Membrane Probes

Deutsche Ausgabe: DOI: 10.1002/ange.201603441
Internationale Ausgabe: DOI: 10.1002/anie.201603441

In Vivo Probe of Lipid II-Interacting Proteins

*Sourav Sarkar, Elizabeth A. Libby, Sean E. Pidgeon, Jonathan Dworkin, and Marcos M. Pires**

Abstract: *β-Lactams represent one of the most important classes of antibiotics discovered to date. These agents block Lipid II processing and cell wall biosynthesis through inactivation of penicillin-binding proteins (PBPs). PBPs enzymatically load cell wall building blocks from Lipid II carrier molecules onto the growing cell wall scaffold during growth and division. Lipid II, a bottleneck in cell wall biosynthesis, is the target of some of the most potent antibiotics in clinical use. Despite the immense therapeutic value of this biosynthetic pathway, the PBP–Lipid II association has not been established in live cells. To determine this key interaction, we designed an unnatural* D*-amino acid dipeptide that is metabolically incorporated into Lipid II molecules. By hijacking the peptidoglycan biosynthetic machinery, photoaffinity probes were installed in combination with click partners within Lipid II, thereby allowing, for the first time, demonstration of PBP interactions in vivo with Lipid II.*

Alarming incidences of drug-resistant bacterial infections have resulted in an urgent need for new antibiotics.[1] Historically, some of the earliest antibacterial agents discovered were molecules that interfere with the biosynthesis of peptidoglycan, the essential mesh-like scaffold that encases bacterial cells. This pathway initiates in the intracellular space of bacterial cells (Figure 1) where a series of enzymatic transformations produce Lipid II, a lipid-linked disaccharide pentapeptide precursor.[2] Upon the translocation of Lipid II to the extracellular space, it is subject of a series of enzymatic transformations by penicillin-binding proteins (PBPs) to tailor the final mesh-like peptidoglycan structure. PBPs are also well-established targets of antibiotics, including β-lactam inactivators (for example, penicillins). Lipid II molecules are present at extremely low levels, thus making them critical limiting precursors in cell wall biosynthesis.[3] Antibiotics such as lantibiotics, glycopeptides, and the recently discovered teixobactin,[4] arrest peptidoglycan synthesis by binding and sequestering Lipid II. Although Lipid II processing by PBPs has been well characterized in vitro, the in vivo association of these two major antibiotic targets has eluded physical characterization to date.[5] Herein, we describe the synthesis of an unnatural dipeptide and demonstrate that it captures non-covalent associations between Lipid II and PBPs. Thus, our probes represent the first method to reveal Lipid II interactions with proteins in live bacterial cells.

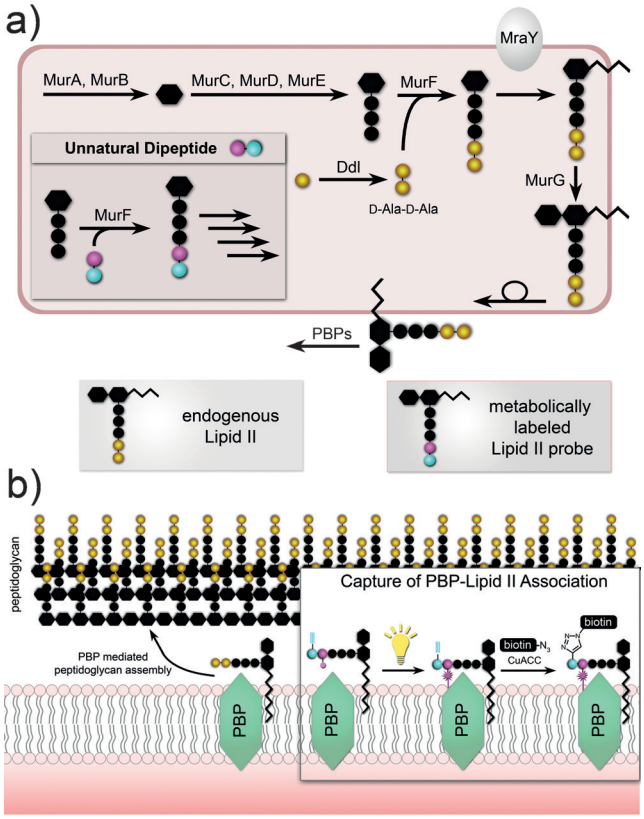


Figure 1. a) Promiscuity by MurF, which ligates the endogenous D-Ala-D-Ala dipeptide with the remaining peptidoglycan precursor, can lead to the introduction of Lipid II probes within the stem peptide. b) Introduction of alkyne and photocrosslinking handles onto the stem peptides can be used to capture PBP–Lipid II association.

A significant hurdle in establishing Lipid II–PBP interactions in vivo has been the inability to capture, retain, and characterize these interactions in their native environments. Our strategy overcomes this roadblock using metabolic incorporation of photoaffinity handles within lipid-anchored peptidoglycan precursor molecules. Recent reports have demonstrated that a number of peptidoglycan-linked enzymes display substrate promiscuity in the processing of unnatural D-amino acids, thus opening the door for incorporation of unnatural substrates.[6] Our research group has exploited this promiscuity for the development of agents with promising therapeutic activities as well as a bacterial profiling platform.[7] In the Lipid II biosynthetic pathway, MurF ligates the stem tripeptide with endogenous D-Ala-D-Ala and,

[*] Dr. S. Sarkar, S. E. Pidgeon, Dr. M. M. Pires
Department of Chemistry
Lehigh University
Bethlehem, PA 18015 (USA)
E-mail: map311@lehigh.edu
Dr. E. A. Libby, Dr. J. Dworkin
Department of Microbiology & Immunology
Columbia University
New York, NY 10032 (USA)

Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201603441.

importantly, displays substrate promiscuity (Figure 1a).[8] We hypothesized that the tolerance of MurF for unnatural dipeptides would allow the introduction of Lipid II probes that capture PBP association by combination of photocrosslinking and bioorthogonal chemical reactions (Figure 1b). The photoaffinity handle can be activated into a highly reactive intermediate upon irradiation that rapidly captures associations through formation of a covalent adduct. Inclusion of an alkyne group provides a handle for adduct isolation by copper-catalyzed azide–alkyne cycloaddition reactions (CuAAC).[9] Activity-based protein profiling (ABPP),[10] a technique pioneered by the Cravatt group, has played a pivotal role in elucidating unexplored protein–lipid associations in live cells.[11] Likewise, we anticipate that ABPP using a metabolic Lipid II probe will unveil unprecedented information about cell wall biosynthesis.

Initially, we synthesized two dipeptide variants (**1** and **2**) that displayed combinations of a minimal alkyne handle and an endogenous alanine residue (Figure 2a). The small sidechain size of D-propargylglycine (D-Pra) was expected to suitably mimic the D-Ala sidechain.[8a] Dipeptides **1** and **2** served to establish the metabolic incorporation of unnatural dipeptides in *Bacillus subtilis* (*B. subtilis*) and to determine positioning preferences within the dipeptide unit in both wild type *B. subtilis* and a genetic derivative (Δ*dacA*) lacking the DacA D,D-carboxypeptidase.[12] Terminal amino acids on the stem peptide can be enzymatically removed by carboxypeptidases during peptidoglycan maturation stages, potentially complicating analysis of incorporation levels. *B. subtilis* cells were incubated with both dipeptides **1** and **2**, subsequently reacted with a fluorescent click partner using CuAAC, and analyzed by flow cytometry. The observed labeling indicates that both dipeptides were well tolerated by MurF (Figure 2b). The lack of significant differences in labeling between dipeptides **1** and **2** in *B. subtilis* (Δ*dacA*) suggests a similar accommodation of the unnatural alkyne handles by MurF. In *B. subtilis* (wild-type), treatment with dipeptide **1** led to much higher levels of labeling compared to dipeptide **2**. This expected difference can be attributed to the protection of D-Pra on the 4th position of the stem peptide from carboxypeptidase and gives support to MurF-mediated incorporation. Competition of the unnatural dipeptides by a co-incubation with the endogenous D-Ala-D-Ala led to considerable suppression of bacterial labeling with **1** and **2**, a finding that is consistent with metabolic incorporation (Supporting Information, Figure S1).

We then investigated the ability of bacteria to incorporate dipeptides displaying both pull-down alkyne and photocrosslinking handles. Since metabolic incorporation of unnatural dipeptides with both amino acids displaying non-native sidechains or sidechains considerably larger than alanine has not been reported previously, we first established whether four additional dipeptides (**3**–**6**) related to **1** and **2** were incorporated. Two main features were assessed: the relative compatibility of phenylazide/benzophenone sidechains to be tolerated as a substrate by MurF and their positioning within the dipeptide. *B. subtilis* cells were treated with each individual dipeptide and labeling levels were quantified as before (Figure 2c). Satisfyingly, treatment with dipeptides **5** and **6** led to a ≈5-fold increase in fluorescence signals. Through these experiments, it was determined that phenylazide sidechains are distinctly better accommodated than benzophenone sidechains. These results are likely due to differences in sidechain size, which may create a steric impediment in binding to the MurF active site (or subsequent proteins that process Lipid II). Alternatively, dipeptides **3** and **4** may have poorer accumulation in the intracellular space of bacteria. In addition, dipeptide-based labeling was observed in varying levels with four additional Gram-positive bacteria (Figure 3). Finally, *Escherichia coli* (*E. coli*) cells were also successfully

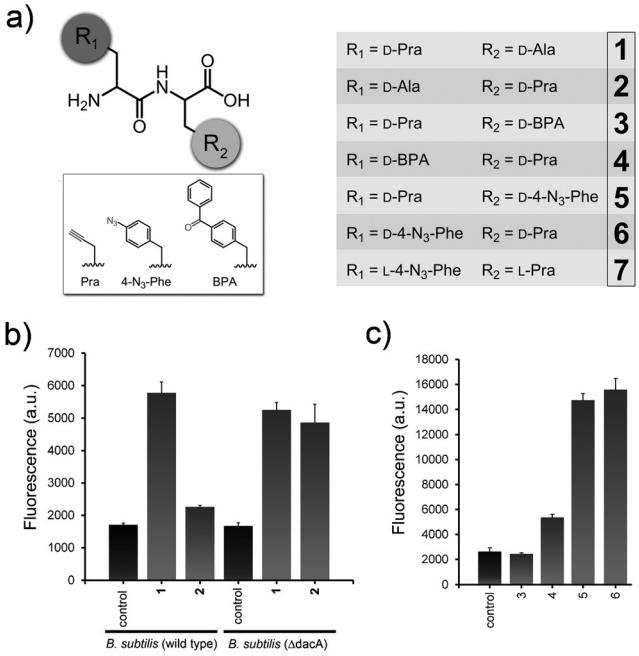


Figure 2. a) Chemical structure of dipeptides **1**–**7**. Alkyne and photocrosslinking handles are incorporated within the sidechains. b) *B. subtilis* (wild-type and Δ*dacA*) were incubated in the presence or absence of dipeptides (5 mM) overnight followed by a click reaction with 6-FAM azide. c) *B. subtilis* (Δ*dacA*) cells were incubated overnight in the presence or absence of dipeptides (10 mM), phenylazide was quenched with UV, and click reacted with 6-FAM azide. Cells were analyzed by flow cytometry. Data are represented as mean + SD ($n = 3$).

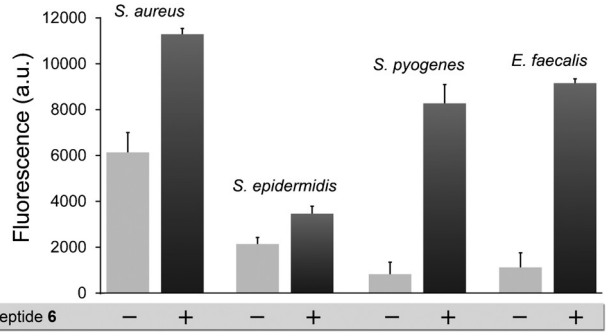


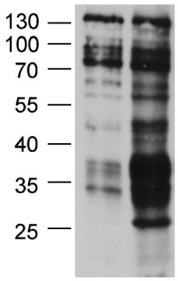
Figure 3. Bacterial cells were incubated overnight in the presence (dark grey) or absence (light grey) of dipeptides (5 mM) and click reacted with 6-FAM azide. Cells were analyzed by flow cytometry. Data are represented as mean + SD ($n = 3$).

labeled with unnatural dipeptide variants, indicating that this strategy is generalizable to Gram-negative bacterial species (Figure S2).

The stereospecificity of the metabolic incorporation of unnatural dipeptides was also evaluated. Treatment of bacterial cells with the enantiomeric dipeptide **7** did not result in an increase in cellular fluorescence (Figure S3), consistent with a MurF-mediated processing of unnatural D-amino acid dipeptides. To confirm that the Lipid II end-product (peptidoglycan) was modified with the unnatural dipeptide, the cells labeled with dipeptide **6** were harvested, their peptidoglycan digested by lysozyme, and the subsequent monomeric units isolated and analyzed by mass spectrometry (Figure S4). Monomeric fragments consistent with a modified disaccharide–pentapeptide were observed, lending further support to dipeptide incorporation into the peptidoglycan. Finally, the site of dipeptide incorporation was performed by fluorescence microscopy. Lipid II is concentrated at the septal region of growing and dividing bacterial cells. Treatment with BODIPY-vancomycin, which preferentially associates with Lipid II molecules, can delineate pools of Lipid II on the bacterial cell surface. A pulse-treatment of *B. subtilis* with dipeptide **6** was followed by incorporation of a fluorescent click partner. Cellular visualization confirmed that the dipeptide was preferentially localized to the septal regions, the primary site of peptidoglycan biosynthesis, supporting dipeptide **6** association with Lipid II (Figure S5). Finally, to demonstrate that Lipid II was being labeled and not the mature peptidoglycan, we generated protoplasts (cells lacking peptidoglycan) using lysozyme. We confirmed that this treatment completely removed the *B. subtilis* peptidoglycan by using flow cytometry to monitor a fluorescently-tagged D-amino acid (D-Dap(NBD)) embedded within the peptidoglycan. The generation of protoplasts verified that fluorescently labelled peptidoglycan was fully released from the bacterial cell (Figure S6). Next, *B. subtilis* cells treated with dipeptide **6** were subjected to the same protoplast procedure and the Lipid II probe was detected by CuACC-mediated fluorophore conjugation (Figure S7). The fluorescence increase in cells treated with dipeptide **6** compared to untreated cells is supportive of probe incorporation within Lipid II molecules. Taken together, these results establish that the dipeptides enter the peptidoglycan biosynthetic pathway.

Finally, we set out to use probe **6** to capture the interaction between lipid-anchored peptidoglycan precursors and PBPs in live cells. *B. subtilis* cells were incubated in the presence of dipeptide **6** and grown to mid-log phase (an active point in peptidoglycan biosynthesis). Photoirradiated samples were subjected to CuAAC reactions with azido-conjugated biotin handles. Biotin-modified adducts (protein/Lipid II-biotin) were detected by Western blotting (Figure 4a). We observed distinct differences in intensity and in the number of bands between non-irradiated and irradiated samples, consistent with specific light-dependent interactions between proteins and probe-labeled lipid-anchored peptidoglycan precursors. The proteins detected in the presence of light are expected to be interacting with lipid-anchored peptidoglycan precursors. Treatment of bacterial cells with the enantiomeric dipeptide **7** (composed of L-amino acids) led to no enrichment of proteins

a)



b)

Protein	ΔObservations	Fold Change	p-value
PBP1a/1b	12.5	3.17	0.08
PrsA	10.25	1.58	0.01
DacA (PBP5)	9	2.33	0.01
PBPC (PBP3)	8.25	4.00	0.03
EzrA	4.5	5.50	0.06
LytE	3.5	15.00	0.05
DivIVA	3.5	5.67	0.08
WprA	3.5	2.27	0.01
MurG	3	3.00	0.10
FtsX	3	2.33	0.13
PBPD (PBP4)	2.75	3.75	0.04
PBPX	2.5	2.11	0.02
MurF	1.5	1.60	0.03

Figure 4. a) *B. subtilis* 168 cells were treated with dipeptide **6** (5 mM) +/− UV light followed by a click reaction with biotin azide. Lysates were run on a SDS-PAGE gel and probed with streptavidin. b) *B. subtilis* 168 cells were treated as in (a), and proteins were pulled down using streptavidin beads. Listed are proteins identified in our experiments that are hypothesized to interact with Lipid II or operate at the cell wall. For each protein, Δ observations (raw change in observed peptides between +/−UV), fold-change in observations, and *p*-values (calculated using paired t-tests) are reported. For criteria for inclusion, see the Supporting Information.

in the presence of light, a finding that is consistent with dipeptide **6** being associated with the cellular membrane (Figure S8).

We then proceeded to identify specific proteins that formed associations with the Lipid II probe by LC-MS/MS. In total, ≈350 proteins were found to be on average more abundant in the presence of UV across 4 experiments. To determine the relative significance of the UV-dependent increase in the signal for each protein, the average raw change (Δ observations) as well as the fold-change across all of the experiments, was considered. To determine the significance of the change in signal +/−UV, paired t-tests were used to calculate *p*-values for each identified protein. We then used these values as criteria to refine the list down to the 85 most significant hits. Among the most significant hits were proteins predicted to interact with Lipid II (for example, many of the known PBPs including PBP1a/1b, DacA, PBPC, PBPD) as well as the dipeptide ligase MurF (Figure 4b, full list available in the Supporting Information). This last observation is consistent with the dipeptide entering the pathway at MurF (Figure 1B).

Of particular note, PBP1a/b was the most abundant protein captured with the Lipid II probe. PBP1a/b is a dual function transglycosylase/transpeptidase, which attaches Lipid II to the existing peptidoglycan scaffold.[13] This association was confirmed using a genetically modified strain of *B. subtilis* that expresses a PBP1a/b-FLAG fusion protein. Immunoprecipitation with anti-FLAG resin followed by Western blot analysis confirmed the association between dipeptide **6** and PBP1a/b (Figure S9).

In conclusion, we exploited the known promiscuity displayed by the MurF ligase and developed D-amino acid dipeptides displaying both pull-down alkyne and photocrosslinking handles. These dipeptides were metabolically incorporated into the stem peptide of peptidoglycan precursors, capturing for the first time in vivo interactions between Lipid II and PBPs. In future investigations, we will expand on

these initial findings to identify previously unrecognized Lipid II binding partners that may serve as new antibiotic targets.

Acknowledgements

This study was supported by Lehigh University (M.P.) and by NIH GM114213 (J.D.). The proteomics analysis was performed by the Proteomics Shared Resource at the Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center.

Keywords: amino acids · cell walls · lipid II · peptides · proteomics

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 8401 – 8404
Angew. Chem. **2016**, *128*, 8541 – 8544

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Received: April 8, 2016
Revised: April 25, 2016
Published online: May 25, 2016