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Glycopeptide antibiotic analogs for selective inactivation and two-photon imaging of vancomycin-resistant strains†

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A novel theranostic divalent vancomycin analog using a planar 1,8-diazapyrene moiety as a rigid scaffold exhibits potent and selective antibacterial activity against Gram (+) bacteria including vancomycin-resistant strains, while having minimal influence on Gram (−) bacteria and mammalian cells. Moreover, this theranostic analog can be also applied for selective two-photon fluorescence imaging of Gram (+) bacteria.

As the drugs of last defence, the glycopeptide based antibiotics, *e.g.* vancomycin (Van), have been extensively used in clinics for effective treatment of lethal bacterial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA),[1] mainly due to their tight binding affinity to the C-terminal D-Ala–D-Ala motif present in bacterial cell wall precursors.[1,2] However, recent emergence of virulent resistant species known as vancomycin-resistant *Enterococci* (VRE) and *Staphylococcus*,[1,3] which can remodel their surface peptidoglycan sequence from D-Ala–D-Ala to D-Ala–D-Lac and substantially decrease the binding affinity ($\sim 10^3$ times loss) of the Van molecule,[1,2,4] has raised serious concerns for human healthcare worldwide and thus urged more efforts to develop alternative treatment strategies to combat the prevalence of antibiotic resistance.[1,3]

So far, some semisynthetic glycopeptide antibiotics, including dalbavancin, oritavancin and telavancin *etc.*, which incorporate lipophilic moieties in Van-like structures, have been utilized to treat infections caused by multi-drug-resistant Gram (+) pathogens.[1,5]

The combination of inhibition of cell-wall synthesis and disruption of membrane integrity has been considered as the major mechanism of action to sustain the potent antimicrobial activity.[6] Alternative multivalent/polyvalent binding scaffolds based on covalently linked Van dimers or oligomers have also been proposed to enhance the potent activities against Van-resistant strains,[7] mostly attributed to their multivalent interactions that circumvent the low affinity between Van and the D-Ala–D-Lac peptide in resistant bacteria.[8] Despite the promising antibacterial activities, the development of new antimicrobial drug analogs that exhibit specific activity against VRE, while avoiding interference with the endogenous microbial population, is of high significance in clinical practice.[9]

Moreover, in line with the urgent demand for new antimicrobial agents to combat the increasing resistance, early diagnosis will also be a key to guarantee patients' survival from resistant bacterial infections. The optical imaging technique based on fluorescent protein variants or small molecule probes offers rapid and direct analysis of a variety of cellular events with promising sensitivity, and has become a powerful tool in studying bacterial infections and monitoring the biological mechanisms in antibiotic resistance.[10] Despite its initial success, several technical barriers still exist regarding nonspecific background signals, light bleaching, limited penetration and possible photodamage, especially for those fluorescent probes requiring short wavelength UV or blue light excitation, which may hamper the biomedical applications of optical imaging in living systems.[11] Compared to the conventional optical imaging modality, two-photon imaging exhibits promising advantages, including minimized auto-fluorescence background, reduced photo-bleaching and phototoxicity of fluorescent dyes, as well as larger penetration depth, and has thus received much attention recently in various bio-applications.[12] Inspired by these unique properties, the rational design of specific theranostic agents that can not only selectively inhibit, but can also be used for non-invasive two-photon imaging of VRE strains to facilitate antimicrobial studies, will be highly desirable.

Herein, we demonstrate a unique and specific divalent bacterial recognition analog using the glycopeptide vancomycin (Van) as an

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affinity ligand conjugated with a planar 1,8-diazapyrene (DP) linkage moiety.[13] The DP molecule was chosen as a bridging scaffold, mainly owing to its essential rigid structure and entropically enhanced affinity for multivalent Van interactions with bacterial surface peptide precursors.[14] In addition, the promising two-photon excitation property of DP is another attractive factor for its effective use in imaging.[13] Such a well-designed divalent Van–DP conjugate provides great advantages to selectively bind to Van-susceptible and VRE bacteria, with minimum interference from Gram (−) *Escherichia coli* (*E. coli*), and can thus be used as a robust theranostic probe to stain and inactivate these Gram (+) bacteria over *E. coli* or mammalian cells. Considering the potential mechanism of bacterial surface perturbation, we also incorporated a lipophilic chain into the divalent Van–DP structure, and the ability of these probes to achieve selective bacterial imaging and inactivation will be systematically investigated.

Fig. 1 illustrates the synthetic process of the divalent Van–DP conjugates. First, the rigid DP linkage moiety **2a** containing two methyl groups was synthesized through the coupling reaction reported previously.[13] After hydrolysis of the ethyl esters in **2a**, the obtained compound (**3a**) was further reacted with Van (**1**) to afford monovalent (**4a**) and divalent (**5a**) Van–DP analogs.[10b] The final products were purified in 21% and 16% yields through reversed-phase HPLC and characterized by NMR and high resolution mass spectrometry (HRMS). As a control, the DP structure with a lipophilic *n*-hexyl chain (**2b**) was also prepared and further conjugated with Van to afford monovalent (**4b**) and divalent products (**5b**). After purification of the Van–DP conjugates (**4a**, **4b**, **5a** and **5b**), their spectroscopic properties were investigated (Fig. S7, ESI†). All the Van–DP conjugates showed similar absorption and emission spectra to the original DP precursors,[13] suggesting conjugation of Van has negligible influence on the photochemical properties of the DP moieties.

The antibacterial activities of the Van–DP conjugates were investigated by standard minimum inhibitory concentration (MIC) assays (Table 1). In this study, six bacterial strains, including Gram (+) *Bacillus subtilis* (*B. subtilis*), Gram (+) Van-sensitive *Enterococcus faecium* (*E. faecium*) and *Enterococcus faecalis* (*E. faecalis*), Gram (+) VRE (*E. faecium* (VanA genotype) and *E. faecalis* (VanB genotype)), and Gram (−) *Escherichia coli* (*E. coli*) DH5α were chosen to

prove our design. As shown in Table 1, similar to Van (**1**), both monovalent (**4a**) and divalent (**5a**) Van–DP derivatives showed effective activities against all the Gram (+) Van-sensitive strains, namely *B. subtilis*, *E. faecium* and *E. faecalis*. Moreover, compared to free Van (**1**), **4a** exhibited slightly increased bacterial inhibition against VRE, suggesting that the rigid and hydrophobic DP moieties in the Van–DP analogs may supply additional affinity for the VRE surface and improve their interactions with peptide precursors in cell-wall structures.[5,6] More importantly, in contrast to Van or **4a**, the divalent Van–DP (**5a**) exhibited more promising activity against VRE. The *in vitro* binding constant analysis further confirmed that **5a** displayed higher affinity for $N^\alpha,N^{\prime\varepsilon}$-diacetyl–L-lysyl–D-alanyl–D-lactate, the sequence mimicking VRE surface peptide precursors, than the monovalent **4a** and Van (**1**) (Table S1, ESI†).[7,8] Moreover, the Van–DP analog **5a** was also found to show higher inhibition activity against the surface peptidoglycan biosynthesis in a VRE pathogen[7d] (Fig. S8, ESI†), suggesting that the DP-based divalent property of **5a** would be the major reason that resulted in the enhanced antimicrobial activities against Gram (+) pathogens, especially for those with resistant functions.[5,6] As a control, there was no significant antimicrobial activity detected when **4a** and **5a** were incubated with *E. coli*, clearly demonstrating the minimum influence of Van–DPs (**4a** and **5a**) towards Gram (−) strains.

Increasing the lipophilicity has become one prevalent strategy in numerous antibiotics to enhance bactericidal activities.[5,6] For comparison, we also evaluated the bacterial inhibition effect with Van–DP derivatives containing a lipophilic chain (*i.e.* **4b** and **5b**). As shown in Table 1, in terms of bactericidal activity against Gram (+) pathogens, **4b** or **5b** exhibited comparable recognition to **4a** or **5a**. However, different from **5a**, divalent Van–DP **5b** was found to have obvious activity against the *E. coli* DH5α strain. Moreover, further comparison was also provided by exploring the cytotoxic properties of **5a** and **5b** against fibroblast NIH3T3 cells, a model of healthy mammalian cells, through standard MTT assays (Fig. S9, ESI†). There was no obvious toxic effect when cells were incubated with **5a**. However, strong cytotoxicity was found when NIH3T3 cells were incubated with **5b**, most likely due to the possibility of lipophilic chain in **5b** to perturb cell membrane structures.

To further investigate the selective recognition of divalent Van–DP derivatives toward different strains, imaging of living bacteria was conducted by fluorescence microscopy. Initially, the divalent Van–DP derivatives (**5a** and **5b**) or original DP molecules (**3a** and **3b**) were incubated with Gram (+) or Gram (−) bacterial strains at 37 °C for 1 h and bacterial imaging was analysed upon the excitation of the DP moiety. The bacterial strains incubated with **3a** or **3b** did not show any significant fluorescence (Fig. S10, ESI†), implying the Van molecule is essential for bacterial staining. Conversely, as shown in Fig. S11 (ESI†), after incubation with **5a** or **5b**, blue fluorescence was observed in both Van sensitive and resistant Gram (+) strains, suggesting the high binding affinity of divalent Van–DPs for the surface of Gram (+) bacteria. In contrast, incubation of *E. coli* DH5α with **5b** led to obvious fluorescence, whereas there was minimum fluorescence observed upon similar cell incubation with **5a**. Furthermore, the divalent Van–DP derivatives were also applied to evaluate the selective imaging of bacteria over mammalian cells.
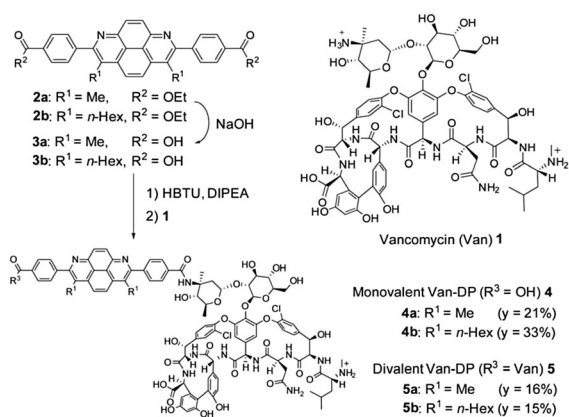

Fig. 1 Scheme for synthesis of Van–DP conjugates.

Table 1   MIC values of Van, DP, and monovalent and divalent Van–DP analogs

	MIC^a (µM)					
	B. subtilis	Van-sensitive *E. faecium*	Van-sensitive *E. faecalis*	Van-resistant *E. faecium*	Van-resistant *E. faecalis*	*E. coli* DH5α
1 (Van)	0.24 ± 0.02	2.7 ± 0.02	3.1 ± 0.21	>64	>64	>64
3a	34 ± 6.4	33 ± 3.0	35 ± 3.1	33 ± 3.1	39 ± 2.9	40 ± 3.3
3b	31 ± 3.3	48 ± 4.2	44 ± 5.2	30 ± 2.1	57 ± 5.4	59 ± 3.7
4a	0.93 ± 0.09	3.4 ± 0.11	3.7 ± 0.15	5.3 ± 1.2	30 ± 1.7	46 ± 2.6
4b	3.3 ± 0.47	2.2 ± 0.13	4.1 ± 0.17	5.7 ± 2.1	14 ± 1.7	35 ± 2.5
5a	0.12 ± 0.02	0.75 ± 0.05	0.65 ± 0.16	0.46 ± 0.05	3.3 ± 0.5	37 ± 5.1
5b	1.1 ± 0.34	1.3 ± 0.24	0.85 ± 0.06	3.2 ± 1.2	4.3 ± 1.2	8.7 ± 2.5

^{a} The MIC is defined as the lowest concentration of the drug necessary to inhibit bacterial growth. Each bacterial strain was incubated in LB with different concentrations of compounds in a 96-well plate for 18 h. The MIC values were determined from OD_{600} values in three separate experiments. The OD_{600} values of the wells in the absence of bacteria were used as the control.

As a proof-of-concept, NIH3T3 cells were chosen and co-cultured with a Gram (+) strain (*i.e.* VanB), and then incubated with **5a** or **5b** at 37 °C for 1 h (Fig. S12, ESI†). The imaging results revealed the great potential of **5a** to selectively recognize Gram (+) bacteria among NIH3T3 cells, whereas **5b** may stain both VanB and NIH3T3 cells, demonstrating the good selectivity of **5a** toward Gram (+) strains and that the lipophilic chains in **5b** would attenuate its selectivity among Gram (+), Gram (−) and mammalian cells.

In order to obtain insight into the different antibacterial properties of **5a** and **5b**, scanning electron microscopy (SEM) was applied to verify the potential bacterial surface disruption after the antibacterial treatment. As shown in Fig. 2 and Fig. S13 (ESI†), all the intact bacteria, including *B. subtilis*, VanB and *E. coli* DH5α, exhibited smooth morphology, indicating that the cell membranes have been well-maintained during sample processing. Incubation of *B. subtilis* and VanB with **5a** or **5b** would lead to a damaged or lysed surface, suggesting that the active mechanisms of **5a** and **5b** may obviously influence Gram (+) cell-wall structures through their higher binding affinities with surface peptide precursors.[7,8,14] In contrast, incubation of *E. coli* DH5α with **5a** would not change the surface morphology, whereas significant collapse damage was observed for *E. coli* DH5α when treated with **5b**, suggesting that the involvement of the lipophilic chain could be one key factor to disturb the bacteria surface, which therefore compromised the selectivity between Gram (+) and Gram (−) bacteria.

More importantly, the selective antibacterial activity of the divalent Van–DP derivatives was further examined by using mixed bacterial cultures with Gram (+) and Gram (−) strains. Typically, Gram (+) *S. aureus* expressing green fluorescent protein (GFP) and
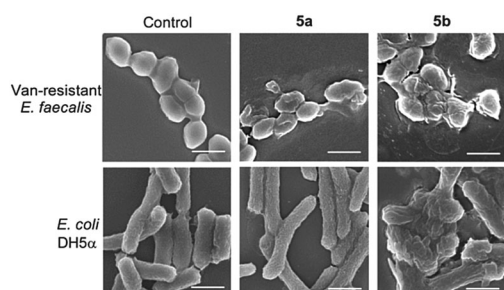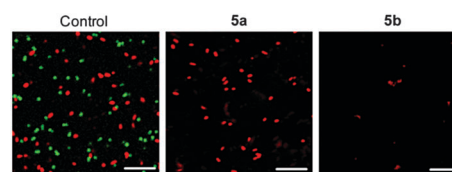

Fig. 3   Fluorescence imaging of the mixture of GFP-expressing *S. aureus* and RFP-expressing *E. coli* treated with **5a** or **5b** (10 µM) at 37 °C for 24 h. Scale bar = 10 µm. (λ_{ex} = 488 nm for GFP and λ_{ex} = 543 nm for RFP, λ_{em} = 515 ± 30 nm for GFP and λ_{em} = 590 ± 60 nm for RFP.)

Gram (−) *E. coli* expressing red-fluorescent protein (RFP) were chosen to incubate with divalent Van–DP **5a** or **5b** (10 µM) at 37 °C for 24 h. The different antibacterial activities were monitored by microscopic analysis (Fig. 3). The bacterial mixture without Van–DP (**5a** or **5b**) treatment was used as a control. Both green and red fluorescence was observed in the bacteria without probe incubation, while only red fluorescence was detected when the mixed bacterial culture was incubated with **5a**. These results demonstrated that divalent Van–DP **5a** could selectively target Gram (+) *S. aureus* over Gram (−) *E. coli* strains. However, after incubation of the mixed bacterial culture with **5b**, there was almost no bright fluorescence observed in the confocal image, suggesting that divalent Van–DP **5b** containing a lipophilic chain would damage the cell surface of both Gram (+) and Gram (−) bacteria and thus inactivate these two pathogens effectively. Unlike most antimicrobial studies, which evaluate the bacterial inhibition and imaging with a single bacterial species, here, our system based on bacterial mixture can prove the selective antibacterial properties of developed drug candidates. Such promising antibacterial activity of **5a** gives great potential to selectively inactivate Gram (+) pathogens including VRE without influencing Gram (−) strains, which will thus afford broad treatment options to minimally perturb other endogenous microbial populations during antibacterial treatment.

Finally, we examined the feasibility to further confirm the selective bacterial recognition through the two-photon imaging strategy.[13] Generally, bacteria *B. subtilis*, VanB or *E. coli* DH5α were incubated with 2 µM of divalent Van–DP (**5a**) at 37 °C for 1 h. Two-photon microscopic imaging was performed upon excitation of **5a** at 760 nm, which was carefully chosen to minimize the bacterial photo-damage caused by UV light, and
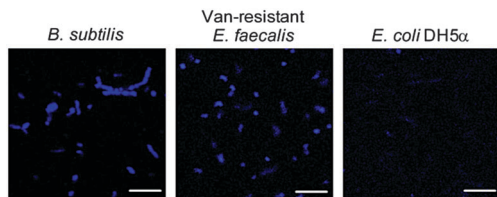

Fig. 2   SEM images of *E. faecalis* (VanB) and *E. coli* DH5α after treatment with **5a** or **5b** (10 µM) at 37 °C for 2 h. Scale bar: 1 µm.

Fig. 4 Two-photon imaging of bacteria stained with **5a** (2 μM) at 37 °C for 1 h. λ_{ex} = 760 nm. Scale bar: 10 μm.

to limit the interference of autofluorescence. As shown in Fig. 4, there was obvious two-photon imaging observed in **5a**-treated *B. subtilis* and VanB, whereas almost no fluorescence was detected in *E. coli* DH5α. These two-photon imaging results are consistent with those of previous one-photon imaging analysis, further proving the concept that the as-developed Van–DP divalent derivative can be used to selectively stain Gram (+) bacteria, including VRE.

In summary, we have successfully constructed a novel theranostic antibacterial analog by conjugation of two Van moieties with a rigid 1,8-diazapyrene (DP) scaffold to achieve selective two-photon imaging and antibacterial treatment against Gram (+) strains including VRE. The divalent Van–DP analog (**5a**) shows promising activity against Van susceptible and resistant pathogens but with minimal influence on Gram (−) strains or mammalian cells. Importantly, the selective treatment of Gram (+) bacteria in a bacterial mixture containing Gram (+) and Gram (−) strains also revealed the promise of rigid divalent **5a** for selective inhibition and imaging of VRE in future biomedical studies. Moreover, the controlled mechanism investigations further demonstrated that integration of lipophilic chains into the Van–DP scaffold may cause significantly poorer selectivity between Gram (+) and Gram (−) strains, most likely due to the non-specific activity towards the cell surface. The insight into the effect of lipophilic chains for selective bacterial recognition may thus provide valuable information to facilitate the design of Van-type antibiotic candidates for bacterial inactivation and imaging in the future.

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