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BRIEF COMMUNICATION

Fluorescence assay to predict activity of the glycopeptide antibiotics

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Abstract

Here, we describe a fluorescent assay developed to study competitive binding of the glycopeptide antibiotics to live bacteria cells. This assay demonstrated that the mechanism of action of the lipoglycopeptide antibiotics strongly depends on the hydrophobicity of the substitutes, with the best antibacterial activity of the glycopeptide antibiotics equally sharing properties of binding to D-Ala–D-Ala residues of the nascent peptidoglycan and to the membrane.

For many years, vancomycin and teicoplanin were the only glycopeptide antibiotics used clinically in treatment of severe infections caused by gram-positive pathogens. However, the spread of glycopeptide-resistant enterococci and staphylococci has led to a renewed interest in the development of novel derivatives of the glycopeptide antibiotics [1–3]. Semisynthetic lipoglycopeptide antibiotics telavancin, dalbavancin and oritavancin, with improved antibacterial activity and pharmacokinetics in comparison to vancomycin and teicoplanin, were recently approved for clinical usage. The improved activity of these lipoglycopeptide antibiotics was associated with lipophilic substitutes introduced to glycopeptides, which enhanced interaction of the antibiotics with cell-wall precursors and with the membrane, leading to inhibition of cell wall synthesis, as well as disruption of cell membrane integrity [4–6]. Because of the complexity of the binding of glycopeptide antibiotics to the cell wall, involving binding to the terminal D-Ala–D-Ala residues of the nascent peptidoglycan, peptidoglycan bridges, interaction with the membrane and antibiotic dimerization [7], direct comparison of the efficiency of binding of different glycopeptide antibiotics to live bacterial cells was lacking.

Fluorescently labelled antibiotics have been used successfully to characterize changes in the cell wall of the bacteria [8, 9]. We decided to use fluorescently labelled glycopeptide antibiotics to compare the binding of the glycopeptide antibiotics to the live bacterial cells. We saturated exponentially growing *Staphylococcus aureus* ATCC29213 cells with fluorescently labelled vancomycin (FL-VAN), available from Sigma-Aldrich, or fluorescently labelled teicoplanin (FL-TEI), labelled with rhodamine B isothiocyanate (Fig. 1a). Protocol of synthesis of FL-TEI is described in Fig. S1. Detailed protocol of the fluorescent assay is described in Supplementary Materials. Then we tracked the release of the fluorescent glycopeptides bound to the cells by treating the cells for 10 min at room temperature with increasing amounts of non-fluorescent glycopeptides: vancomycin—VAN (V2002, Sigma-Aldrich), teicoplanin—TEI (T0578, Sigma-Aldrich), dalbavancin—DALB (HY-17586, MedChemExpress), oritavancin—ORI (SML1586, Sigma-Aldrich) and recently published novel lipoglycopeptide antibiotic derivatives with substitutes to the primary amino function of teicoplanin pseudoaglycon: MA79, MA72, ERJ390, LTS3, SZZS-12 [10–12] (Fig. 1b, Fig S1). As a proof of concept of the experiment, we showed nearly 100% release of the FL-VAN by *N*-acetyl-D-Ala–D-Ala dipeptide (D-Ala–D-Ala) (Sigma-

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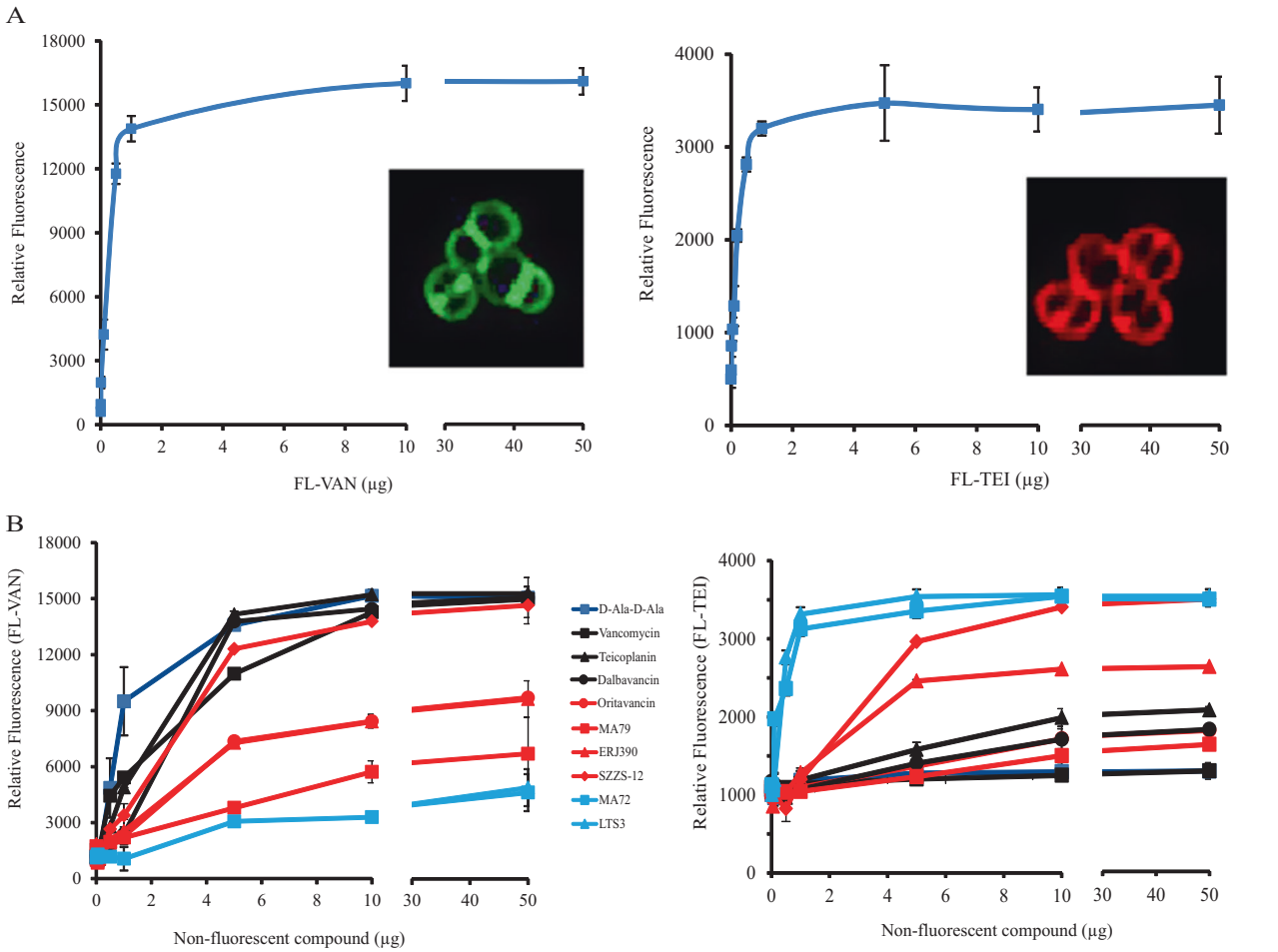
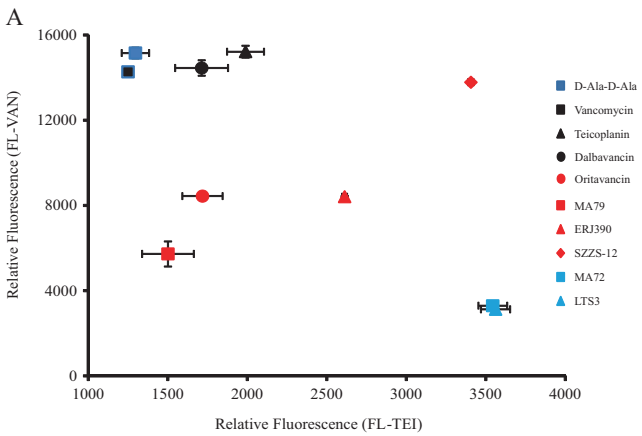


Fig. 1 **a** Saturation of *S. aureus* ATCC 29213 with fluorescent vancomycin (FL-VAN) and teicoplanin (FL-TEI). Fluorescent microscopy pictures of the *S. aureus* ATCC 29213 cells, stained with 1 µg of FL-VAN or FL-TEI, are added inside saturation plots. **b** Release of bound fluorescent vancomycin (FL-VAN) or fluorescent teicoplanin (FL-TEI) from *S. aureus* ATCC 29213 cells by addition of D-Ala–D-Ala, non-fluorescent vancomycin, teicoplanin, dalbavancin, oritavancin, MA79, ERJ390, SZZS-12, MA72 and LTS3



B

MIC (mg/L)	ATCC BAA1721 MSSA	ATCC 29213 MRSA	ATCC 35984 *S.epidermidis*	ATCC 700802 *E.faecalis* VanB	ATCC 15376 *E.faecalis* VanA
VAN	0,5	1	2	>64	>64
TEI	0,25	0,5	8	8	>64
DALB	0,125	0,125	0,125	0,25	64
ORI	0,0625	0,125	0,125	0,0625	0,125
MA79	0,25	0,5	0,5	0,125	0,125
ERJ390	0,25	0,25	0,5	0,125	1
SZZS-12	0,125	0,125	0,125	0,25	0,125
MA72	>8	>8	>8	>8	>8
LTS3	>8	>8	>8	>8	>8

Fig. 2 **a** Relative fluorescence of FL-VAN versus FL-TEI, released by non-fluorescent glycopeptides from live *S. aureus* cells, saturated by FL-VAN or FL-TEI. **b** MICs of glycopeptides for five bacteria strains, measured by microbrowth dilution method. Glycopeptide antibiotics inactive against VanA resistant enterococci are marked in black, glycopeptide antibiotics active against VanA resistant enterococci are marked in red. Inactive glycopeptide antibiotics are marked in blue

Aldrich) (Fig. 1b). Incubation of *S. aureus* in the presence of increasing concentrations of the antibiotics for 10 min at room temperature had no effect on the number of colony-forming units in the bacterial suspension. Plotting the relative fluorescence values of the released FL-VAN versus FL-TEI after incubation of the cells with non-fluorescent glycopeptide antibiotics divided the antibiotics into three groups (Fig. 2a), which correlated with the in vitro antibacterial activities of these antibiotics (Fig. 2b). Lipoglycopeptide antibiotics with low antibacterial activity, MA72 and LTS3, had low ability to compete out FL-VAN, but the highest ability to compete out FL-TEI. On the contrary, the glycopeptide antibiotics vancomycin, teicoplanin and dalbavancin, which efficiently competed out FL-VAN but not FL-TEI, had potent in vitro antibacterial activity against staphylococci and glycopeptide-sensitive enterococci, but were not active against VanA-resistant enterococci. Teicoplanin was not a good competitor of FL-TEI, probably due to the rhodamine B isothiocyanate attached to the primary amino function (Fig S1). This side group could provide additional sites of interactions with the bacterial cell surface, which could not be efficiently outcompeted by nonfluorescent teicoplanin, putting teicoplanin into the group of antibiotics effectively competing out only FL-VAN and unable to overcome VanA resistance. Lipoglycopeptides MA79, ERJ390, SZZS-12 and oritavancin had balanced properties of competing out both FL-VAN and FL-TEI, with MA-79 having the lowest ability to compete out FL-VAN and FL-TEI, and SZZS-12 being the most efficient. Independently of the level of the balanced ability to compete out FL-VAN and FL-TEI, all four glycopeptide antibiotics maintained high antibacterial activity against glycopeptide-sensitive and/or -resistant staphylococci and enterococci.

Finally, the in vitro activities of MA79, ERJ390 andSZZS-12, covering the whole group of the antibiotics with balanced FL-VAN and FL-TEI competing properties, were compared to the in vitro activities of vancomycin, teicoplanin, dalbavancin and oritavancin against an extended panel of clinical isolates of staphylococci and enterococci. The MIC values of the glycopeptide antibiotics for the 113 strains, measured by microbroth dilution method according to EUCAST guidelines (ISO 20776), are summarized in Table S1. All MIC values and a short description of the origin of the strains are present in Table S2. The novel glycopeptide antibiotics were active against vancomycin-sensitive and -resistant staphylococci and enterococci, with MIC_{50} values slightly lower than that of oritavancin. MIC values for *Enterococcus faecium* strains with VanA resistance were dramatically increased for vancomycin, teicoplanin and dalbavancin, but not for oritavancin, MA79, ERJ390 and SZZS-12, indicating that binding of the MA79, ERJ390, SZZS-12 and oritavancin to the D-Ala–D-Ala, the primary target of vancomycin, is not crucial for their observed antibacterial activity. It has been already demonstrated that oritavancin can inhibit bacteria cell-wall synthesis even with the inactivated D-Ala–D-Ala binding site [13].

In conclusion, our data indicate that competitive release of FL-VAN (binding to peptidoglycan) is required for antimicrobial activity of the glycopeptide antibiotics against vancomycin-sensitive staphylococci and enterococci antibiotics (vancomycin, teicoplanin and dalbavancin). Competitive release of FL-VAN (binding to peptidoglycan) and FL-TEI (binding to membrane) is necessary for antimicrobial activity of the glycopeptide antibiotics against vancomycin-resistant enterococci (MA79, ERJ390, SZZS-12 and oritavancin). Lipoglycopeptide antibiotics with highly lipophilic substituents (MA72 and LTS3) compete out efficiently FL-TEI (membrane binding), but not FL-VAN (binding to peptidoglycan), and do not have antibacterial activity. Altogether, this suggests that binding of the glycopeptide antibiotics to peptidoglycan and membrane is important for antibacterial activity against vancomycin-resistant bacteria. Our fluorescence assay provides the qualitative, but not quantitative, tool to compare binding of the glycopeptide antibiotics to the live bacterial cells, as well as helps to predict in vitro antibacterial activity of the glycopeptide antibiotics. Further studies are required for quantitative correlation between efficiency of competitive binding of glycopeptide antibiotics and their in vitro antibacterial activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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