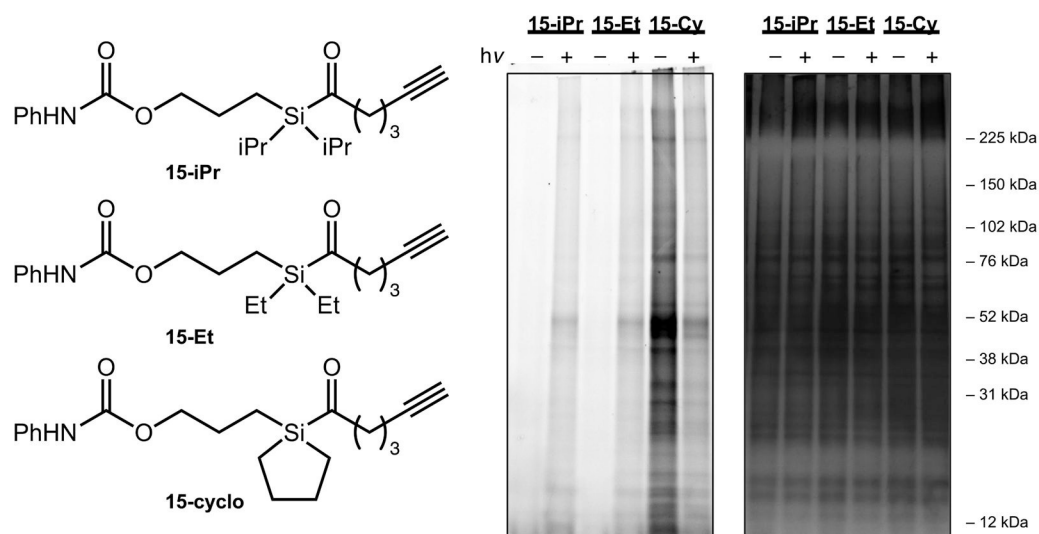


Figure 2. Evaluation of labeling by photoaffinity probes.

Evaluation of labeling by proof-of-concept photoaffinity probes (15-iPr, 15-Et, and 15-cyclo) in K562 cell lysate by in-gel fluorescence (left image) following Cu click reaction with Rh-N₃ after irradiation at 365 nm or incubation in the dark. Protein loading was confirmed following silver staining (right image). Gels are representative from n=3 biologically independent replicates per group.

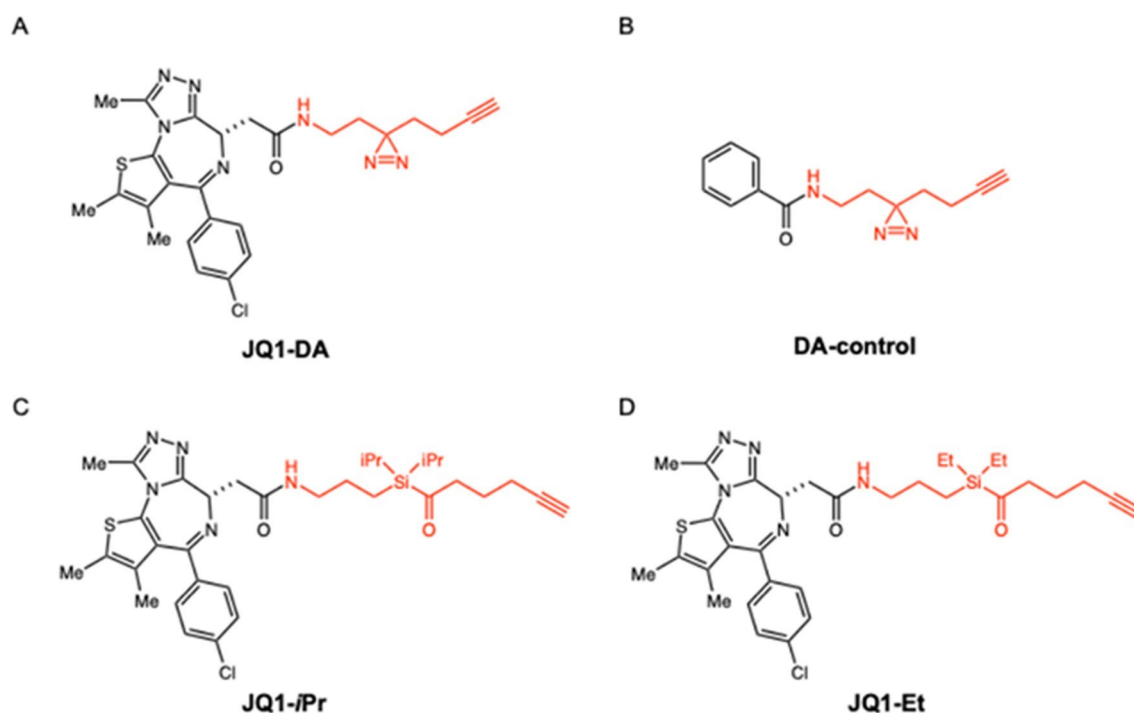


Figure 3. Structures of PAL probes used in evaluating the (+)-JQ1 interactome using chemoproteomic approaches. **(A)** Minimalist diazirine JQ1 probe; **(B)** minimalist diazirine non-targeting control; **(C,D)** isopropyl- and ethyl-substituted acyl silane JQ1 probes.

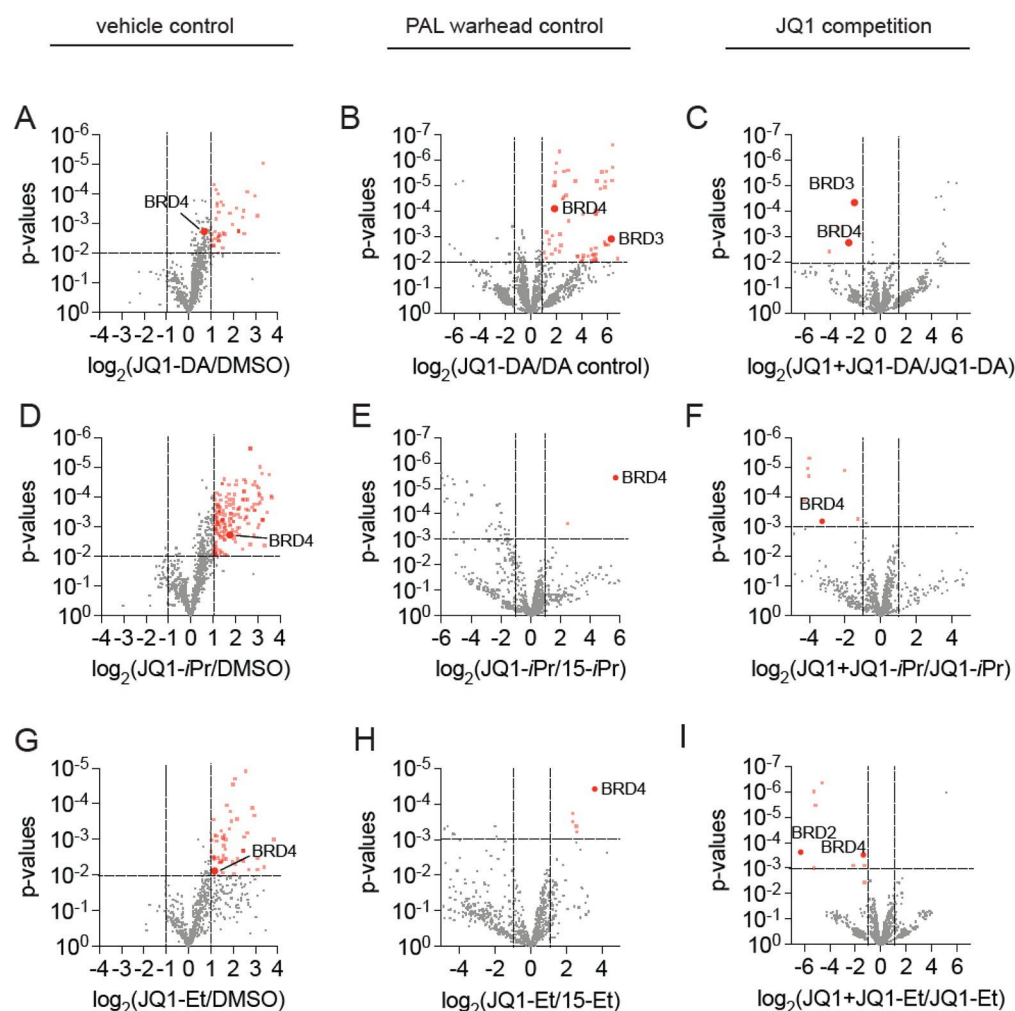


Figure 4. Chemoproteomic profiling with (+)-JQ1 PAL probes in live cells.

In all experiments, K562 cells were treated with DMSO vehicle or 1 μ M probe for 1 hour and subjected to irradiation at 365 nm for 20 minutes. Subsequent lysates were subjected to CuAAC with an azide-functionalized biotin handle, after which probe-modified peptides were avidin-enriched, tryptically digested, eluted, and analyzed by TMT-based quantitative proteomics. (A,D,G) Proteins enriched by JQ1-based probes **JQ1-DA**, **JQ1-iPr**, or **JQ1-Et** over DMSO vehicle. (B,E,H) Enrichment of JQ1-based probes **JQ1-DA**, **JQ1-iPr**, or **JQ1-Et** over their counterpart non-targeting control probes **DA control**, **15-iPr**, or **15-Et**. (C,F,I) Enrichment from cells pre-treated for 0.5 hours with 50 μ M (+)-**JQ1**, then treated with **JQ1-DA**, **JQ1-iPr**, or **JQ1-Et**, over cells treated with **JQ1-DA**, **JQ1-iPr**, or **JQ1-Et** alone. Data are from $n=3$ biologically independent replicates/group. Full datasets are available in Tables S1-S8.

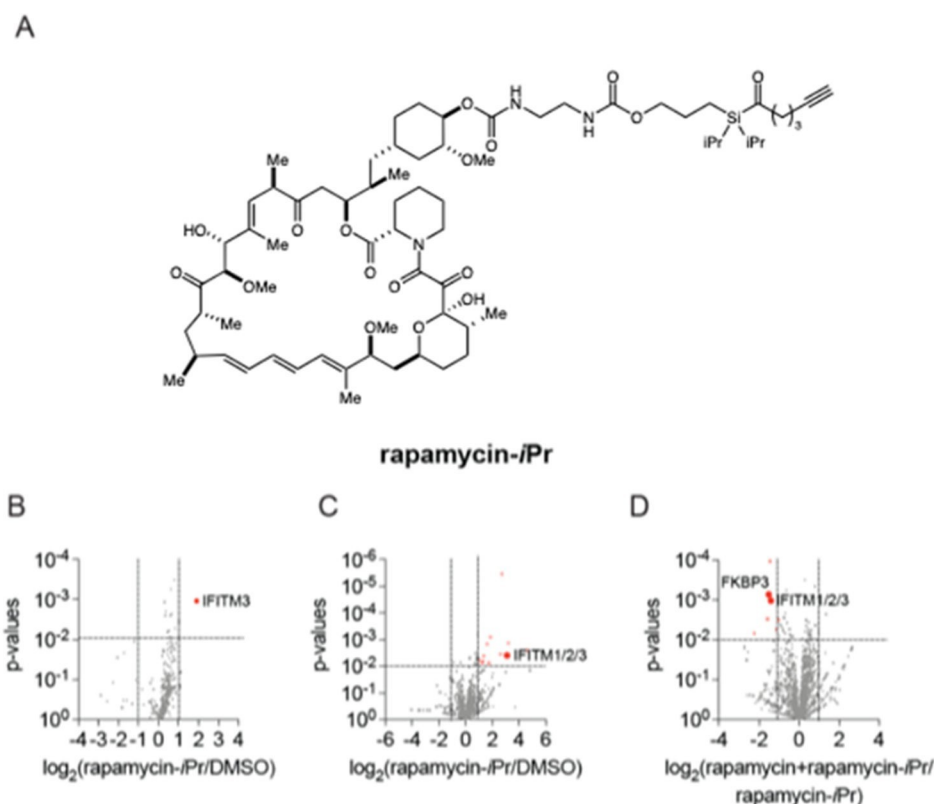
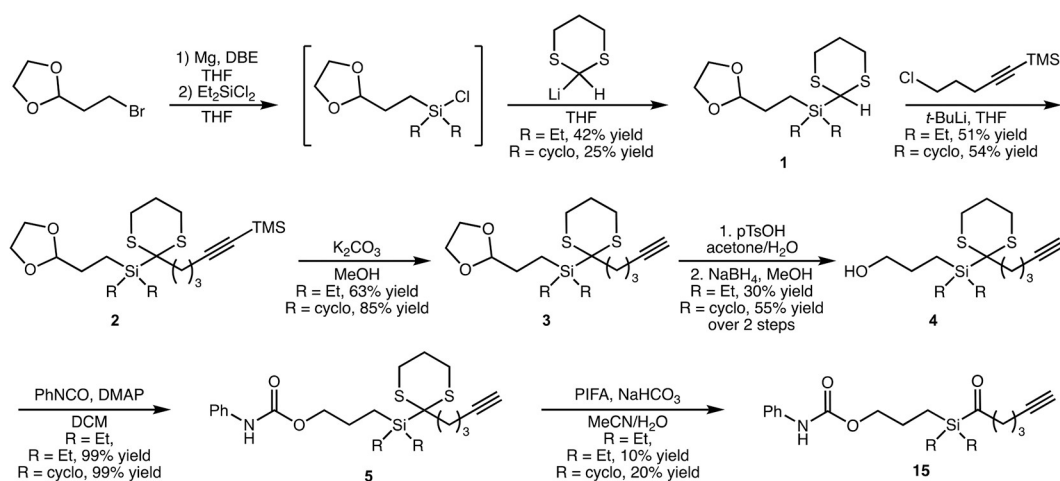
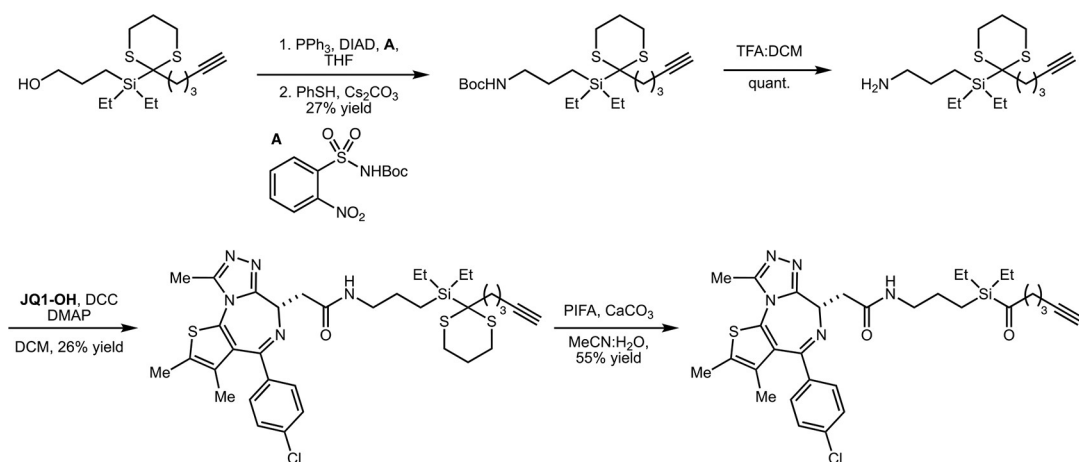


Figure 5. Chemoproteomic profiling with rapamycin PAL probe in K562 cells or lysates. (A) Structure of rapamycin-based acyl silane PAL probe. (B-D) K562 cells or lysates were treated with DMSO vehicle or 1 μM **rapamycin-*i*Pr** for 1 hour and subjected to irradiation at 365 nm for 20 minutes. Subsequent lysates were subjected to CuAAC with an azide-functionalized biotin handle, after which probe-modified peptides were avidin-enriched, tryptically digested, eluted, and analyzed by TMT-based quantitative proteomics. (B) Proteins enriched by **rapamycin-*i*Pr** over DMSO vehicle when treated in whole cells. (C) Proteins enriched by **rapamycin-*i*Pr** over DMSO vehicle when treated in cell lysates. (D) Enrichment from cell lysates pre-treated for 0.5 hours with 50 μM rapamycin, then treated with **rapamycin-*i*Pr** over lysates treated with **rapamycin-*i*Pr** alone. Data are from n=3 biologically independent replicates/group. Full datasets are available in Tables S9-S11.



Scheme 1. Synthesis of proof-of-concept photoaffinity probes containing diversified silane substitution.



Scheme 2. Synthetic route towards fully-functionalized probe JQ1-Et.