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Small molecule inhibitors of peptidoglycan synthesis targeting the lipid II precursor

Adeline Derouaux [a], Samo Turk [b], Nick K. Olrichs [c], Stanislav Gobec [b], Eefjan Breukink [c], Ana Amoroso [a], Julien Offant [d], Julieanne Bostock [e], Katherine Mariner [e], Ian Chopra [e], Thierry Vernet [d], Astrid Zervosen [f], Bernard Joris [a], Jean-Marie Frère [a], Martine Nguyen-Distèche [a], Mohammed Terrak [a,*]

[a] Centre d'Ingénierie des Protéines, Université de Liège, Allée de la chimie, B6a, B-4000, Sart Tilman, Liège, Belgium
[b] University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia
[c] Department Biochemistry of Membranes, Bijvoet Center, Institute of Biomembranes, Utrecht University, Utrecht 3584 CH, The Netherlands
[d] Institut de Biologie Structurale (UMR 5075, Université Joseph Fourier, CNRS, CEA), Grenoble, France
[e] Institute of Molecular and Cellular Biology and Antimicrobial Research Centre, University of Leeds, Leeds LS2 9JT, UK
[f] Centre de Recherches du Cyclotron, Allée du 6 Août, 8, B30, Université de Liège, 4000 Liège, Belgium

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ABSTRACT

Bacterial peptidoglycan glycosyltransferases (GTs) of family 51 catalyze the polymerization of the lipid II precursor into linear peptidoglycan strands. This activity is essential to bacteria and represents a validated target for the development of new antibacterials. Application of structure-based virtual screening to the National Cancer Institute library using eHits program and the structure of the glycosyltransferase domain of the *Staphylococcus aureus* penicillin-binding protein 2 resulted in the identification of two small molecules analogues 5, a 2-[1-[(2-chlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl]ethanamine and 5b, a 2-[1-[(3,4-dichlorophenyl)methyl]-2-methyl-5-methyl-sulfanylindol-3-yl]ethanamine that exhibit antibacterial activity against several Gram-positive bacteria but were less active on Gram-negative bacteria. The two compounds inhibit the activity of five GTs in the micromolar range. Investigation of the mechanism of action shows that the compounds specifically target peptidoglycan synthesis. Unexpectedly, despite the fact that the compounds were predicted to bind to the GT active site, compound 5b was found to interact with the lipid II substrate via the pyrophosphate motif. In addition, this compound showed a negatively charged phospholipid-dependent membrane depolarization and disruption activity. These small molecules are promising leads for the development of more active and specific compounds to target the essential GT step in cell wall synthesis.

1. Introduction

The increase of bacterial resistance to antibiotics has resulted in a decline of available efficient antibacterial treatments. Therefore, the discovery and development of new antibiotic classes able to cure infections due to resistant pathogens are urgently needed.

Peptidoglycan is an essential polymer and the main constituent of the bacterial cell wall. Its biosynthesis requires several steps and offers many unexplored targets for the development of new antibacterial drugs [1,2]. The last two reactions in peptidoglycan biosynthesis are performed by bifunctional penicillin-binding proteins (PBPs) and result in the assembly of the cell wall polymer from the monomeric intermediates [3]. This takes place outside the plasma membrane and relies on the activity of the glycosyltransferase (GT) module of bifunctional PBPs which uses the lipid II precursor to synthesize glycan chains and their transpeptidase module which catalyzes the cross-linking of two glycan chains via the peptide side chains. Inhibition of either of these two reactions leads to bacterial cell death. β-Lactam antibiotics target the transpeptidation reaction but antibiotic therapy based on inhibition of the GTs has not yet been developed. The only well characterized inhibitor targeting the GTs is the natural product moenomycin, a potent antibacterial phosphoglycolipid active at nanomolar concentrations [4]. Despite intensive studies of its structure–function properties it has not been developed for use in human chemotherapy because of poor pharmacokinetic properties

Abbreviations: PBP, penicillin-binding protein; GT, glycosyltransferase; PG, peptidoglycan; CF, carboxyfluorescein; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; 11-PP, undecaprenylpyrophosphate; 11-P, undecaprenylphosphate; LUV, large unilamellar vesicle.

* Corresponding author. Tel.: +32 4 3663332; fax: +32 4 3663364.
E-mail address: mterrak@ulg.ac.be (M. Terrak).

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related to its C25 lipid chain. A delipidated moenomycin is inactive and the sugar moiety can only be reduced to three saccharide units while retaining good antibacterial activity [4].

Recently the X-ray structures of PBPs or GT modules and their complexes with moenomycin A have been determined [5–8]. They shed light on the GT domain fold, which exhibits some similarities with that of λ-lysozyme and confirmed the catalytic mechanism of glycan chains elongation and the mode of action of moenomycin. The GT domain contains an extended enzymatic cleft which can accommodate six sugar units. It is divided into two sub-sites, a donor site for the elongating chain and an acceptor site for the lipid II substrate. Between the sub-sites a flexible region is proposed to play an important role in the translocation of the product from the acceptor site to the donor site through a folding and unfolding process [9]. The enzymatic cavity is bordered by several conserved residues and harbors the two glutamate residues essential for catalysis [10].

Inhibition of the GT reaction can be accomplished either by a compound binding to the enzyme, like moenomycin which occupies the donor site mimicking the elongating glycan chain [5], or by agent binding to the lipid II substrate [11]. Natural products such as nisin are known to target the peptidoglycan precursor. Nisin binds to the pyrophosphate motif of lipid II, sequestrates the substrate and induces pore formation in the bacterial membrane.

The potential of membrane damaging agents as antibacterials has been validated in the case of the cyclic lipopeptide daptomycin, a drug used in the treatment of certain infections caused by Gram-positive bacteria. Its antibacterial activity against *Staphylococcus aureus* including methicillin-resistant strains has been shown to be the result of membrane potential disruption [12].

In this paper we report the discovery of new peptidoglycan GT inhibitors through the use of structure-based virtual screening of small molecules from the National Cancer Institute library. Selected hits were evaluated for their ability to inhibit in vitro the GTs activity of *Escherichia coli* PBP1b in the presence of lipid II substrate, followed by the determination of their effect on bacterial growth. The active compounds were then submitted to several assays to demonstrate their specificities and modes of action.

2. Materials and methods

2.1. Chemicals

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG), 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(3-lysyl(1-glycerol))] (lysyl-DOPG) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids Inc. Nisin A was produced, isolated, and purified as described [13]. Moenomycin A, was a gift from Aventis (France). The fluorescent dye 3,3′-diethylthiodicarbocyanine iodide ($DiSC_2(5)$) was from Molecular Probes Inc. Lipid I, lipid II and Dansyl-lipid II were synthesized and purified as described elsewhere [14]. Undecaprenylphosphate and undecaprenylpyrophosphate were obtained by phosphorylation of undecaprenol [15] that was isolated from *Laurus nobilis* as described [16]. Radiolabelled [^{14}C]*meso*-diaminopimelic acid (A_2pm)-labelled lipid II was prepared essentially as previously described [17].

The small molecules tested in this study were obtained from the National Cancer Institute and solubilised in DMSO at a concentration of 100 mM or 10 mM depending on the solubility of the compound. The identities of the active compounds 5 and 5b (NSC no. 17383, and NSC no. 17382 respectively) were verified by mass spectrometry and NMR. The data (not shown) were consistent with the expected mass and structure for both compounds.

2.2. Proteins expression and purification

Five model proteins of the GT51 family were prepared and used in this study. *E. coli* PBP1b, *S. aureus* MtgA and PBP2, and the PBP1a from *Thermotoga maritima* MSB8 were produced and purified as previously described [5,17–19]. The gene encoding (A68-Q723) PBP2 of *Enterococcus hirae* was cloned into pET28a (+) expression plasmid (Novagen) and the His Tag PBP2 was expressed in *E. coli* BL21 (DE3). The cells were grown at 37 °C to an optical density of 0.8 at 600 nm, protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside and incubation was continued overnight at 18 °C. The cells were resuspended in 25 mM HEPES pH 7.5, 500 mM NaCl and the PBP2 was purified in one step on a HisTrap column (GE, Heathcare) (unpublished results). The total protein yield was about 15 mg/l of culture and the purity was over 90% as judged by on SDS–PAGE.

2.3. Virtual screening

The virtual screening was performed using the programme eHiTS 6.0 from SimBioSys Inc. eHiTS (http://www.simbiosys.com/ehits) [20,21] was used for the active site detection and docking. Open Babel (http://openbabel.org) was used for manipulating the ligands with various chemical formats. PyMol from DeLano Scientific was used for visual inspection of the results and the graphical representations.

The 3D structures of the compounds from the NCI Diversity Set were obtained from the NCI webpage (http://dtp.nci.nih.gov/dw/testmasters/chem3d.html). The 1990 highly diverse compounds of the NCI Diversity Set represented a broad chemical spectrum of the whole NCI database. However, no special preparation of the 3D structures was applied since eHiTS automatically evaluates all the possible protonation states for the ligands and enzymes. The crystal structure of *S. aureus* PBP2 as a complex with moenomycin (PDB entry 2OLV) [5] was initially prepared with eHiTS. The program automatically detected the ligand in the complex and selected the part of the enzyme within a 7 Å margin around the ligand as the active site. The NCI Diversity Set was then docked into the active site. The scoring was according to the eHiTS_Score that is included in the eHiTS software package. Two-dimensional similarities search was performed using a ZINC built-in engine and the entire NCI database containing more than 250,000 compounds (Tanimoto similarity index was set to 0.75).

2.4. In vitro glycosyltransferase activity and GT inhibition assays

2.4.1. Fluorescence assay

GT activity was monitored using the continuous fluorescence assay method [22]. For the simultaneous screening of numerous conditions this technique was adapted to a 96-well plate format (Greiner Bio-One). The standard reaction was carried out at 30 °C in 50 μl of 50 mM Hepes pH 7.5, 200 mM NaCl, 0.2% decyl PEG, 10 mM $CaCl_2$, 20% DMSO, 10 μM dansyl-lipid II, 1 unit of muramidase (Cellosyl) and 100 nM *E. coli* PBP1b. The data were collected for 30 min using a Victor 3 fluorimeter (Perkin Elmer) with excitation at 355 nm and emission at 536 nm.

For the other GT enzymes the reaction conditions were adapted for optimal activity as follows, with only variable conditions given: 2 μM *S. aureus* MtgA was used in the presence of 20 μM dansyl-lipid II, 10% DMSO and 10 mM $MnCl_2$. 2.5 μM *S. aureus* PBP2 was used in the presence of 20 μM dansyl-lipid II and 50 mM sodium acetate pH 5. *E. hirae* PBP2 and *T. maritima* PBP1a were used at 300 nM and 150 nM respectively.