Effect of a small molecule Lipid II binder on bacterial cell wall stress

Abstract: We have recently identified small molecule compounds that act as binders of Lipid II, an essential precursor of bacterial cell wall biosynthesis. Lipid II comprised a hydrophilic head group that includes a peptidoglycan subunit composed of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) coupled to a short pentapeptide moiety. This headgroup is coupled to a long bactoprenol chain via a pyrophosphate group. Here, we report on the cell wall activity relationship of dimethyl-3-methyl(phenyl)amino-ethenylcyclohexylidene-propenyl-3-ethyl-1,3-benzothiazolium iodide (compound 5107930) obtained by functional and genetic analyses. Our results indicate that compounds bind to Lipid II and cause specific upregulation of the vancomycin-resistance associated gene vraX. vraX is implicated in the cell wall stress stimulon that confers glycopeptide resistance. Our small molecule Lipid II inhibitor retained activity against strains of Staphylococcus aureus mutated in genes encoding the cell wall stress stimulon. This suggests the feasibility of developing this new scaffold as a therapeutic agent in view of increasing glycopeptide resistance.

Keywords: defensin, Lipid II, antibiotics, bacterial membrane, vancomycin

Introduction

Bacteria, and in particular Gram-positive species, surround themselves with cell walls, structures that are essential for survival and growth.1 Interference with the biosynthesis of the cell wall has been a successful antibacterial strategy, and numerous classes of antibiotics have been developed that affect assembly. Penicillin and vancomycin are arguably the two most well-known examples of such therapeutics. Penicillin represents the class of β-lactam agents that interfere with cross-linking of peptide stems of peptidoglycan units outside of the bacterial cell.2 Vancomycin specifically binds to the l-Ala–l-Ala motif of the pentapeptide moiety of Lipid II, an essential precursor of peptidoglycan transport and assembly and acts at the membrane interface. Resistance against agents such as next-generation β-lactams and last-resort antibiotics including vancomycin is evolving at an alarming rate.3 Thus, there is a great need for alternative strategies to combat multidrug-resistant organisms.

We recently identified an interaction between a class of antimicrobial peptides in humans, termed defensins, and bacterial Lipid II.4 Based on these initial findings, we further characterized this interaction in molecular detail and identified small molecule Lipid II inhibitors for the first time.5 In this report, we further characterize one small molecule Lipid II inhibitor, termed compound 5107930. Compound 5107930 was examined for its effects on bacterial cell wall stress, binding to Lipid II and antibacterial killing in the context of glycopeptide resistance.
Materials and methods

Materials

Unless stated otherwise, chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). *Staphylococcus aureus* ATCC 29213 was obtained from Microbiologics (St. Cloud, MN, USA). Dimethyl-3-methyl(phenyl)amino-ethenylcyclohexylidene-propenyl-3-ethyl-1,3-benzothiazolium iodide was purchased from Chembridge Ltd. (San Diego, CA, USA). *S. aureus* strains carrying deletions in the cell wall stress stimulon (CWSS) were generously provided by M. Senn and C. Qublier (Institute for Medicinal Microbiology, University of Zurich) and were described earlier.6

Antibacterial activity assay

Determination of the minimal inhibitory concentrations (MICs) by dilution was carried out by broth dilution according to CLSI standards.7

Lipid II purification

Short-chain water-soluble Lipid II containing a lipid tail of three isoprene units was generated and purified essentially as described.8

Surface plasmon resonance

Binding experiments were carried out on a BIAcore T100 system (BIAcore, Inc., Piscataway, NY, USA) at 25°C. The assay buffer was 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, 150 mM NaCl, 0.05% surfactant P20, and pH 7.4 (±3 mM EDTA). 3-Lipid II (50 response units) was immobilized on CM5 sensor chips using the amine-coupling chemistry recommended by the manufacturer.

RNA isolation

*Staphylococcus aureus* strain 29213 was exposed to compound 5107930 for 30 min at 10 µM or left untreated. Total mRNA was subsequently isolated using the RNEasy mini kit (Qiagen; Germantown, MD, USA) according to manufacturer’s instructions.

Sequencing

The mRNA samples were sequenced using the HiSeq platform (Illumina; San Diego, CA, USA) for the four *S. aureus* samples (one control replicate and three treated replicates) in order to generate 101 bp reads. These paired end reads were populated into two separate FASTQ format files, and the quality of the reads was tested using the FastQC toolkit to ensure quality of the sequencing reads. The reads were trimmed based on the base quality score if necessary.9

Read alignment using bowtie

The sequenced reads were used as input to Bowtie version 0.12.7 to align the reads to the *S. aureus* 29213 reference genome for each sample.10 The output from Bowtie was obtained as binary alignment map (BAM) format files that consist of information on the alignment of each individual read within the reference genome. In the alignment phase, we allowed two mismatches over the entire read alignment. The BAM alignment files obtained from the Bowtie alignment tool were analyzed to generate the alignment statistics for each sample, namely, the total number of reads, the number of mapped reads, the percent of total mapped reads, and the percentage of reads that mapped to genic and intergenic regions. The coverage analysis aided in determining the success of the sequencing process, the coverage across the reference genome, and the presence of any outliers in the samples.

Differential expression analysis

The alignment BAM files from Bowtie are further utilized to compute gene expression levels and test each gene for differential expression.11 The reference annotation for *S. aureus* 29213 in GFF3 format was used. The number of reads that mapped to each gene described in the annotation was calculated using the python package HTSeq. The read count represents the expression of the gene. Differential gene expression analysis was conducted using the DESeq R package. The DESeq analysis resulted in the determination of potential differentially expressed genes (DEGs) when compared between the control samples and the treated samples. The read counts for each sample were normalized for sequencing depth and distortion caused by highly DEGs. Then, the negative binomial (NB) model was used to test the significance of differential expression between the control and the treated conditions. The DEGs were deemed significant if the normalized gene expression value was greater than the 10th percentile in either of the conditions and showed >1.5× fold-change difference (upregulated or downregulated) between the conditions.

Results and discussion

In our initial study, we found that compound 5107930 interacted with Lipid II and selectively killed Gram-positive organisms, most notably *S. aureus*.3 We confirmed the binding of our compound to Lipid II by surface plasmon resonance and found that it binds to Lipid II with a 2 µM binding affinity (Figure 1). Binding to Lipid II and a preferential killing of Gram-positive organisms by this compound could indicate targeting of the bacterial cell wall. To further investigate this possibility, we
examined the mRNA expression profile of *S. aureus* exposed to compound 5107930. Analysis of DEGs with a fold-change cut-off at 1.5× (i.e., absolute (Log2 fold change) ≥ 0.58) revealed a list of 490 genes that were upregulated or downregulated compared to control expression (Figures 2 and 3; Table S1). Downregulated expression was predominantly located to genes involved in protein translation, including ribosomal proteins L6, L18, L24 and S18, and translation elongation factor P. Upregulated gene expression was primarily located to bacteriophage S13 loci as well as the stress response protein GrpE. Most notably, compared to control, exposure to compound 5107930 for 30 min resulted in highly significant (a *p* value of $2.6 \times 10^{-14}$) and robust (Log2 fold change of 3.55) overexpression of the *vraX* gene (Table S1).

The *vraX* gene encodes a 55-aa protein with a putative signal sequence, suggesting that the protein is exported. Overexpression of Vrax has been reported upon exposure to agents that act against the bacterial cell wall, including antimicrobial peptides, bacitracin, fosfomycin, vancomycin, daptomycin and teicoplanin. Expression of Vrax is regulated by vancomycin-resistance associated regulator (VraR), which together with vancomycin-resistance associated sensor (VraS), orfI and yvqF, forms a four-component CWSS. Both VraR and VraS are overexpressed upon exposure to vancomycin, and overexpression results in decreased vancomycin susceptibility. Deletion of *vraR* and *vraS* was shown to result in increased susceptibility to vancomycin. In addition,
VraRS contributes to decreased susceptibility to daptomycin in clinical isolates.\textsuperscript{19} To test if the VraRS regulon is involved in susceptibility to compound 5107930, we determined the MICs against \textit{S. aureus} strains deleted for each individual gene of the stimulon (Table 1). We find that compound MIC against strains deleted for \textit{vraR}, \textit{vraS}, \textit{orf1}, or \textit{yvqF} remained unaltered, whereas the MIC of vancomycin varied slightly, but not significantly. Our findings are in agreement with previous observations in clinical isolates.\textsuperscript{19} To test if the VraRS regulon is involved in susceptibility to compound 5107930, we determined the MICs against \textit{S. aureus} strains deleted for each individual gene of the stimulon (Table 1). We find that compound MIC against strains deleted for \textit{vraR}, \textit{vraS}, \textit{orf1}, or \textit{yvqF} remained unaltered, whereas the MIC of vancomycin varied slightly, but not significantly. Our findings are in agreement with previous findings that showed little effect on vancomycin sensitivity upon CWSS deletion.\textsuperscript{4} This study and our findings differ from previous studies that showed decreased susceptibility to daptomycin in strains deleted for \textit{vraR} gene of the stimulon (Table 1). We find that compound MICs against \textit{S. aureus} strains deleted for each individual gene of the cell wall stress stimulon (Table 1). We find that compound MICs against \textit{S. aureus} strains deleted for each individual gene of the cell wall stress stimulon (Table 1). We find that compound MICs against \textit{S. aureus} strains deleted for each individual gene of the cell wall stress stimulon (Table 1). We find that compound MICs against \textit{S. aureus} strains deleted for each individual gene of the cell wall stress stimulon (Table 1). We find that compound MICs against \textit{S. aureus} strains deleted for each individual gene of the cell wall stress stimulon (Table 1). We find that compound MICs against \textit{S. aureus} strains deleted for each individual gene of the cell wall stress stimulon.\textsuperscript{18} A possible explanation for these differences could be that the strains we examined have the methicillin-resistant background.\textsuperscript{6} In conclusion, we show that compound 5107930 is a small Lipid II binder with cell wall targeting specificity. Importantly, we did not observe overexpression of the \textit{vraR} or \textit{vraS} genes upon exposure to this compound as reported for many other antibacterial agents. Compound 5107930 thus represents a new chemical scaffold for potential optimization as an antibacterial agent that acts against strains with decreased vancomycin or daptomycin susceptibility due to changes in the cell wall stress regulon.

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**Disclosure**

The authors report the following competing interests: US patent number 8,796,323 and US nonprovisional patent application number 13/911,234. The authors report no other conflicts of interest in this work.

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**Table 1** Minimal inhibitory concentration (µg/mL) of compound 5107930 and vancomycin against \textit{Staphylococcus aureus} strains with cell wall stress stimulon deletions

| Strain          | 5107930 | Vancomycin |
|-----------------|---------|------------|
| S. aureus BB270 | 16      | 2          |
| S. aureus BB270Δorf1 | 16 | 2          |
| S. aureus BB270ΔvraR | 8    | 1          |
| S. aureus BB270ΔvraS | 16 | 2          |
| S. aureus BB270ΔyvqF | 8   | 3          |

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