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Letter

Diazirine Photoprobes for the Identification of Vancomycin-Binding Proteins

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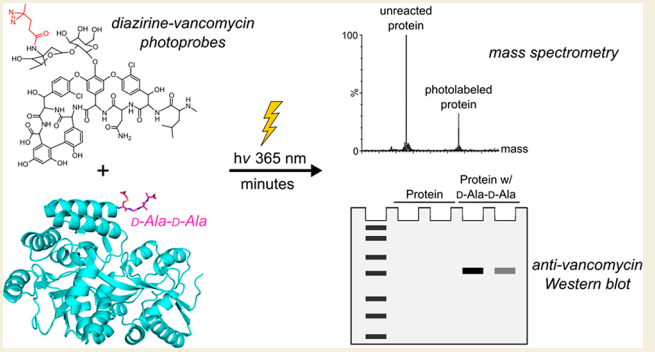
ABSTRACT: Vancomycin's interactions with cellular targets drive its antimicrobial activity and also trigger expression of resistance against the antibiotic. Interaction partners for vancomycin have previously been identified using photoaffinity probes, which have proven to be useful tools for exploring vancomycin's interactome. This work seeks to develop diazirine-based vancomycin photoprobes that display enhanced specificity and bear fewer chemical modifications as compared to previous photoprobes. Using proteins fused to vancomycin's main cell-wall target, D-alanyl-D-alanine, we used mass spectrometry to show that these photoprobes specifically label known vancomycin-binding partners within minutes. In a complementary approach, we developed a Western-blot strategy targeting the vancomycin adduct of the photoprobes, eliminating the need for affinity tags and simplifying the analysis of photolabeling reactions. Together, the probes and identification strategy provide a novel and streamlined pipeline for identifying vancomycin-binding proteins.



KEYWORDS: *vancomycin, photoaffinity probes, diazirine, antivancomycin antibody, Western blotting, proteomics*

INTRODUCTION

Vancomycin is a glycopeptide antibiotic that is used in healthcare settings across the globe.[1] It has historically served as an antibiotic of last resort to treat persistent infections caused by Gram-positive microbes.[2−4] However, decades of clinical use have led to the emergence of vancomycin resistance in various human pathogens, most notably vancomycin-resistant *Enterococci* (VRE).[5−8] VRE infections cause thousands of deaths each year and lead to extended hospital stays and increased treatment costs. Accordingly, VRE have been identified as high-priority pathogens for the development of new therapeutic approaches.[9−12]

Efforts to develop novel VRE-targeted therapies will benefit from a thorough understanding of all of vancomycin's interaction partners. Vancomycin binds the D-alanyl-D-alanine (D-Ala-D-Ala) moiety of the cell-wall precursor Lipid II, thereby interfering with peptidoglycan synthesis.[13−16] D-Ala-D-Ala is the substrate for the transpeptidase enzyme, and by sequestering this peptide, vancomycin inhibits the cross-linking reaction required to form a rigid cell wall, leaving the bacteria susceptible to lysis.[17] VRE elude vancomycin's effects by remodeling D-Ala-D-Ala to D-alanyl-D-lactate or D-alanyl-D-serine, neither of which is efficiently recognized by the antibiotic.[18−25] The VRE resistance phenotype is regulated by VanS, a membrane-bound sensor histidine kinase.[26] VanS detects the presence of the antibiotic and responds by initiating a signaling pathway that culminates in the expression of cell-wall remodeling enzymes.[26−28] However, it remains unclear whether VanS proteins sense vancomycin by binding directly to the antibiotic, or through more indirect mechanisms.[6,29,30] Complicating matters, nine different types of VRE have been described and the corresponding VanS proteins vary significantly in sequence, raising the possibility that some VanS proteins may bind directly to vancomycin, while others do not.[6,31] Furthermore, the vancomycin-protein interactome may extend beyond the VanS proteins, as vancomycin has been suggested to interact with additional proteins, including an autolysin and an ABC transporter.[32] Thus, there is a need for new tools that can aid in identifying and characterizing vancomycin's molecular partners.

One tool that has found wide application in profiling interacting partners is photoaffinity labeling.[33] This approach has already been applied to vancomycin, having been used to probe the interaction between the antibiotic and the VanS protein from *Streptomyces coelicolor*, and for proteomic studies aimed at identifying novel interaction partners.[32,34] In both of these examples, the vancomycin photoprobes contained

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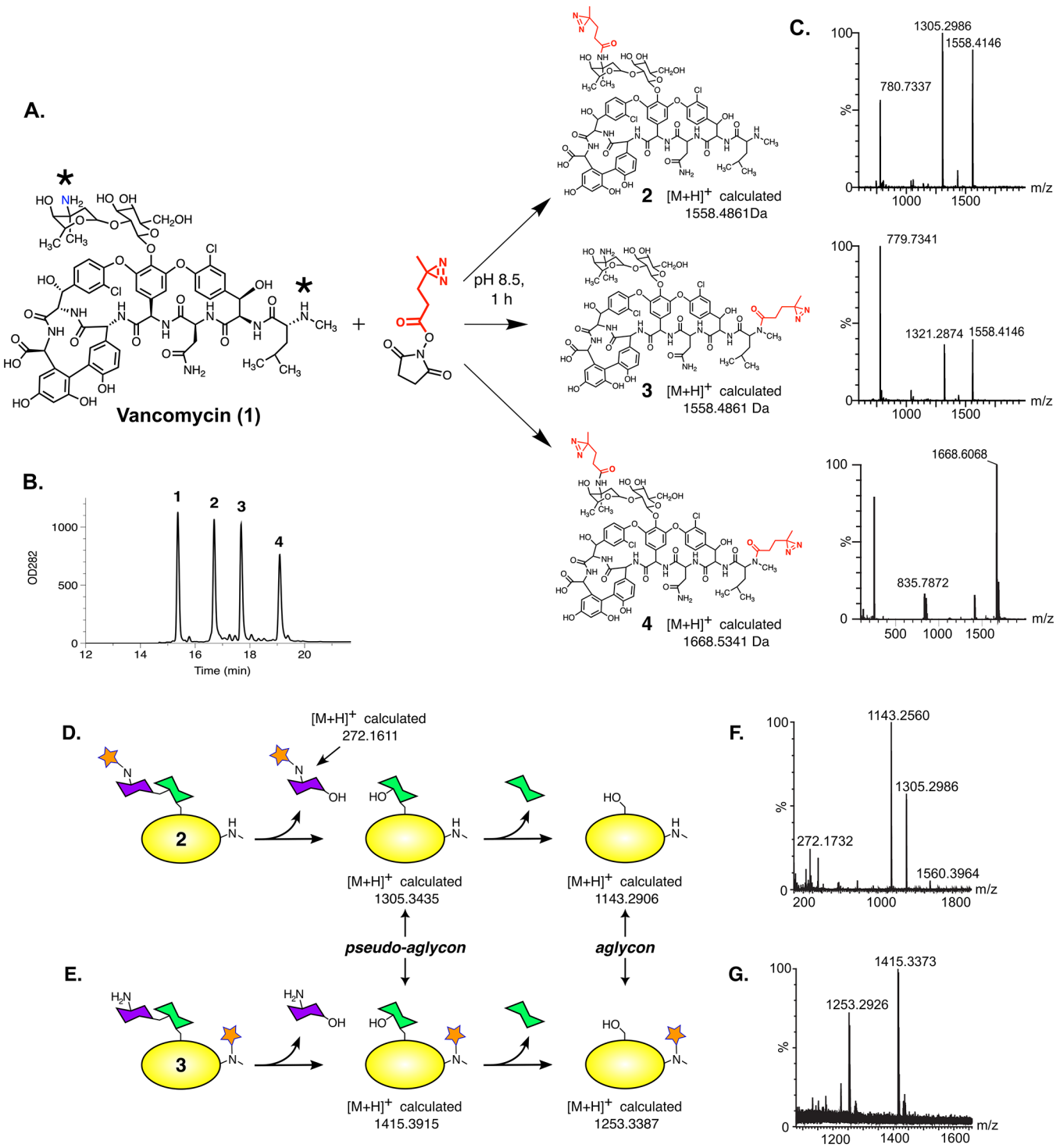

Figure 1. Preparation of vancomycin-diazirine photolabeling reagents. (A) Reaction of vancomycin with a diazirine-NHS ester yields three products. The NHS ester is expected to react at two positions (indicated by asterisks): A primary amine on the vancosamine sugar and the secondary amine at vancomycin's N-terminus. (B) The products and starting material were separated using RP-HPLC. (C) Mass spectrometry was used to confirm the presence of the adduct in the three products. For compound **3**, in addition to the singly and doubly charged versions of the molecule, an additional peak is seen at 1321.2874, which likely represents a breakdown product of the labeled pseudoaglycon (see Figure S2). (D, E) Schematic drawings showing the anticipated acid hydrolysis products and their predicted masses for photoprobe **2**, in which the diazirine group is attached to the vancosamine sugar (panel D), or photoprobe **3**, with the diazirine attached to the N-terminus (panel E). The diazirine is represented by the orange star. (F, G) Mass spectra of the acid-hydrolysis products.

benzophenone groups as the photoactive species. Benzophenones are relatively bulky, and thus can potentially cause steric interference with interacting partners; they also require long periods of UV irradiation for effective protein labeling, which can damage the target molecule and increases the possibility of nonspecifically labeling bystander proteins.[33] We therefore chose to explore diazirine-containing photoprobes. Diazirines are smaller and more flexible than benzophenones, decreasing

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the risk of steric clashes in protein-binding interfaces; they are also less hydrophobic. Importantly, diazirines require less UV exposure for activation, reducing the radiation burden on the sample.[35,36] Upon UV irradiation, diazirines form highly reactive and unstable carbenes, which last for only picoseconds before being quenched by water.[37−39] These short half-lives ensure that the carbenes are rapidly extinguished before they can diffuse and react with off-target proteins.

Previously used vancomycin photoprobes also included biotin or alkyne handles to facilitate the isolation of the photolabeled species. Such handles are useful when isolating targets from complex cellular mixtures but contribute additional bulk that can disrupt interactions. Encouragingly, in the examples cited above these modifications did not impair vancomycin's antibiotic activity, nor did they alter the antibiotic's ability to bind to D-Ala-D-Ala *in vitro*; however, it is still possible that they might interfere with other biologically relevant interactions. We therefore questioned whether it would be possible to avoid incorporating additional tags, such as biotin, into the photoprobe. Instead, we chose to rely upon direct immunodetection of vancomycin using commercially available antibodies. Together, these strategies have allowed us to produce novel and effective vancomycin photoprobes, which improve our ability to identify vancomycin-interacting proteins and complement the existing repertoire of tools.

RESULTS AND DISCUSSION

Development and Identification of Diazirine-Containing Vancomycin Photoprobes

Targeting vancomycin's amine groups has proven to be a straightforward synthetic strategy for introducing additional groups onto the antibiotic.[40] Vancomycin contains a primary amine on its vancosamine sugar and a secondary amine at its methylated N-terminus (Figure 1A); modifications at both positions are known to preserve antibiotic activity, making these plausible sites for introducing the photolabel. We used a diazirine derivative containing an NHS ester to acylate the antibiotic, producing multiple species that could be separated using reversed-phase high-pressure liquid chromatography (RP-HPLC) (Figure 1B). Mass spectrometry (MS) revealed that two of the isolated compounds had masses consistent with singly labeled species, presumably reflecting attachment of the diazirine at either the vancosamine or the N-terminus (compounds **2** and **3**, respectively; Figure 1C). An additional species was obtained, having a mass consistent with the doubly labeled product (**4**), which was not pursued further.

We next sought to determine the locations of the diazirine group in the two singly labeled species. One of the species provided a clue about its identity in its native mass spectrum, which showed a peak for the pseudoaglycon lacking the diazirine adduct, suggesting it was compound **2** (Figure 1C; see Figure S1 for a definition of the pseudoaglycon and aglycon; formation of the pseudoaglycon frequently occurs during ionization of vancomycin[41]). Conversely, the other singly labeled species contained a peak at 1321.2874 in its native mass spectrum, consistent with a degradation product derived from the pseudoaglycon of compound **3** (Figure S2). The MS/MS data were consistent with these assignments (Figure S3).

To provide independent confirmation of the identities of the two singly labeled species, we used acid hydrolysis to remove one or both of the antibiotic's sugar molecules (Figure 1D and E; Figure S1). In the case of **2**, removal of the sugars generates pseudoaglycon and aglycon species lacking the diazirine, whereas for **3** both species retain the diazirine. Compound **2** was readily identified, as it gave the unlabeled pseudoaglycon and aglycon species, along with a molecule having the appropriate mass for a diazirine-labeled vancosamine sugar (Figure 1F). Likewise, hydrolysis of the other singly labeled sample yielded pseudoaglycon and aglycon species containing the diazirine group, confirming its identity as **3** (Figure 1G).

Antibacterial Activity of Diazirine-Vancomycin Photoprobes

We measured the antibacterial activities of probes **2** and **3** against two species of Gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecium*. Minimum inhibitory concentrations (MICs) in planktonic cultures showed that both probes retained antimicrobial activity, albeit at slightly lower levels than that of vancomycin itself (Table 1). The lower activity of probe **3** versus that of **2** is consistent with published structure−activity data for vancomycin, which reveals that N-acylation at the antibiotic's N-terminus is significantly more likely to perturb activity than comparable modifications of the vancosamine amino group.[42−44]

Table 1. MIC Values (μg/mL) for Vancomycin and Photoprobes 2 and 3

species	vancomycin	probe **2**	probe **3**
S. aureus (ATCC 25923)	0.5	4	16
E. faecalis (ATCC 19433)	0.5	4	32

Diazirine-Vancomycin Photoprobes Specifically Label D-Ala-D-Ala-Containing Proteins

To test whether our photoprobes could label known vancomycin-binding proteins, we created constructs in which D-Ala-D-Ala peptides were fused to the C-termini of different proteins that served as carriers. Specifically, we used native chemical ligation to couple an L-Cys-L-Lys-D-Ala-D-Ala peptide to C-terminal thioesters of maltose-binding protein (MBP), T4 lysozyme (T4L), and ubiquitin (Ub).[45] Following coupling, the cysteine was alkylated with iodoacetic acid. The resulting protein-peptide conjugates contain a C-terminal sequence that closely mimics the peptide moiety of Lipid II. All three of these protein-peptide conjugates bind vancomycin with low-micromolar affinities, and they have been used to determine crystal structures of vancomycin and other glycopeptide antibiotics bound to their peptide targets.[45,46] Notably, in the absence of the D-Ala-D-Ala-containing peptide, none of the three protein carriers bind to vancomycin.

For photolabeling reactions, proteins were mixed with either photoprobe **2** or **3**, and then irradiated with 365-nm UV light, after which the products were analyzed by MS. As expected, no photolabeling was observed using control proteins lacking the D-Ala-D-Ala peptide (Figure S4). All of the D-Ala-D-Ala-containing constructs were photolabeled, albeit with varying efficiencies. Substantial labeling of MBP-D-Ala-D-Ala (MBP-DADA) was observed with **2**, whereas labeling by **3** was only roughly half as efficient (Figure 2A). **2** was similarly more efficient than **3** in labeling T4L-D-Ala-D-Ala (T4L-DADA) and Ub-D-Ala-D-Ala (Ub-DADA; Figure 2B and C); overall, labeling was most efficient for MBP-DADA.

We also observed varying photolabeling efficiencies within a given sample. For example, one of our MBP-DADA

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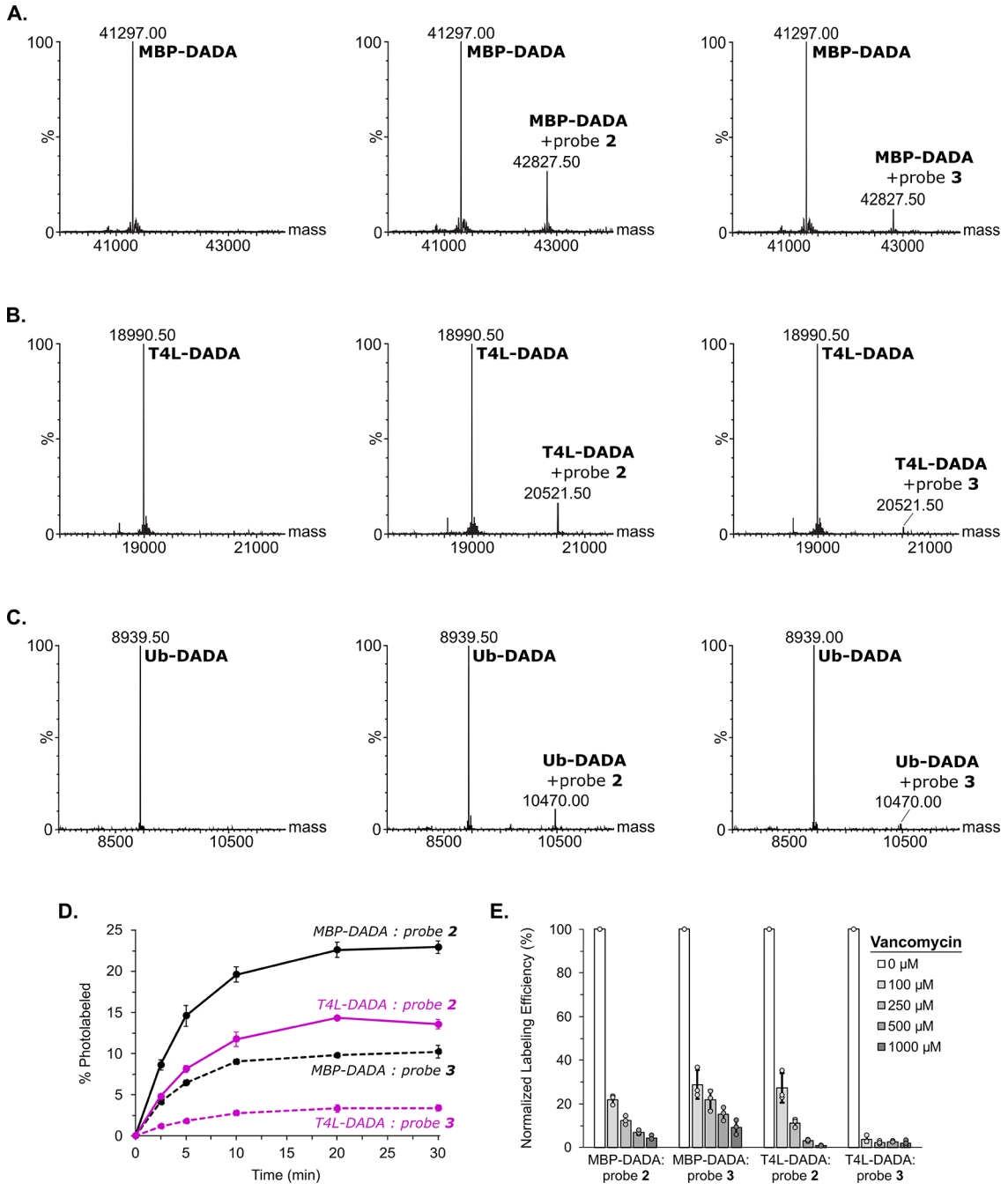

Figure 2. Photolabeling of protein-peptide conjugates containing D-Ala-D-Ala. (A) MBP-DADA (predicted mass 41297.91), (B) T4L-DADA (predicted mass 18990.75), and (C) Ub-DADA (predicted mass 8939.26) were mixed with buffer (left), photoprobe **2** (middle), or photoprobe **3** (right) and irradiated with UV light for 30 min. (D) Time-course of photolabeling reactions for MBP-DADA and T4L-DADA. The percentage of photolabeled protein was determined by the ratio of the unlabeled and photolabeled peak intensities in the mass spectra. Error bars represent one standard deviation (n = 3). (E) Competition experiments with vancomycin. Titration series for MBP-DADA and T4L-DADA were created containing either photoprobe **2** or **3** with increasing amounts of vancomycin. Samples were irradiated with UV light for four min, after which the efficiency of photolabeling was determined by mass spectrometry. Efficiency was normalized to the value observed in the absence of vancomycin. Error bars represent one standard deviation (n = 3). Protein and probe concentrations in photolabeling experiments were 20 and 60 μM, respectively.

preparations was incompletely alkylated by iodoacetic acid, resulting in a sample containing roughly equal amounts of the S-carboxymethyl-modified cysteine and the unmodified cysteine (Figure S5). When this preparation was labeled with probe **2**, the strong majority of the labeled species corresponding to the unmodified cysteine, implying that photolabel **2** bound preferentially to peptides containing an unalkylated cysteine. In contrast, probe **3** showed no preference with the photolabeled species being split equally between proteins containing modified and unmodified cysteines.

Kinetic experiments with the MBP-DADA and T4L-DADA constructs indicated that under these conditions, photolabeling reached a maximum by 20 min, with significant levels of

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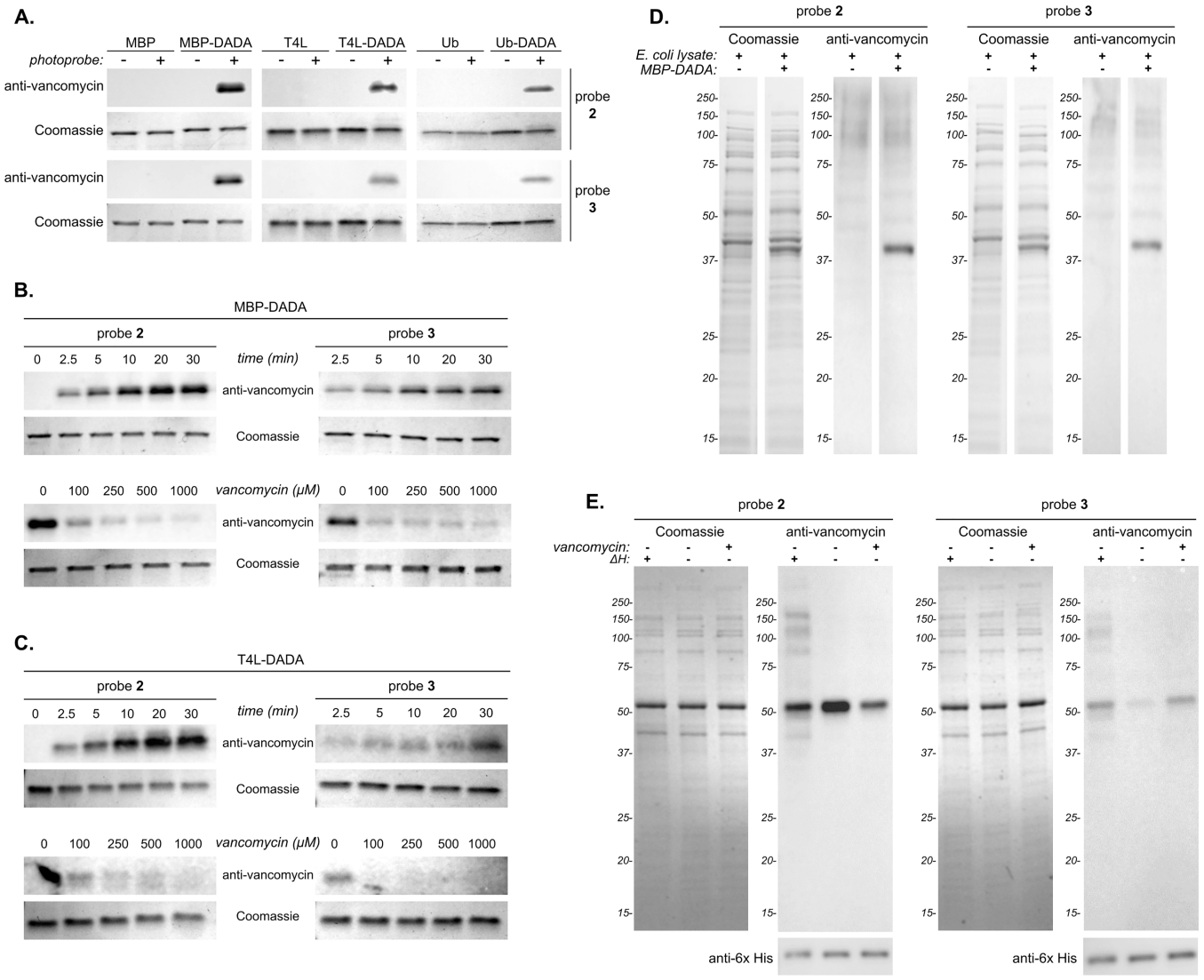

Figure 3. Photolabel Western blot using an antivancomycin antibody. (A) Western blots of photolabeling reactions for DADA-containing proteins, as well as control proteins lacking DADA. (B, C) Western blots showing time courses of photolabeling (upper panels) and competition experiments with vancomycin (lower panels) for MBP-DADA (panel B) and T4L-DADA (panel C). Coomassie-stained gels are shown as loading controls. In panels A-C, protein and probe concentrations in photolabeling experiments were 20 and 60 μM, respectively. (D) Photolabeling of MBP-DADA spiked into *E. coli* cell lysates. Lysates were prepared from BL21(DE3) cells containing empty vectors, and MPB-DADA was added to a final concentration of 50 ng/μL prior to labeling. For each probe, Coomassie-stained gels are shown at left, and antivancomycin Western blots are shown at right. (E) Identification of GtfD in *E. coli* cell lysates. Lysates were prepared from BL21(DE3) cells expressing His_6-tagged GtfD, and photolabeled with probes **2** and **3**; in control experiments, lysates were boiled (ΔH) prior to labeling. In competition experiments, 1 mM vancomycin was added to the lysates prior to labeling. To confirm that the labeled band corresponds to GtfD, blots were stripped and reprobed with an anti-His_6 antibody. For panels D and E, lysates were adjusted to a total protein concentration of 1 mg/mL, and 60 μM probe concentrations were used.

labeling being observed after only 2.5 min (Figure 2D). This is significantly shorter than the 2-h exposures required for benzophenone-containing vancomycin photoprobes,[32,34] which should presumably translate into an enhanced specificity for the new diazirine probes. To test the specificity of labeling, we asked whether unlabeled vancomycin could compete with the photoprobes. For both compounds **2** and **3**, the addition of unlabeled vancomycin reduced the photolabeling efficiency in a dose-dependent manner for both MBP-DADA and T4L-DADA (Figure 2E), suggesting that both photoprobes bind specifically to the vancomycin-binding site. Overall, these results demonstrate that the diazirine photoprobes are able to efficiently and specifically label known vancomycin-binding partners. They also reveal that photolabeling efficiency depends on the location of the photoreactive group, highlighting the utility having multiple probes in which the diazirine is attached at different positions.

Antivancomycin Western Blot for Detecting Photolabeled Proteins

We anticipate that some vancomycin-binding partners, such as VanS, will be membrane-bound. Membrane-protein preparations typically include lipids and detergents, which complicates mass spectrometric analysis. Therefore, we sought to develop an alternate technique for detecting photolabeled proteins. Because our photoprobes introduce a covalent vancomycin adduct, we reasoned that an antivancomycin antibody could be used to identify labeled proteins via standard immunodetection

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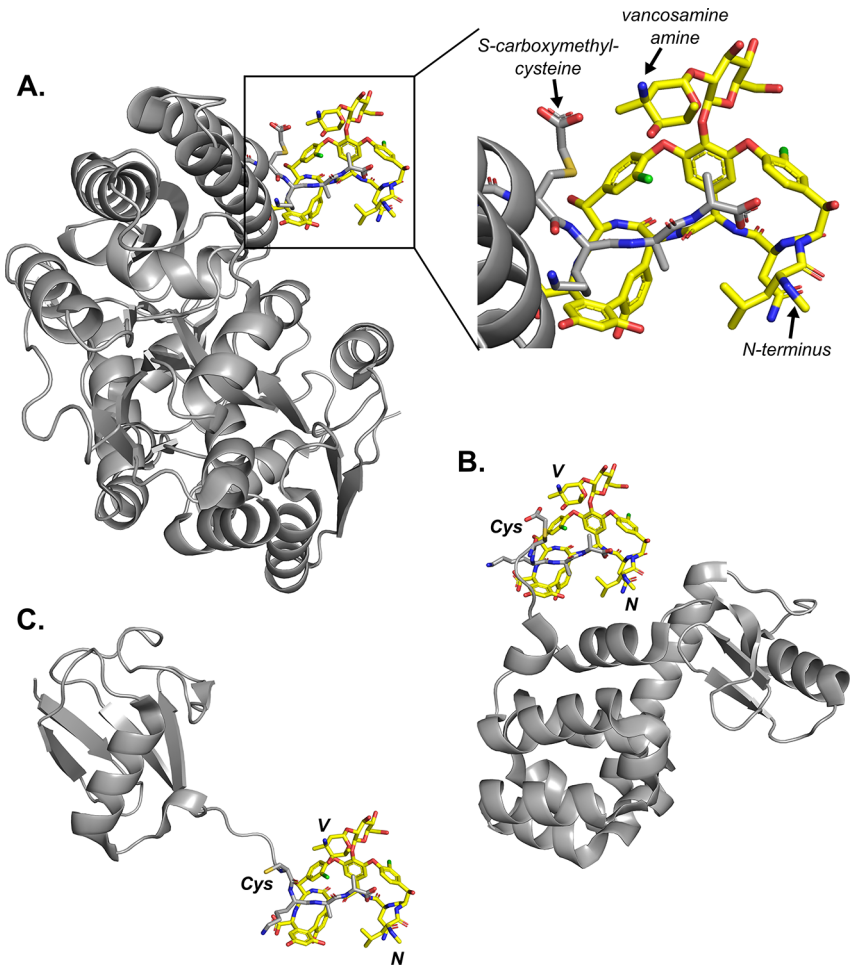

Figure 4. *Structural modeling of photoprobe interactions with protein-peptide conjugates.* (A) Model of the complex of vancomycin bound to MBP-DADA (left). The inset shows a close-up view of the interaction region, highlighting relative positions of the two diazirine attachment sites and showing the modified cysteine residue in the protein-peptide conjugate. (B) Crystal structure of T4L-DADA bound to vancomycin (PDB ID: 3RUN). (C) Model of the complex of Ub-DADA bound to vancomycin. In all panels, vancomycin is shown as yellow sticks, while the protein-peptide conjugates are colored gray, with the fused Cys-Lys-D-Ala-D-Ala peptides being shown as sticks. The positions of the vancosamine amine and N-terminus of vancomycin are indicated by V and N, respectively, while the cysteine of the ligated peptide is indicated by Cys.

methods. Direct detection of the vancomycin molecule would have the additional advantage of abolishing the need for other tags (e.g., biotin or click handles), thereby reducing the size and complexity of the photoprobe. For these reasons, we used a commercial antivancomycin antibody to develop a novel Western-blot strategy for identifying photoprobe-labeled proteins.

Proteins were subjected to photolabeling, after which they were analyzed by denaturing SDS-PAGE and Western blotting. The labeling patterns observed in the Western blots agreed remarkably well with those seen using MS. Thus, all three D-Ala-D-Ala conjugates were seen to be labeled by **2** and **3** (Figure 3A), whereas proteins lacking the D-Ala-D-Ala group were not labeled by either compound. Labeling by probe **2** was much more efficient than that by probe 3 (Figure S6), consistent with the weaker intensities observed for probe **3**-labeled peaks in the MS data.

Kinetic and competition profiles obtained by Western blotting were also consistent with those observed using MS (Figure 3B and C). For both photoprobes, labeling of MBP-DADA and T4L-DADA was detectable within 2.5 min of UV exposure and reached a plateau around 20 min. In both cases, a dose-dependent reduction in the Western-blot signal was seen when increasing amounts of free vancomycin were added to the photolabeling reactions, reinforcing that the signal is due to the specific binding of the probes.

We next tested whether specific recognition of MBP-DADA could be achieved in the context of a complex proteome by spiking the MBP-DADA protein into an *E. coli* cell lysate. Despite the presence of the endogenous *E. coli* proteins, for both probes the predominant signal observed in the Western blot corresponded to that of the MBP-DADA protein (Figure 3D).

Finally, we examined whether our photoprobes would be able to identify a vancomycin-binding protein that does not contain the D-Ala-D-Ala binding epitope. We chose GtfD, which is an enzyme from the vancomycin biosynthetic pathway. GtfD catalyzes the addition of the vancosamine sugar to the vancomycin pseudoaglycon;[47,48] it interacts with the pseudoaglycon with modest affinity ($K_m \sim 20\ \mu M$).[49] We therefore reasoned that GtfD represents a good test case of a protein that binds vancomycin specifically but not extremely tightly. We expressed the enzyme in *E. coli*, and labeled the crude lysate with probes **2** and **3**. Both probes labeled GtfD at levels that yielded clear signals in the Western blot, with probe **2** labeling the enzyme more efficiently than probe **3** (Figure

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3E); lower levels of labeling by **3** may reflect steric constraints around the antibiotic's N-terminus when it is complexed to the enzyme.[49] In the case of Probe **2**, heating the cell lysate prior to labeling reduced the signal, indicating that labeling requires properly folded protein; in addition, for this probe, vancomycin competitively reduced the signal. In the case of probe **3**, the overall signal was not strong, but the signals for the heat-treated and vancomycin-treated samples were reproducibly slightly stronger than those of the untreated lysate. The reasons for these observations are not immediately clear. As noted above, the structure of GtfD suggests that steric considerations should limit binding of **3**, and so perhaps heat treatment allows the structure to open somewhat, helping the probe to enter the active site. The vancomycin effect could conceivably reflect a role for antibiotic dimerization in recognition by the enzyme; at the probe concentrations used, the probe alone should be monomeric, but adding high concentrations of unlabeled vancomycin is expected to drive formation of labeled/unlabeled heterodimers. In any case, the differential recognition of GtfD by the two compounds further highlights the utility of having multiple probes with photolabels located at different sites.

Taken together, these results demonstrate that the Western-blot protocol described herein is a quick and sensitive method for identifying proteins labeled by vancomycin photoprobes and is applicable both to purified proteins and to proteins in complex cell lysates.

Structural Analysis of Photoprobe Interactions with D-Ala-D-Ala Proteins

Differences between the labeling efficiencies of probes **2** and **3** will likely reflect differences in binding to the D-Ala-D-Ala target, which are reflected in the different MIC values of the two compounds (Table 1). However, for a given probe, there are differences in labeling efficiency for different protein-peptide conjugates. To explore the structural basis for these differences, we constructed models for photoprobes **2** and **3** bound to each of the three protein-peptide conjugates used. A crystal structure is already available for T4L-DADA bound to vancomycin (PDB ID 3RUN);[45] for MBP-DADA and Ub-DADA, we used crystal structures of the proteins bound to teicoplanin and dalbavancin, respectively, as starting points for the modeling (PDB IDs 3VJF and 3RUL).[45,46]

In the case of MBP-DADA, the primary amine on the vancosamine sugar lies close to MBP's C-terminal α helix, explaining why a diazirine attached at this position (probe **2**) robustly labels this protein (Figure 4A). On the other hand, the antibiotic's N-terminus is oriented away from the body of MBP and projects outward into solvent; hence, a diazirine attached at this position (probe **3**) will be farther from the protein, consistent with the lower efficiency of labeling associated with this probe-protein pair. The structure also offers an explanation for the preferential labeling of a protein with an unalkylated cysteine by probe **2**: The *S*-carboxymethyl group on the modified cysteine lies immediately adjacent to the vancosamine sugar and thus may interfere sterically with the diazirine group; in contrast, the smaller unmodified cysteine side chain offers less steric hindrance, suggesting **2** will bind preferentially to the unalkylated protein.

In the crystal structure of T4L-DADA bound to vancomycin, vancomycin's N-terminus is close to the protein's first α helix, suggesting a potential steric clash with the diazirine of probe **3** (Figure 4B), possibly explaining its poor labeling efficiency for this protein. Finally, in the case of Ub-DADA, the structure reveals that the protein's C-terminus extends outward, away from the body of the molecule; thus, when vancomycin binds to the D-Ala-D-Ala group, diazirine groups attached to either the antibiotic's N-terminus or its vancosamine sugar lie far from the core of the ubiquitin molecule, explaining why Ub-DADA was less efficiently labeled by both probes, as compared to the other D-Ala-D-Ala conjugates (Figure 4C).

The structural mode by which vancomycin recognizes D-Ala-D-Ala probably also contributes to the stronger labeling of the protein-peptide conjugates by probe **2** versus that by probe **3**. When D-Ala-D-Ala is bound to vancomycin, its C-terminus lies next to the antibiotic's N-terminal amine. By definition, the C-terminal residues are the ones farthest away from the remainder of the protein; this likely biases probe **3** toward positions that are more removed from the bulk of the protein than is true for probe **2**. Overall, then, our structural knowledge of how vancomycin binds to the protein-peptide conjugates is consistent with the observed photolabeling patterns. Importantly, the structural context underscores the importance of close spatial proximity between the photoreactive group and the protein target as well as good structural complementarity in the probe-target pair.

In conclusion, we report novel diazirine-vancomycin photoprobes that can specifically label known vancomycin-binding proteins while requiring only a fraction of the UV burden needed for previous probes; ultimately, this should reduce sample damage and decrease the chance of labeling off-target proteins. We prepared two complementary photoprobes having reactive groups located at different positions, increasing the likelihood that at least one probe will be able to engage a given target without steric clashes. Chances of such clashes are further minimized by the small size of the diazirine groups and by our antibody strategy for recognizing the vancomycin adduct, which allowed us to generate probes having minimal modifications to the vancomycin molecule. In addition, we have developed a new Western-blotting approach that provides a simple and powerful way to identify the proteins labeled by our photoprobes; by eliminating the dependence upon mass spectrometry, this approach is likely to prove especially advantageous when studying vancomycin interactions with membrane proteins. Going forward, we anticipate that this antibody-based approach will also prove to be useful for proteomic studies, enabling the isolation of photolabeled proteins without the need for an affinity tag.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomedchemau.3c00067.

Detailed methods for photoprobe synthesis and characterization, protein purification and preparation of protein-peptide conjugates, photolabeling, mass spectrometry, and Western-blot assays (PDF)

Structural model of MBP-DADA bound to vancomycin (PDB)

Structural model of T4L-DADA bound to vancomycin (PDB)

Structural model of Ub-DADA bound to vancomycin (PDB)

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Author Contributions

CRediT: **Photis Rotsides** conceptualization, investigation, methodology, writing-original draft, writing-review & editing; **Paula J Lee** investigation, methodology; **Nakoa Webber** investigation, methodology; **Kimberly C Grasty** investigation; **Joris Beld** formal analysis, investigation, methodology, writing-review & editing; **Patrick J Loll** conceptualization, funding acquisition, project administration, supervision, writing-original draft, writing-review & editing.

Notes

The authors declare no competing financial interest.

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