

Multifunctional divalent vancomycin: the fluorescent imaging and photodynamic antimicrobial properties for drug resistant bacteria†

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A simple and specific divalent vancomycin–porphyrin has been developed. This divalent vancomycin–porphyrin conjugate indicates promising properties in fluorescent imaging and photodynamic inactivation of vancomycin-sensitive and vancomycin-resistant enterococci (VRE) bacterial strains.

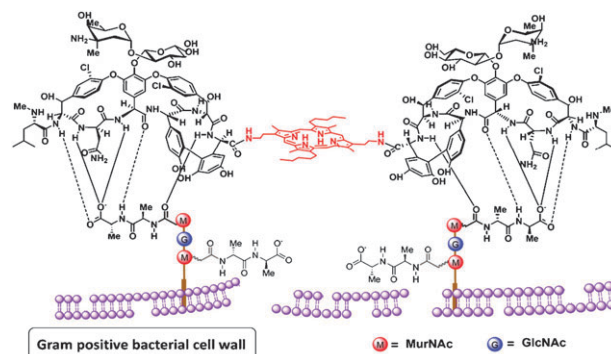
Vancomycin (Van) is a powerful glycopeptide antibiotic to treat methicillin-resistant Gram-positive infections through their specific binding affinity to the C-terminal L-Lys-D-Ala-D-Ala motif present in bacterial cell wall precursors.¹ However, bacteria having resistance to vancomycin known as vancomycin-resistant enterococci (VRE) recently emerged as a serious threat to public health, which is typically due to the mutation of peptidoglycan sequence from D-Ala-D-Ala to D-Ala-D-Lac, resulting in the substantial decrease of binding affinity ($\sim 10^3$ times loss) to Van molecules.¹ Extensive studies done by Griffin *et al.*,² Nicolaou *et al.*,³ Williams *et al.*⁴ and Whitesides *et al.*⁵ revealed that covalently linked dimers and oligomers of Van could serve as promising approaches to enhance the potent activities against VRE based on the polyvalent/multivalent interactions to circumvent the low affinities binding between Van and D-Ala-D-Lac peptide precursors in resistant bacteria.⁶ However, recent reports also indicated that the increased binding affinity may not always lead to substantial activities with effective minimum inhibitory concentration (MIC) against VRE organisms.^{4,7} Thus, the search for alternative treatment approaches against VRE bacterial infections is still highly desirable.

One promising alternative for the microbiological control is based on photodynamic antimicrobial chemotherapy (PACT),⁸ which involves the use of photosensitizers to generate reactive oxygen species (ROS, *e.g.* singlet oxygen (1O_2)) upon light exposure at a suitable wavelength. These reactive oxygen species are cytotoxic and are capable of destroying the cell walls and membranes, thus resulting in cell death.⁹ To date, PACT has been demonstrated to be effective against a variety of Gram-positive and Gram-negative bacteria.^{8,9} One possibility to minimize side effects and further improve

the efficiency of PACT in clinics is the use of affinity ligands that can efficiently target photosensitizers to areas of bacterial infections. Several affinity ligands based on antibodies,¹⁰ protein cage,¹¹ polypeptide,¹² nanoparticles,¹³ and bacteriophage¹⁴ have been reported to successfully direct lethal photosensitizers to antibiotic-resistant bacteria. However, the development of simpler and economical novel targeting molecules capable of specifically directing photosensitizers to drug resistant bacteria remains necessary and is of great significance since most of the current approaches are complicated, require tedious manipulation and may suffer from difficulty in synthesis, self-aggregation or possible immunogenicity.^{10–14}

In this study, Van antibiotic was employed as the affinity ligand, and porphyrin, a commonly used photosensitizer due to its clinical significance in both PACT^{8,15} and noninvasive fluorescent imaging of living cells *in vitro* and *in vivo*,¹⁶ was chosen as the bridging moiety to generate Van conjugated multivalent/polyvalent dimeric system (Scheme 1). The divalent Van–porphyrin conjugate possesses several unique advantages including (i) ease of preparation, (ii) the rigid structure of the porphyrin linker supplies entropically enhanced binding and steric hindrance necessary for multivalent/polyvalent interactions between the disubstituted Van and VRE strains,^{5,6} (iii) selective adhesion of the divalent Van to bacterial surfaces leading to the enhanced photodynamic inactivation of Van-sensitive and VRE strains which are more potent than the MICs of Van itself, and (iv) the divalent Van serves as a promising fluorescent probe to label and monitor bacterial strains in a highly effective manner.

Scheme 2 illustrates the synthetic pathway for preparing the divalent Van derivative. Typically, the commercially available Van (**1**) reacted with porphyrin derivative (**2**), to afford Van carboxamide (**3b**) by employing HBTU as the coupling reagent. The divalent conjugate was purified in 53.6% yield



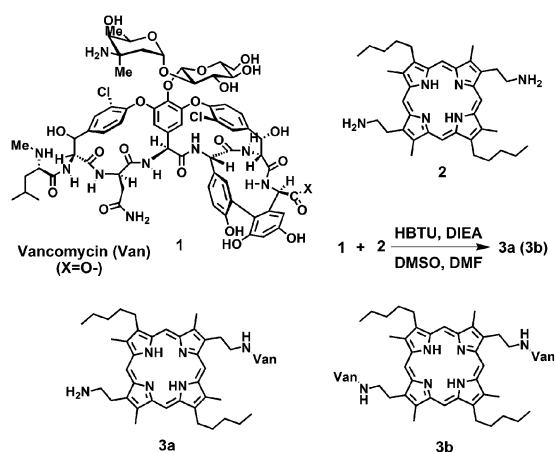
Scheme 1 Interaction of divalent vancomycin and Gram-positive bacterial cell wall.

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Scheme 2 The structures and synthetic scheme of Van-porphyrin derivatives.

by reversed-phase HPLC and characterized by ^1H NMR spectroscopy and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Similarly, the monovalent Van adduct (**3a**) with porphyrin was also prepared in 52.9% yield by using excess amount of porphyrin (see ESI †). After obtaining the Van-porphyrin derivatives (**3a–b**), their photochemical properties were investigated. The UV-Visible and fluorescence spectroscopy results indicated that the monovalent and divalent Van derivatives exhibited absorption bands of both Van (~ 280 nm) and porphyrin moieties (B band around 400 nm, Q bands between 500 and 620 nm). The emission spectra of the Van-porphyrin precursors showed no difference from those of porphyrin molecule (Fig. S1, ESI †), suggesting Van conjugations have no effect on the fluorescent property of porphyrin. Moreover, all the precursors could produce singlet oxygen upon white light illumination (Scheme 1 and Fig. S2, ESI †).

The *in vitro* antibacterial activities of Van-porphyrins were first investigated by standard broth microdilution assays.^{2–6} In a typical study, three bacterial strains: Van-sensitive strain, *Bacillus subtilis* (ATCC 33677) and two Van resistant enterococci (VRE) including *Enterococcus faecium* (VanA genotype, ATCC 51559) and *Enterococcus faecalis* (VanB genotype, ATCC 51299) were chosen as model organisms. Both monovalent (**3a**) and divalent (**3b**) Van derivatives showed effective MIC activity against Van sensitive *B. subtilis* which was similar to the parent Van molecule (Table S1, ESI †). However, **3a** and **3b** demonstrated distinct decrease in their antimicrobial activities against VRE (Table S1, ESI †). The exact nature of the mechanism regarding the low activity of **3b** is unclear at this moment. Although previous studies indicated the enhanced affinities of divalent Vans for the bacterial cell wall precursors,^{2–6} the binding affinity between Van derivatives and cell wall precursors may not correlate well with the potent MIC activity against VRE.⁷

The binding affinity of Van-porphyrins towards various bacteria was further identified by fluorescent imaging technique. Typically, the bacterial strains were incubated with Van-porphyrin derivatives at 37 °C for 1 hour in a culture media. The bacterial imaging was conducted upon the excitation of the Q bands of porphyrin under fluorescent microscope.¹⁷

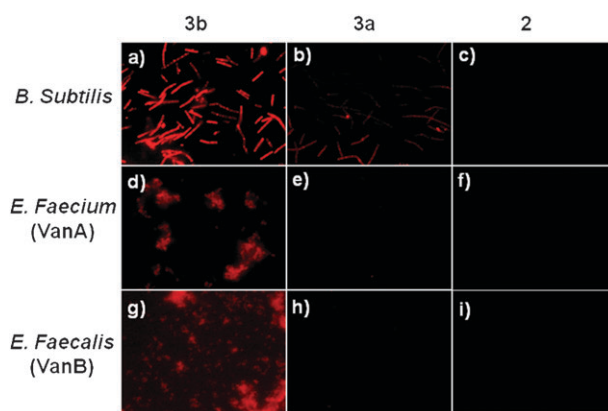


Fig. 1 Fluorescent imaging of bacterial staining with Van-porphyrin derivatives. (a)–(c), *B. subtilis* loaded with 2 μM of **3b**, **3a**, and **2**, respectively; (d)–(f), *E. faecium* (VanA) with 10 μM of **3b**, **3a**, and **2**; (g)–(i), *E. faecalis* (VanB) same as VanA. Ex = 535/50 nm; Em = 610/75 nm.

As shown in Fig. 1c, incubation of porphyrin (**2**) itself with *B. subtilis* would not lead to obvious fluorescence. However, upon the specific targeting of Van affinity ligand, both **3a** and **3b** (2 μM) revealed obvious fluorescent signals in *B. subtilis* (Fig. 1a and b). Compared to **3a**, **3b** exhibited stronger fluorescence, suggesting the higher binding association of **3b** to the surface of *B. subtilis*. Similar bacterial imaging was also carried out by incubating VRE with 2 μM of **3a** and **3b**, separately. There was no significant fluorescence observed in these strains (Fig. S3, ESI †) and the effective fluorescent imaging could only be detected when a higher concentration of **3b** (10 μM) was used (Fig. 1d and g), indicating the lower binding affinity of Van-porphyrins to the bacterial cell walls of VRE as compared to the Van-sensitive bacterial strain. However, when compared to **3a**, the multivalent/polyvalent interactions found in the divalent Van-porphyrin (**3b**) significantly improved the association between **3b** and the drug resistance bacteria. In addition, Fig. 1 shows that incubation of **3b** with VanA type VRE (Fig. 1d) displayed a lower fluorescent signal as compared to **3b** incubated with VanB (Fig. 1g). This suggested a higher affinity between the divalent derivative and VanB strain. There was no obvious fluorescent signal observed in control *E. coli* imaging experiment indicating the lowest binding affinity between Van derivatives and Gram-negative strain (data not shown).

In order to further explore the photodynamic inactivation of VRE by Van-porphyrins, the PACT treatment was performed in the dark and upon white light exposure by a traditional surface plating approach.^{11,17} In this study, photosensitizers including **2**, **3a** and **3b** were incubated with VanA and VanB, separately. Upon white light irradiation, the bacteria lethality was evaluated by counting the number of colony forming units (cfu) on the LB agar plate. Fig. 2 displayed the bacterial lethality of VRE under different photosensitizer concentrations. It was found that increasing concentrations of photosensitizers enhanced the bacterial killing efficiency for both VanA and VanB. Among the three photosensitizers used, **3b** showed the highest antibacterial activity against VRE throughout the whole concentration range. About 95% bacterial lethality could be observed in **3b** (2 μM) incubated VanB upon irradiation with 60 J cm^{-2} of

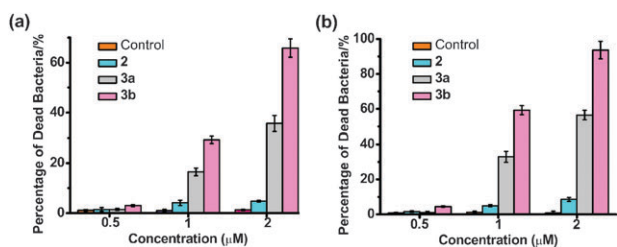


Fig. 2 Photodynamic inactivation of bacterial strains towards different concentrations of compounds **2**, **3a** and **3b**. (a): *E. faecium* (VanA); (b): *E. faecalis* (VanB). The white-light dose was 60 J cm^{-2} (exposure for 2 min at a fluence rate of 500 mW cm^{-2}). Bacteria treated with compound **3b** but no light illumination as control groups.

white light, whereas, a smaller killing efficiency ($\sim 66\%$) was detected for VanA suspension when exposed to the same dose of light. The photodynamic inactivation of both VanA and VanB were further investigated in the presence of different doses of white light (Fig. S4, ESI[†]) while maintaining a fixed concentration ($2 \mu\text{M}$) of photosensitizers. Light irradiation of both VRE strains but no photosensitizers incubation would not induce obvious bacterial damage which was used as the control. There was no significant bacterial lethality detected for **2** incubated VanA and VanB strains upon light exposure. On the other hand, **3a** and **3b** revealed the effective photodynamic inactivation of VanA and VanB upon exposing the bacteria to different doses of light and more significant bacterial reduction (e.g. $>99\%$) could be achieved when higher doses of irradiation was applied (Fig. S4, ESI[†]). This clearly showed that Van acted as an efficient affinity ligand and aided in targeting the porphyrin moiety to the VRE surfaces which resulted in an effective drug resistant bacterial lethality upon PACT treatment. Compared to **3a**, **3b** displayed a substantially enhanced potency against VRE. The significant bacterial lethality achieved for VanA ($\sim 66\%$) and VanB ($\sim 95\%$) when $2 \mu\text{M}$ of **3b** was incubated with VRE strains and irradiated with 60 J cm^{-2} of white light was more potent than the MIC values of Van itself on VanA (~ 44 times) and VanB (~ 22 times) separately (Table S1, ESI[†]). Moreover, the photodynamic inactivation was also carried out by incubating *B. subtilis* and *E. coli* with different concentrations of **2**, **3a** and **3b**. Similarly, **3b** displayed the highest potency against *B. subtilis* among the three photosensitizers. More than 95% bacterial lethality was observed when $0.5 \mu\text{M}$ **3b** incubated bacteria was exposed to 60 J cm^{-2} of white light, which was more effective (~ 4 times) than the value of **3b** in MIC measurements (Table S1, Fig. S5, ESI[†]). There was almost no lethality observed in *E. coli* for **2**, **3a** and **3b** (Fig. S6, ESI[†]). These results unequivocally demonstrated that the porphyrin conjugated divalent Van could serve as an effective photoactive antibacterial reagent against Van-sensitive and VRE strains due to the stronger association between **3b** and the bacteria as a result of efficient multivalent/polyvalent interactions. This is consistent with the results observed in the bacterial imaging measurements.

In summary, this work presents a simple and novel photo-therapeutic reagent by conjugating the photosensitizer, porphyrin with two Van moieties. This divalent Van–porphyrin exhibits a relatively higher binding affinity to bacterial surface and retains potent PACT activities against vancomycin-sensitive

and VRE bacteria when compared to Van and porphyrin alone. Apart from the enhanced photodynamic antimicrobial activity, the red fluorescent emission of Van–porphyrin conjugate can be used to carry out noninvasive imaging study in living bacterial strains. So far, photodynamic therapy based on some porphyrin photosensitizers has obtained clinical approval in many countries for treating various types of diseases.⁹ We expect that this multifunctional divalent vancomycin provides the possibilities for the photodynamic inactivation of antibiotic-resistant bacteria. It may also act as a useful fluorescent probe to image bacteria or other cells in an effective manner.

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