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In vivo probe of Lipid II-interacting proteins

Dr. Sourav Sarkar^a, Dr. Elizabeth A. Libby^b, Sean E. Pidgeon^a, Dr. Jonathan Dworkin^b, and Dr. Marcos M. Pires^a

^aDepartment of Chemistry, Lehigh University, Bethlehem, PA 18015 (USA)

^bDepartment of Microbiology & Immunology, Columbia University, NY 10032 (USA)

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.

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Lipid II Probe: Lipid II is a critical intermediate in the biosynthesis of bacterial cell walls and it is also the purported target of several antibiotic agents. Unnatural dipeptides were used to gain entry into the biosynthetic pathway to deliver a dual-functioning probe that can report on potential Lipid II-interacting proteins.

Keywords

cell wall; lipid II; peptides; amino acid; proteomics

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 produces 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 (e.g., 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, demonstrate that it is show 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.

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 photo-affinity 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, 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 *via* 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 via 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 (*dacA*) lacking the DacA D,D-carboxypeptidase.^[12] Terminal amino acids on the stem peptide can be enzymatically

removed by carboxypeptidase 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). Lack of significant differences in labeling between dipeptides **1** and **2** in *B. subtilis* (*ΔdacA*) is suggestive of 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 (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 ~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 to varying levels with four additional Gram-positive bacteria (Figure 3). Finally, *Escherichia coli* (*E. coli*) cells were also successfully 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 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 via CuACC mediated fluorophore conjugation (Figure S7). The fluorescence increase in cells treated with dipeptide **6** compared to untreated cells is supportive of the probe incorporation within Lipid II molecules. 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 control enantiomer of dipeptide **6** (composed of L-amino acids) led to no enrichment of proteins in the presence of light, a finding that is consistent 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 (observations) as well as the fold change across all experiments were 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 ~85 most significant hits. Among the most significant hits were proteins predicted to interact with lipid II (e.g., many of the known PBPs including PBP1a/1b, DacA, PBPC, PBPD) as well as the dipeptide ligase MurF (Figure 4b). This last observation is consistent with the dipeptide entering the pathway at MurF (Fig. 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 novel D-amino acid dipeptides displaying both pull-down alkyne and photocrosslinking handles. These dipeptides are 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 novel antibiotic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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.

Figure 2.

a) Chemical structure of dipeptides **1-7**. Alkyne (red) and photocrosslinking (green) handles are incorporated within the sidechains. b) *B. subtilis* (wild type and *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* (*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).

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. Shown are known, hypothesized lipid II, or cell wall interacting proteins found consistently in 4 experiments. 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 materials and methods.