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Small molecule inhibitors of peptidoglycan synthesis targeting the lipid II precursor

Running title: Small molecules targeting lipid II

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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-methylsulfanylindol-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.

Keywords: Glycosyltransferase; Peptidoglycan; Antibacterial; Lipid II; Penicillin-Binding Protein.

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 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. MATERALS 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]. Radiolabeled [^{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 n° 17383, and NSC n° 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 per litre 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 7Å 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.

2.4.2. Radioactive assay.

The radioactive assay was performed in 30 µl in the same conditions as the fluorescence assay using 4 µM [^{14}C]lipid II (0.126 µCi nmol^{-1}) instead of the fluorescent substrate and by omitting the muramidase. The reaction was stopped with moenomycin (10 µM) and the products were separated by TLC in 2-propanol-ammonium hydroxide-H_2O (6:3:1; V/V/V) and analyzed using a Typhoon Trio+ Imager and the ImageQuant TL software (GE Healthcare).

2.4.3. GT inhibition assay.

The IC_{50} values of compounds 5 and 5b were determined using a fluorescence based assay.

The initial rate of the reaction was determined in the presence of various concentrations of inhibitor (20-1500 µM) and plotted versus the inhibitor concentration using the Sigma plot program (Systas Software). The IC_{50} value is the inhibitor concentration that decreases the initial rate by a factor of 2.

2.5. MIC determination and effect of 5b on bacterial viability

The *S. aureus, Enterococcus faecalis, Listeria innocua, Micrococcus luteus, Streptococcus epidermis, Streptococcus pneumoniae* and *Pseudomonas aeruginosa* strains listed in table 2 were obtained from the American Type Culture Collection (ATCC). The *E. coli* strains 1411 and SM 1411 were reported previously [23].

Minimum inhibitory concentration (MIC) determinations were carried out using the Clinical and Laboratory Standard Institute (CLSI, 2009) broth microdilution method. Compounds were solubilised in 100% DMSO at a concentration of 10 mg/ml, and twenty-fold diluted in Mueller-Hinton broth (MHB), just before utilization.

For bacterial viability experiments *S. aureus* ATCC 25923 colonies were grown overnight on Mueller-Hinton agar (MHA) plates and suspended in MHB (≅1.10^8 CFU/mL). Compound 5b was added at 1, 2 and 4 times the MIC and mixed. Samples were taken at different time intervals of incubation at 37° C, serially diluted and plated on MHA in triplicate. The colonies were counted after incubation at 37° C for 18h.

2.6. Pathway reporter assays in Bacillus subtilis

We have employed a *Bacillus subtilis* reporter system which differentiates inhibitors of cell envelope, DNA, RNA, protein and fatty acid biosynthesis by quantifying the upregulation of a specific luciferase reporter construct in each case as described [24, 25].

The assay based on the LiaRS two-component system in *Bacillus subtilis* that senses

stress on the cell wall caused by compounds interfering with lipid II cycle was used according to published methods [26, 27]. The *B. subtilis* strain BFS2470 carries a β-galactosidase reporter gene under the control of *liaI* promoter. The effect of antibiotics or compounds spotted on a Whatman disc is revealed by blue/white selection method after overnight incubation at 37°C on agar-plate containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal).

2.7. LIVE/DEAD bacterial viability assay

The membrane damage inflicted by 10 minutes exposure to a panel of agents on *S. aureus* SH1000 was analysed using the LIVE/DEAD *Bac*light™ bacterial viability kit as reported [46]. Compound 5b and several control antimicrobial agents were tested at 4x MIC in comparison to a drug free control.

2.8. Preparation of large unilamellar vesicles (LUVs)

Large unilamellar vesicles were prepared essentially as described [28]. Desired amounts of lipid solutions in chloroform were mixed and evaporated under a gentle stream of nitrogen. The lipid film was subsequently dried for 20 min under vacuum. The film was hydrated by the addition of the buffer of choice under mechanical agitation and submitted to ten freeze-thaw cycles using liquid nitrogen and a water bath. The lipid suspension was then extruded ten times through a polycarbonate membrane filter with a pore size of 200 nm (Whatman International) [29]. The final phospholipid concentration was determined by phosphate analysis according to Rouse *et al*. [30].

2.9. Vesicles binding assay

DOPC with or without 1 mol% Lipid II vesicles were prepared as described above in 10 mM

MES-KOH, 15 mM K_2SO_4 at pH 7. Vesicles (1 mM lipid Pi) were incubated with 5 μM and 20 μM 5b, respectively, for 15 min at room temperature. The mixture was centrifuged in a TLA 120.2 rotor using a Beckman Ultracentrifuge (TL-100) for 1.5 h at 100 krpm and 20 °C. The amount of 5b before centrifugation and in the supernatant and pellet was determined by monitoring its intrinsic fluorescence on a Cary Eclipse fluorescence spectrophotometer (Varian Inc.). The percentage of 5b in the supernatant and pellet was determined by comparing the maximal value (350 nm) of fluorescence emission intensity.

2.10. Carboxyfluorescein leakage assay in large unilamellar vesicles

Carboxyfluorescein (CF)-loaded LUVs were prepared in 50 mM MES-KOH, 100 mM K_2SO_4, pH 6.5 (K+-buffer) as described above with the addition of 50 mM CF. Following the extrusion step, the vesicle suspension was applied to Sephadex G-50 spin columns equilibrated with K+-buffer to remove free CF. The final phospholipid concentration was determined by phosphate analysis according to Rouser *et al*. [30]. Vesicles were resuspended in K^+-buffer to a concentration of 25 μM and 5b was added at the desired concentration 1 min prior to the addition of nisin A (50 nM, final conc.). High-salt experiments were conducted in K^+-buffer supplemented with 0.5M NaCl. The nisin-induced CF leakage from the vesicles was monitored with excitation and emission wavelengths set at 430 nm and 513 nm, respectively. Triton X-100 was added 1 min after the addition of nisin, to a final concentration of 0.2% (w/v) to fully disrupt the lipid vesicles and the corresponding fluorescence was taken as 100% leakage. The 5b-induced reduction of leakage is expressed as a percentage of the leakage observed in the absence of 5b relative to the total release of carboxyfluorescein after addition of Triton X-100. The leakage results in an increase of fluorescence as the dye self-quenching is reduced.

2.11. Membrane depolarization assay

The cytoplasmic membrane depolarization activity of 5b was determined with the membrane potential-sensitive dye $DiSC_2(5)$ using *Staphylococcus simulans* and *Micrococcus flavus* grown in LB broth at 37ºC and 30ºC, respectively. Bacterial cells in the mid-logarithmic phase were centrifuged at 5000 rpm, washed in 5 mM HEPES (pH 7.8), and resuspended in the same buffer to an optical density at 600 nm of 0.05 in a 1 cm cuvette. A stock solution of $DiSC_2(5)$ was added to a final concentration of 0.4 μM and quenching was allowed to occur at room temperature for approximately 1 min. The desired concentration of 5b was added and changes in fluorescence due to the disruption of the membrane potential gradient across the cytoplasmic membrane were continuously recorded with a Cary Eclipse spectrofluorometer at an excitation and emission wavelengths of 622 and 670 nm, respectively.

3. RESULTS

3.1. Structure-based virtual screening and identification of GT inhibitors hits

We set out using a virtual screening approach based on the published *S. aureus* PBP2 structure in complex with moenomycin (PDB entry: 2OLV) [5] to identify small molecule inhibitors of the GT activity and to reduce the number of compounds to be tested using a direct *in vitro* assay based on lipid II substrate (see Materials and Methods). This approach has been used successfully in the identification of inhibitors of other targets of the cell wall synthesis pathway [31, 32].

The selected compounds were first evaluated with *E. coli* PBP1b then with other GT including *S. aureus* PBP2 [5, 17]. In the first step of screening, the 30 highest ranked compounds (predicted pKd values between -3.61 and -4.37) were selected for biochemical

testing. The number of compounds tested was further reduced to 21 as the remaining compounds were insoluble. Soluble compounds were then tested *in vitro* at 0.2 and 1 mM final concentrations for their ability to inhibit *E. coli* PBP1b GT activity in a 96-well microtiter plate assay with fluorescent lipid II. Compounds which inhibited PBP1b activity or interfered with the fluorescence assay were additionally tested in a radioactivity-based assay. One compound (5, ranked as the fifth best by eHits) (Figure 1A), a 2-[1-[(2-chlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl]ethanamine, was found to inhibit the GT activity of *E. coli* PBP1b (IC_{50} of 59 µM). In the second step, a search based on two-dimensional similarities to compound 5 identified an analogue of compound 5 called 5b (Figure 1A), about 2 times more active than compound 5 against *E. coli* PBP1b (IC_{50} of 29 µM) and was the focus of further characterisation.

The eHiTS-predicted binding mode of compound 5b is presented in Figure 1B. The model shows that compound 5b partly overlaps with co-crystallized moenomycin rings C and E, but is buried deeper because of favourable hydrophobic interactions. On the other hand moenomycin does not form hydrophobic interactions in the hydrophobic shelf due to its hydrophilic character.

Interestingly, some indole derivatives were shown to inhibit lysozyme, a structural homologue of GT [33]. The docking model of indole 3-carbinol into the active site of lysozyme [34] shows some similarities with the predicted binding of compounds 5/5b to the GT. Compounds 5/5b at 1mM concentrations do not inhibit lysozyme and have no effect on the penicillin-binding activity of the second domain of *E. coli* PBP1b or the *Actinomadura* R39 DD-peptidase (data not shown). This indicates that compounds 5 and 5b do not exhibit a promiscuous behaviour. Two other assays based on the use of a detergent [35, 36] and aggregation of the green fluorescent protein (GFP) [37] also support this observation (data not shown).

3.2. Evaluation of compounds 5 and 5b for GTs inhibition and antibacterial activity.

Both 5 and 5b were able to inhibit the GT reaction catalyzed by the five GTs tested. The IC_{50} values of compound 5b for *E. coli* PBP1b, *S. aureus* PBP2 and MtgA, *T. maritima* PBP1a and *E. hirae* PBP2 were determined using the fluorescence assay and were in the micromolar range (21-56 µM) (Table 1).

Furthermore, both 5 and 5b exhibit antibacterial activity against several important human Gram-positive pathogens with MIC values as low as 4 µg/ml (Table 2). Interestingly the compounds displayed MIC values of 4-8 µg/ml against methicillin–resistant *S. aureus* strains (MRSA) which pose a major problem in hospitals. The two GT inhibitors were less active against Gram-negative bacteria, as observed for other indole and tryptamine containing compounds [38-40]. The MIC values of compounds 5 and 5b against *E. coli* 1411 were 64 and 16 µg/ml respectively. They decreased to the values observed in Gram-positive organisms after polymyxin B nonapeptide (PMBN) treatment, or in an *acr*AB efflux mutant (Table 2). These data suggest that uptake of the compounds into *E. coli* is affected both by efflux and poor permeation through the outer membrane.

The effect of different concentrations of compound 5b on the growth and survival of *S. aureus* was evaluated by measuring the number of living cells and turbidity over time. The killing curves at 1x MIC (10 µM) and 2x MIC show that 5b causes an approximately 1 $\log_{10}$ and 1.3 $\log_{10}$ reduction of viability respectively after 90 min (Figure 2). At time zero (during sample mixing) the cell count was slightly lower than the control. At 4x MIC, the killing curve shows that 5b causes an immediate decrease in the bacterial count, more important than that observed at 1x and 2x MIC (1x $\log_{10}$ approximately), followed by a slower phase yielding an approximately 5.4 $\log_{10}$ reduction of viability after 90 minutes (Figure 2) and no viable cells were observed after 120 min (data not shown) showing that the compound is bactericidal. Similarly, the cell turbidity (Absorbance at 600nm) at 4x MIC also decreased

(36%) immediately after addition of 5b, then remained unchanged (data not shown). It seems that a two-phase process is taking place upon addition of 5b to the cells: a rapid phase occurs immediately after addition of the compound followed by a slower one.

3.3. Mode of action of compounds 5 and 5b

3.3.1. Compounds 5 and 5b induce cell wall biosensors in Bacillus subtilis.

Compounds 5 and 5b were predicted to specifically target peptidoglycan synthesis in bacterial cells. In order to characterize their mode of action we have employed a *Bacillus subtilis* reporter assay which differentiates inhibitors of cell wall, DNA, RNA, protein and fatty acid biosynthesis by quantifying the upregulation of specific luciferase reporter gene constructs in each pathway [24, 25]. Using this system, only the cell wall biosensor expression was induced in the presence of compounds 5 and 5b showing that they specifically inhibit cell wall biosynthesis as initially hypothesised (Table 3).

Two possible mechanisms for inhibition of the GT reaction include direct binding to the enzyme, or binding to the lipid II substrate. Experiments using fluorescence and microcalorimetry were not conclusive on whether 5b binds a GT enzyme (data not shown). Therefore, we checked whether 5 and 5b could interfere with the lipid II. First we used the *Bacillus subtilis* reporter strain BFS2470 (β-galactosidase reporter system) sensing cell wall antibiotics that interfere with the lipid II cycle [26, 27]. Using a disk diffusion assay with different antibiotics or compounds 5 or 5b on agar-plates containing X-Gal, the following observations were made (Figure 3A): compounds 5, 5b and nisin (as a positive control) produced a blue colour at the edge of the inhibition halo indicating an interaction of compounds 5 and 5b with the lipid II. In contrast, moenomycin and ampicillin did not induce β-galactosidase and the blue colour around the inhibition zone. These results indicate that compounds 5 and 5b could interact with lipid II.

3.3.2. Compound 5b interacts with lipid II.

To test for a possible specific interaction between 5b and lipid II, a competition assay was set up with the lantibiotic nisin, which uses lipid II for its pore-forming activity [14, 41, 42]. This experiment was performed with compound 5b which is more active than 5 on GT activity. A similar approach had previously been used with vancomycin in the study that determined the inhibition of the lipid II-dependent pore-forming activity of nisin by vancomycin in intact cells [43] and in a model membrane setup as is used here (data not shown) [44]. The addition of nisin to carboxyfluorescein-loaded vesicles containing lipid II causes leakage of the fluorescent dye from the vesicles, due to lipid II-dependent pore formation by nisin (CF loaded vesicles without lipid II showed negligible leakage after adding nisin, Figure 4). When 50 nM nisin was added to 25 μM DOPC vesicles containing 0.1 mol% lipid II, a leakage of ~75% was observed. In the presence of 2.5 μM 5b, CF leakage was significantly decreased by ~36%. Moreover, increasing concentrations of 5b resulted in higher reductions and almost complete inhibition of leakage at 15 μM (1.5x MIC) (Figure 3B). These results suggest that 5b binds to lipid II, thereby preventing an interaction of the latter with nisin.

To get insight into the binding site of 5b on lipid II, we first tested whether 5b could also inhibit the lipid I-dependent pore-forming activity of nisin. Nisin does not discriminate between lipid I (lacking the GlcNAc moiety) and lipid II. Leakage due to pore formation of nisin with lipid I was diminished to an equal extent by the presence of 5b as with lipid II (data not shown). This suggests that the GlcNAc moiety of lipid II is not an essential requirement for the interaction. If 5b specifically interacts with lipid II, structurally related compounds should compete for binding. In order to test possible interaction of 5b with the (pyro)phosphate moiety of lipid II, the inhibition effect of 5b on nisin-lipid II pore formation was analyzed in the presence of the peptidoglycan biosynthetic intermediates undecaprenylphosphate (11-P) or undecaprenylpyrophosphate (11-PP), respectively (Figure

3C). Nisin activity in the presence and absence of 5b was analyzed in CF-loaded DOPC vesicles containing 0.1 mol% lipid II and 2 mol% of either 11-P, 11-PP or, as a control, the anionic lipid DOPG. Strikingly, in the 11-PP containing vesicles, nisin induced CF-release was not significantly reduced in the presence of 2.5 μM 5b. In comparison, 5b was still able to cause significant inhibition of nisin activity in the presence of 11-P or DOPG, albeit somewhat less profound than in their absence (Figure 3C). This indicates that the excess of 11-PP competes with lipid II for binding 5b and that the pyrophosphate moiety is important for the interaction. To investigate this in more detail, NMR measurements were performed using undecaprenylpyrophosphate in detergent micelles in the presence and absence of 5b. ^{31}P NMR revealed a change in chemical shift ($\Delta\delta$P=-0.4 ppm) for one of the phosphate groups in the presence of 5b (Figure 3D). No significant shifts were observed when 5b was added to undecaprenylphosphate-containing Triton-X100 micelles (data not shown). These results confirm the importance of the pyrophosphate moiety for the interaction of 5b with lipid II.

3.3.3. Membrane disruptive effects of 5b

The vesicle binding assay using ultracentrifugation showed that 5b was present exclusively in the pellet in both lipid II-containing and pure DOPC large unilamellar vesicles (LUVs) while 5b alone was present in the supernatant (data not shown). This shows that 5b has an affinity for the lipid vesicles, regardless of whether lipid II is present or not. To gain insight into the effect of 5b on membrane, leakage experiments were subsequently conducted with LUVs containing anionic phospholipids in the absence of lipid II. Vesicles composed of only the zwitterionic lipid DOPC displayed negligible leakage even in the presence of 40 μM 5b (Figure 4A). Strikingly, in the presence of the negatively charged phospholipid DOPG leakage was detected at 5 μM concentration and almost total CF-leakage was obtained at 20 μM of 5b (Figure 4A). This suggests that 5b has a general membrane effect independent of

lipid II, and this disruption of membrane integrity is anionic lipid-dependent.

To investigate if 5b exerts similar effects on bacterial membranes that are known to contain relatively large amounts of negatively charged lipids, the depolarization effect of 5b on the plasma membrane in intact cells of *Staphylococcus simulans* and *Micrococcus flavus* was determined using the membrane-potential sensitive fluorophore $DiSC_2(5)$. This dye inserts into the cytoplasmic membrane in the presence of a membrane potential resulting in self-quenching of fluorescence. The addition of 5b to a final concentration of 1 μM caused a major release of the dye from the membrane in *M. flavus* (Figure 4b) showing that the membrane potential was dissipated upon binding of 5b. *S. simulans* also showed significant membrane depolarization at 5μM concentration of 5b. Nisin was more effective in dissipating the membrane potential in *M. flavus* (Figure 4b), which is in relative agreement with the difference in MIC values for both compounds (~5 nM nisin for *M. flavus* versus ~10 μM 5b for most Gram-positive strains tested).

These results are supported by experiments performed with the LIVE/DEAD *Bac*Light™ bacterial viability assay [45]. The membrane damage inflicted by a 10 minute exposure to compound 5b on *S. aureus* was analysed. Cells treated with the test agent 5b at 4x MIC only maintained 33% membrane integrity (67% damage), which does suggest that the membranes of *S. aureus* cells were permeabilized by the addition of 5b.

4. DISCUSSION

Peptidoglycan assemblage can be efficiently disrupted either through the inhibition of the glycosyltransferase or the transpeptidase enzymatic activities or by sequestration of the lipid II, thus, preventing its use as a substrate.

In our effort to identify small molecules inhibitors of the GT reaction, we have

discovered two structurally related indole derivative compounds (5 and 5b) inhibiting several GTs in vitro and having good antibacterial activity in the micromolar range. These compounds specifically induce peptidoglycan pathway stress sensors in *Bacillus*, confirming that bacterial cell wall synthesis is the target.

Moreover, we have found that compound 5b binds to the lipid II precursor. This is reminiscent of several lipid II-targeting antibiotics such as vancomycin and related (second generation) glycopeptides [46], and the lantibiotics mersacidin and nisin [11]. Undecaprenylpyrophosphate competes with lipid II for interaction with 5b. As undecaprenylphosphate and DOPG did not significantly compete with lipid II for 5b, the pyrophosphate moiety appears to be essential for the interaction with 5b. Nisin also binds the pyrophosphate of lipid II, and this could explain the effective inhibition of nisin-lipid II pore assembly by 5b. The data obtained from NMR analysis of undecaprenylpyrophosphate in the presence and absence of 5b further strengthens the hypothesis that the pyrophosphate moiety is critical for complex formation of 5b with lipid II. The amine residue of 5b has independently been shown to be essential for its glycosyltransferase inhibiting activity, suggesting its crucial involvement in the interaction with the pyrophosphate group of lipid II. The bacitracin antibiotic has also been shown to inhibit PG synthesis by sequestration of undecaprenyl pyrophosphate and was known to specifically bind to the pyrophosphate moiety of the lipid carrier [47].

Compound 5b at low concentration caused membrane depolarization in *M. flavus* and *S. simulans* as did nisin. Several lines of evidence also indicate that compound 5b affects in vitro membrane integrity in a negatively charged lipid dependent way in the absence of lipid II. These results suggest that membrane disruption could contribute to its antibacterial activity. Indeed, the effect of 5b on *S. aureus* viability shows that a two phase process contributes to its bactericidal activity. The first and immediate effect could be the result of

membrane integrity disruption. The second and slower phase (4x MIC) is more likely the consequence of cell wall synthesis inhibition as was previously observed for the kinetics of inhibition of *S. aureus* by moenomycin [48]. Therefore, it appears from our data that the identified compounds have two modes of action, namely, inhibition of cell wall synthesis and membrane depolarization/disruption. This mode of action resembles that of nisin which binds and sequesters lipid II preventing its use by the peptidoglycan polymerases and inducing pores formation in the bacterial membrane and membrane depolarization. The properties described for 5b could also be shared by compound 5 due to close similarity between the two compounds.

Bacterial resistance to antibiotics requires a continuing effort to find new antibacterial compounds able to cope with emerging resistant strains. The glycosyltransferase step in peptidoglycan biosynthesis is a validated target for new antibacterial development. The small antibacterial molecules described here are much simpler agents compared to known natural products targeting lipid II. Considering the importance of lipid II and the pyrophosphate motif for the whole enzymatic machinery of the bacterial cell wall synthesis pathway, this simple mode of binding to lipid II may limit the occurrence of resistance to such promising compounds. Finally, this work provides the basis for the development of more active and specific analogues of compounds 5/5b to target the essential GT step in cell wall synthesis using small molecule inhibitors.

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Figure legends

Figure 1. Structures of the compounds 5 and 5b and the predicted binding model of compound 5b to *S. aureus* PBP2 active site. A. Structures of the compounds 5, 2-[1-[(2-chlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl]ethanamine and 5b, 2-[1-[(3,4-dichlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl]ethanamine. **B.** Superimposition of the computer model of compound 5b (blue) on the X-ray structure of moenomycin (magenta lines, moenomycin rings are labeled from A to F) bound to *S. aureus* PBP2 (pdb code: 2OLV). Amino acids that interact with compound 5b are presented as sticks. Hydrophobic residues are shown as semi-transparent yellow surfaces. Hydrogen bonds between the free amino group of compound 5b and the Asp156 side chain are represented as dotted lines.

Figure 2. Effect of compound 5b on bacterial viability.
Cell viability after exposure of *S. aureus* ATCC 25923 to 1, 2, and 4 times the MIC of compound 5b.

Figure 3. Interaction of compound 5 and 5b with lipid II.
A. Effect of compounds 5, 5b and control agents on the lipid II cycle. The control antibiotics or compounds were spotted at the indicated amount on a Whatman disc on an agar-plate containing X-Gal and inoculated with the *Bacillus subtilis* strain BFS2470. The picture was taken after overnight incubation at 37°C.
B. Effect of 5b on nisin pore formation in DOPC LUVs containing 0.1 mol % lipid II. Compound 5b reduced the leakage of carboxyfluorescein induced by nisin.
C. Competition of undecaprenylpyrophosphate (11-PP), undecaprenylphosphate (11-P) and

DOPG with lipid II (LII) (20 fold molar access over lipid II) for 5b (2.5 µM) binding. The averages of two independent experiments are shown with the error bars indicating the spread between values. Experiments were also conducted in the absence of 5b as the leakage reduction is depicted as a percentage of the leakage without 5b (the latter was similar in all three cases).

D. ^{31}P-NMR of undecaprenylpyrophosphate in Triton-X100 micelles in the presence or absence of 1 mM 5b.

Figure 4. Membrane disruption effect of compound 5b.

A. Comparison of the disruptive effect of compound **5b** on DOPC/DOPG (1:1 mol/mol) and DOPC vesicles.

B. Effect of 5b on the plasma membrane potential of *Staphylococcus simulans* and *Micrococcus flavus* using $DiSC_2(5)$. Dye release was monitored at excitation and emission wavelengths of 622 and 670 nm, respectively.

List of 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

Table 1. IC_{50} values of compound 5b for various GTs.

IC_{50} (μM)	
	Compound 5b
E. coli PBP1b	29 ± 12
S. aureus PBP2	20.7 ± 1
T. maritima PBP1a	56 ± 7
E. hirae PBP2	33 ± 6
S. aureus MtgA	27 ± 9

The IC50 of compound 5 for E. coli PBP1b was 59 ± 3.3 μM. For comparison, the IC_{50} value of moenomycin for S. aureus PBP2 was 1.3 ± 0.3 μM.

Table 2. Susceptibility of a panel of bacteria to compounds 5, 5b and moenomycin.

	MIC (µg/ml)		
	5	5b	moenomycin
E. faecalis ATCC 29212	16	4 (10 µM)	4 (0.6 µM)
L. innocua ATCC 33090	8	4	4
S. aureus ATCC 25923 (MSSA)	8	4	0.5
S. aureus ATCC 43300 (MRSA)	8	4	0.5
S. aureus PL1 (MRSA)	8	4	0.5
M. luteus ATCC 9341	8	4	> 64
S. epidermidis ATCC 12228	8	4	4
S. pneumoniae D39	16	4	nd
E.coli 1411	64	16	nd
E.coli 1411 + PMBN (4 µg/ml)	16	8	nd
E.coli SM1411 (Δ*acrAB*)	16	4	nd
Pseudomonas aeruginosa	> 250	> 250	nd

PMBN, polymyxin B nonapeptide. nd, not determined.

Table 3. Induction of *B. subtilis* antibiotic biosensors in response to a panel of antimicrobial agents.

Antimicrobial Agent	Upregulated biosynthetic pathway				
	Cell Wall	***Protein***	***RNA***	***DNA***	***Fatty Acid***
Vancomycin	+	-	-	-	-
Flucloxacillin	+	-	-	-	-
5 or 5b	+	-	-	-	-
Nisin	-	-	-	-	-
Mersacidin	+	-	-	-	-
Cetyltrimethylammonium bromide	-	-	-	-	-
Tetracycline	-	+	-	-	-
Anhydrotetracycline	-	+	-	-	-
Fusidic Acid	-	+	-	-	-
Rifampicin	-	-	+	-	-
Rifamycin SV	-	-	+	-	-
Ciprofloxacin	-	-	-	+	-
Trimethoprim	-	-	-	+	-
Triclosan	-	-	-	-	+

+/- Induced/uninduced biosensor in response to a range of concentrations of antimicrobial agent (0.005-20µg/ml).

Figure1

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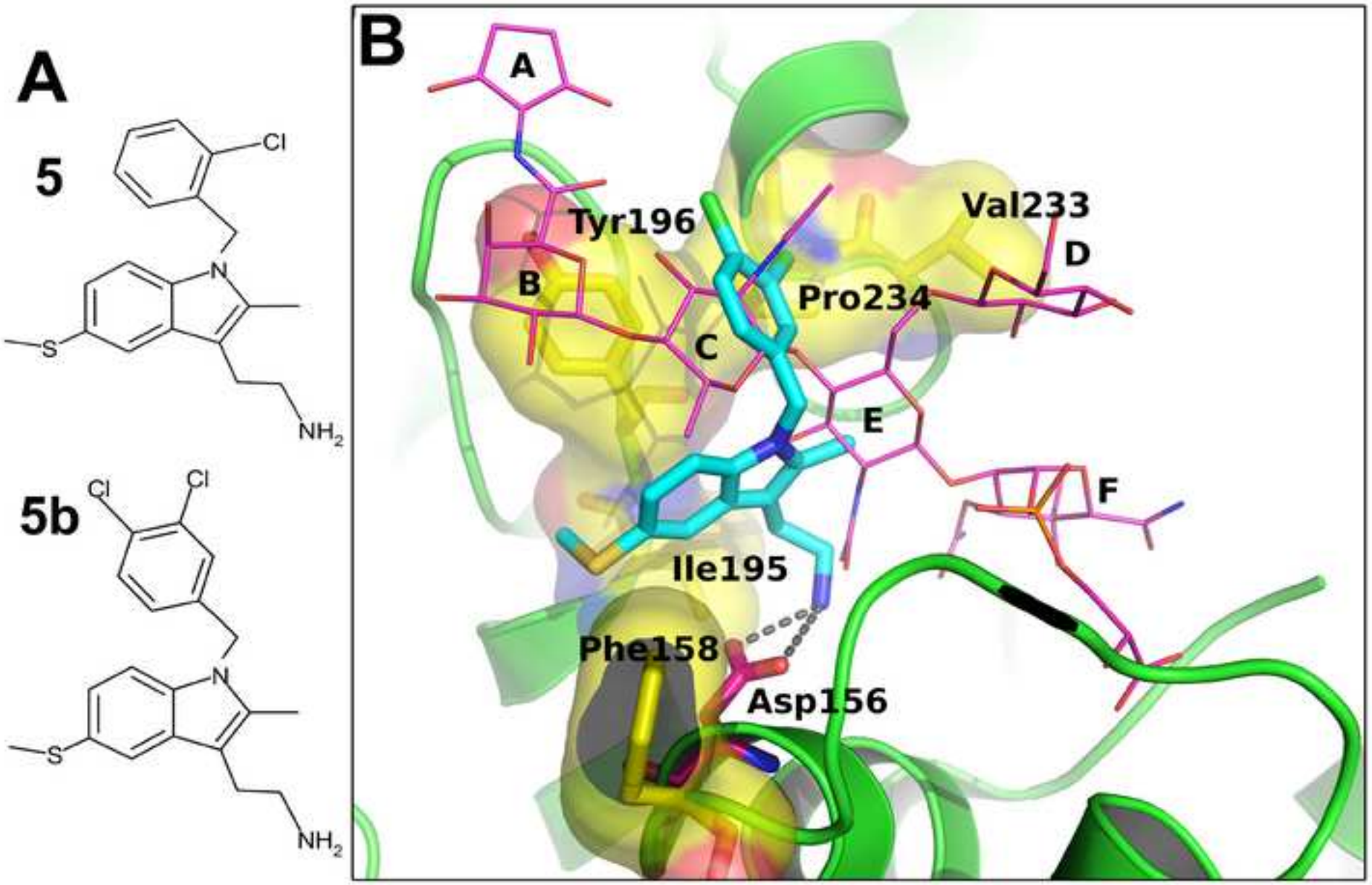

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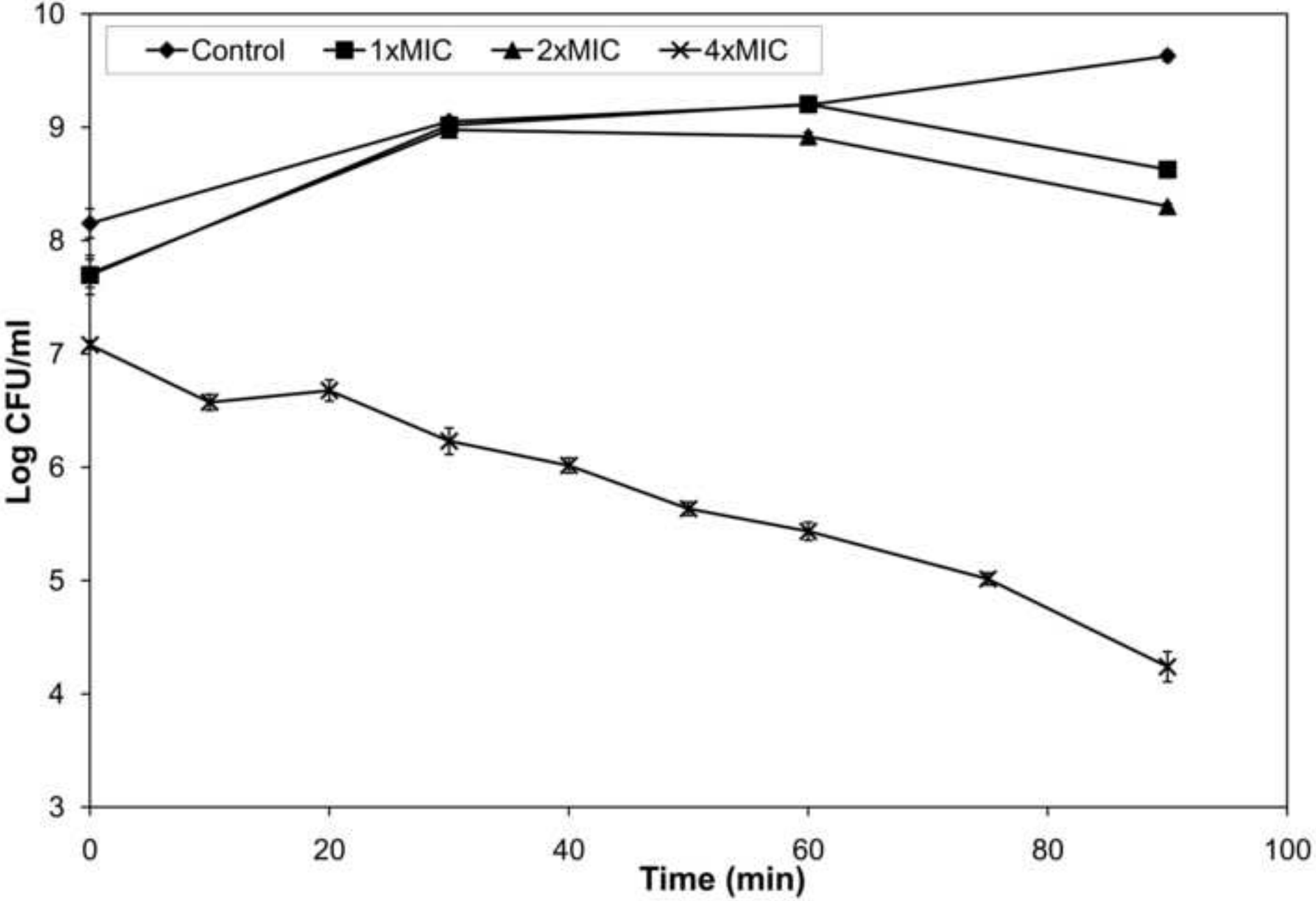

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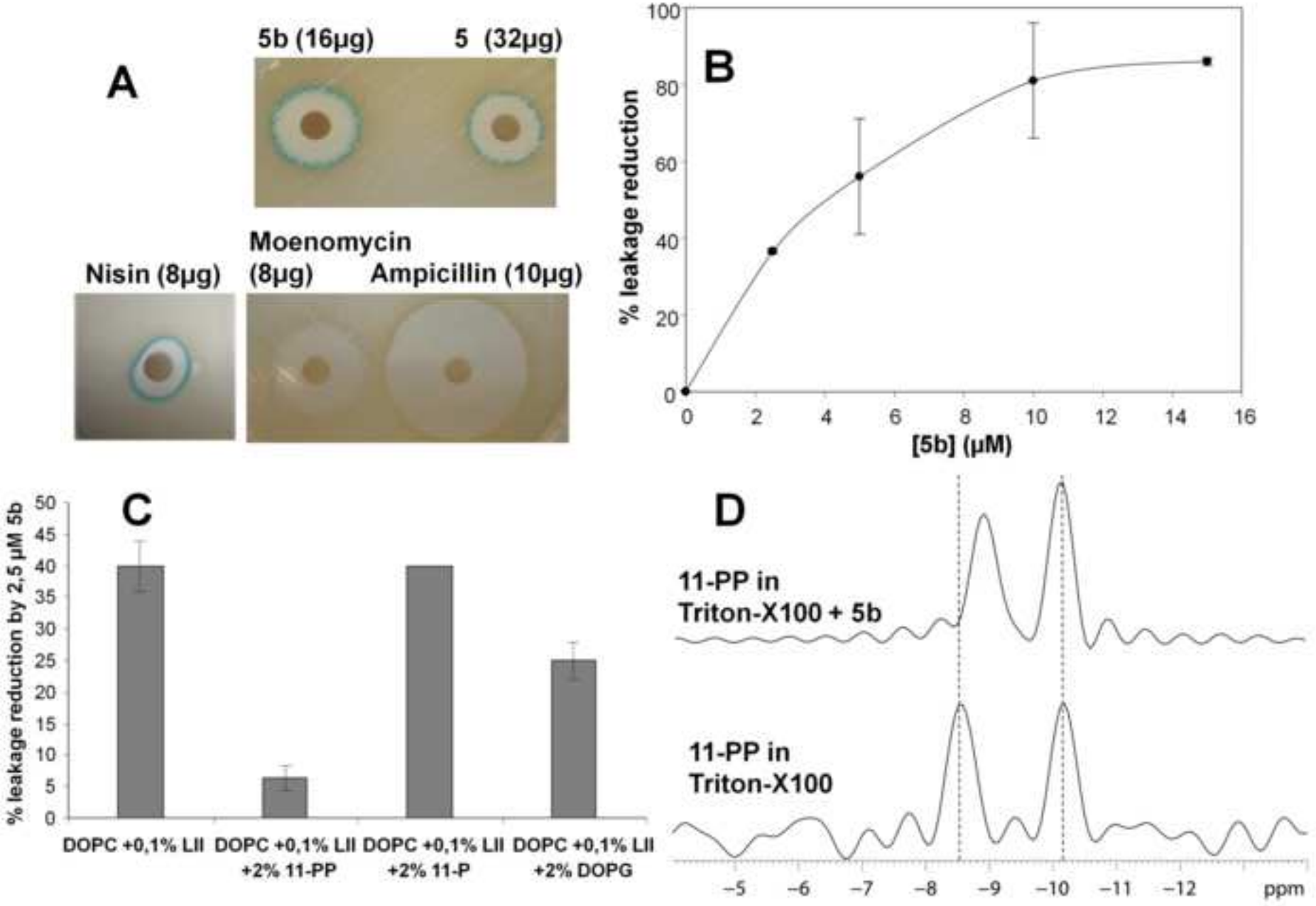

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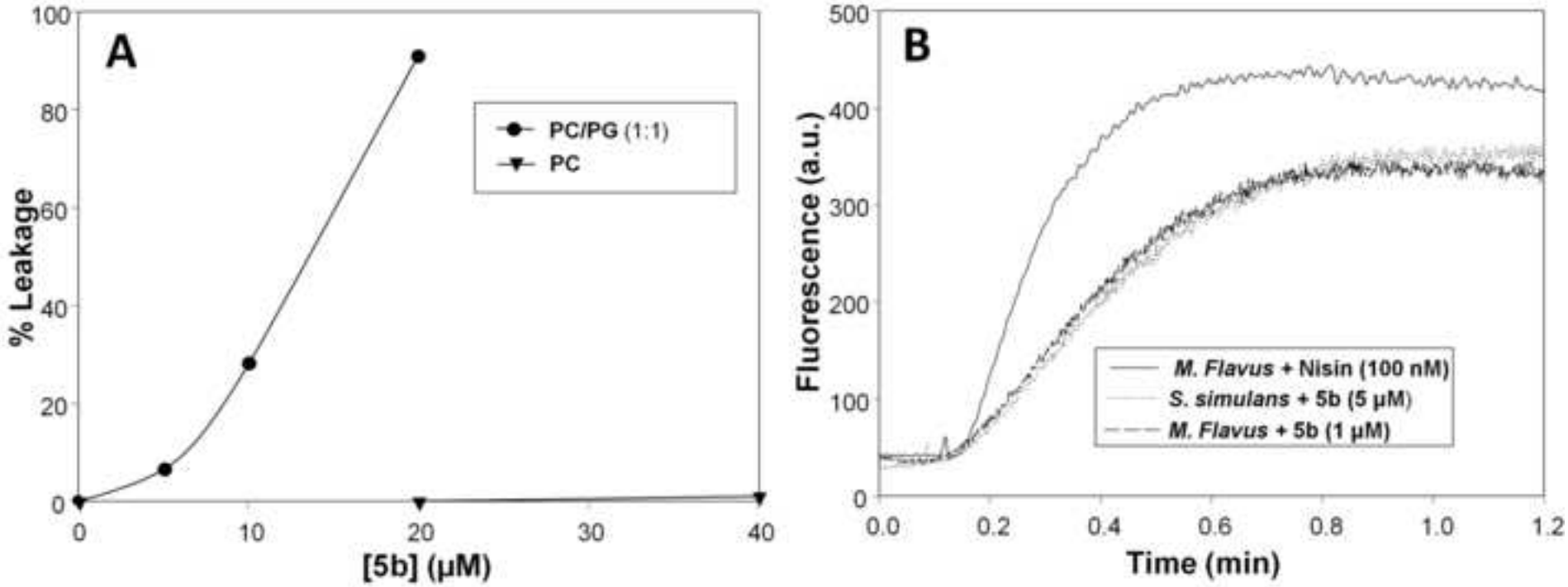

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