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Photoinactivation of vancomycin-resistant *Enterococci* and *Bacillus subtilis* by a novel norvancomycin–rhodamine B conjugate

Cite this: *New J. Chem.*, 2013, **37**, 575

Received (in Montpellier, France) 9th October 2012,
Accepted 17th December 2012

DOI: 10.1039/c2nj40906b

www.rsc.org/njc

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In order to accumulate photosensitizers on the cell walls of vancomycin-sensitive and vancomycin-resistant bacterial strains for labeling or/and photoinactivation of bacteria upon light irradiation, a simple and novel norvancomycin–rhodamine B (Van–Rh) conjugate was synthesized, characterized and confirmed using MALDI-TOF mass spectrometry. The properties of Van–Rh in photodynamic inactivation and fluorescent imaging of vancomycin-sensitive and vancomycin-resistant *Enterococci* (VRE) strains were investigated. The photodynamic assay indicated that Van–Rh effectively inactivated *Bacillus subtilis* (ATCC 6633), clinical isolates of VRE and *Enterococcus faecalis* (ATCC 51299, Van B genotype) with inactivation rates of 71, 54 and 47% at 9 μM upon 3 min of light exposure, respectively. Van–Rh enabled the fluorescent imaging of *B. subtilis* at 5 μM and two VRE strains at 20 μM, but not *E. coli*. The phototoxicity and binding affinity of rhodamine B were enhanced by conjugation with norvancomycin as an affinity ligand.

1. Introduction

Vancomycin, called the "antibiotic of last resort", is often used for the treatment of methicillin-resistant Gram-positive infections and in patients who are allergic to penicillins.[1,2] However, during the last two decades, as vancomycin has become a frontline drug for the treatment of clinical infections, there has been a pronounced increase in the occurrence of vancomycin-resistant microbial pathogens such as vancomycin-resistant *Enterococci* (VRE), which is typically due to the alteration of a peptidoglycan sequence from D-Ala-D-Ala to D-Ala-D-Lac, resulting in an overall 1000-fold reduction in affinity for vancomycin.[3,4] To date, serious infections with VRE strains have very limited effective antimicrobial therapy, and alternative treatment approaches against VRE infections are still highly desirable.[5]

One promising therapy for antibiotic resistant bacterial infections is based on photodynamic antimicrobial chemotherapy (PACT), which relies on wavelength-specific light activation of photosensitizers (PSs) to generate active oxygen species.[6] These active oxygen species are capable of destroying the cell walls and membranes, thus resulting in cell death.[7,8] The photodynamic approach to killing bacteria is clearly a rapidly emerging alternative to current antimicrobial regimens and it is unlikely that bacteria could develop resistance to the photodynamic action of active oxygen species.[9] Nevertheless, there are potential problems associated with PACT, one of which is a lack of specificity. The PSs may bind to, or be taken up by other cells or host cells. Since subsequent irradiation of such cells could lead to their destruction, it is desirable to lead the PSs to the target cells.[10]

The rhodamine dyes are widely used as fluorescent probes and molecular markers in chemistry and biology because of their excellent photophysical properties. A previous study demonstrated that increasing the membrane binding or cell uptake could enhance the phototoxicity of rhodamines.[11] Vancomycin, a cup-shaped glycopeptide antibiotic, specifically binds to the D-Ala-D-Ala end of the peptidoglycan pentapeptide of Gram-positive bacterial strains *via* five hydrogen bonds.[12,13] This makes it possible to employ the vancomycin as an affinity ligand to accumulate rhodamines on cell walls of bacterial strains, consequently, enhancing the phototoxicity. Based on this idea, in this work, a novel norvancomycin–rhodamine B (Van–Rh) was constructed, and its properties in fluorescent imaging and photodynamic inactivation of vancomycin-sensitive and VRE bacterial strains were investigated.

2. Results and discussion

2.1 Synthesis of Van–Rh

The synthetic pathway of Van–Rh is shown in Scheme 1. Rhodamine B reacted with *N*-hydroxysuccinimide in the

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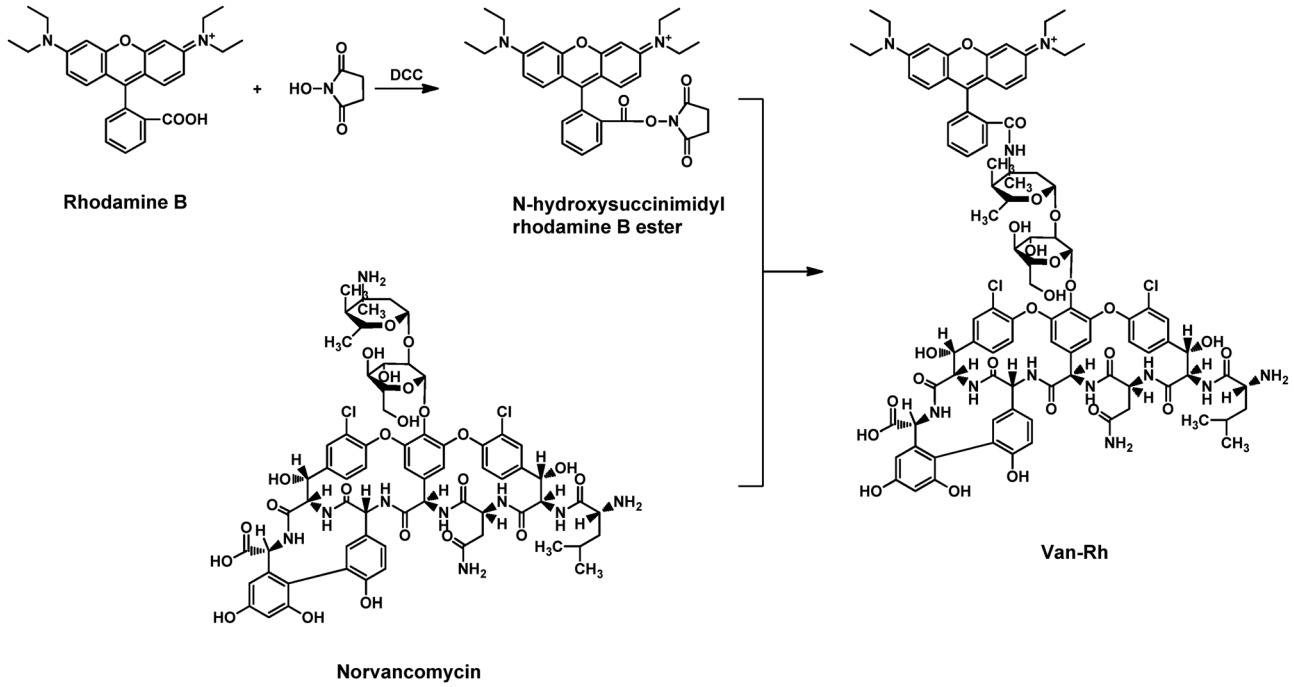

Scheme 1 Conjugation of rhodamine B and norvancomycin.

presence of catalyst DCC to provide *N*-hydroxysuccinimidyl rhodamine B ester (RB-S) with a yield of 34%. Norvancomycin hydrochloride reacted with RB-S in an alkaline medium for 18 h to completely hydrolyze the ester groups. The resulting crude product was purified using silica gel followed by Sephadex G-15 filtration to obtain Van–Rh as a deep purple solid with a total yield of 19%. The identity of Van–Rh was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The peak at *m*/*z* 1862.19 M^+ corresponding to Van–Rh was clearly observed as shown in Fig. 1.

Recently, several affinity ligands based on antibodies,[10] protein cages,[14] peptides,[15] nanoparticles,[16] and bacteriophages[17] have been reported to successfully direct the PSs to antibiotic-resistant bacterial strains. However, these molecules are relatively difficult to be synthesized and may suffer from self-aggregation, possible immunogenicity or complication in labeling. Vancomycin is an excellent affinity ligand because it (1) specifically binds to the D-Ala-D-Ala end of the peptidoglycan pentapeptide of Gram-positive bacterial strains; (2) selectively leads PSs onto the cell walls of Gram-positive bacteria, but not to other cells or host cells; (3) easily conjugates with PSs by its amino or carboxyl groups. In this work, synthesis of the conjugate norvancomycin–rhodamine B was achieved in aqueous solution using RB-S as the intermediate, and the product Van–Rh was easily purified by Sephadex G-15 filtration.

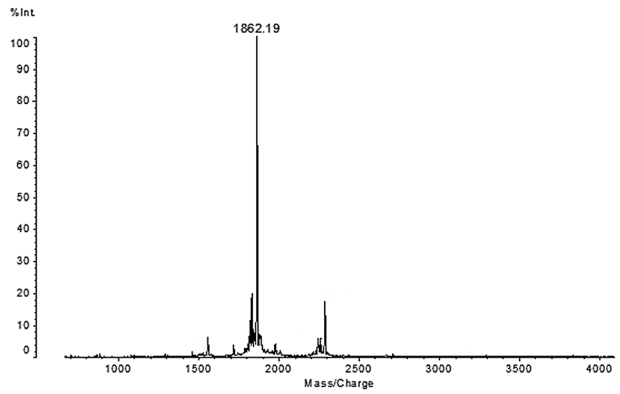

Fig. 1 MALDI-TOF mass spectrum of Van–Rh.

2.2 Spectroscopic characterization

Van–Rh was characterized by UV-Vis and fluorescence spectra. The UV-Vis spectroscopy showed that the maximum absorption wavelength of norvancomycin hydrochloride and rhodamine B in aqueous solution was 200 and 553 nm, respectively. Van–Rh exhibited absorption bands of both norvancomycin and rhodamine B, but the absorbance decreased by about 50% in comparison with that of rhodamine B (Fig. 2a). As shown in Fig. 2b, the λ_{em} for rhodamine B was 579 nm. Van–Rh exhibited native fluorescence characteristic of rhodamine B with a maximum absorption at 575 nm. Also, a nearly half decrease in fluorescence intensity was observed. Van–Rh was treated with 2 N trifluoroacetic acid at 45 °C for 8 h to cleave its glycosidic linkage. The cleaved products were characterized by MALDI-TOF-MS and peaks at *m*/*z* 749.69 $[M + H]^+$ and 1130.97 $[M + H]^+$ were found, indicating that rhodamine B was conjugated with the amino of a disaccharide group.

2.3 MIC determination

The antibacterial activity of Van–Rh was investigated *in vitro* by determination of MIC values according to the Clinical and Laboratory Standards Institute (CLSI) macrodilution (tube)

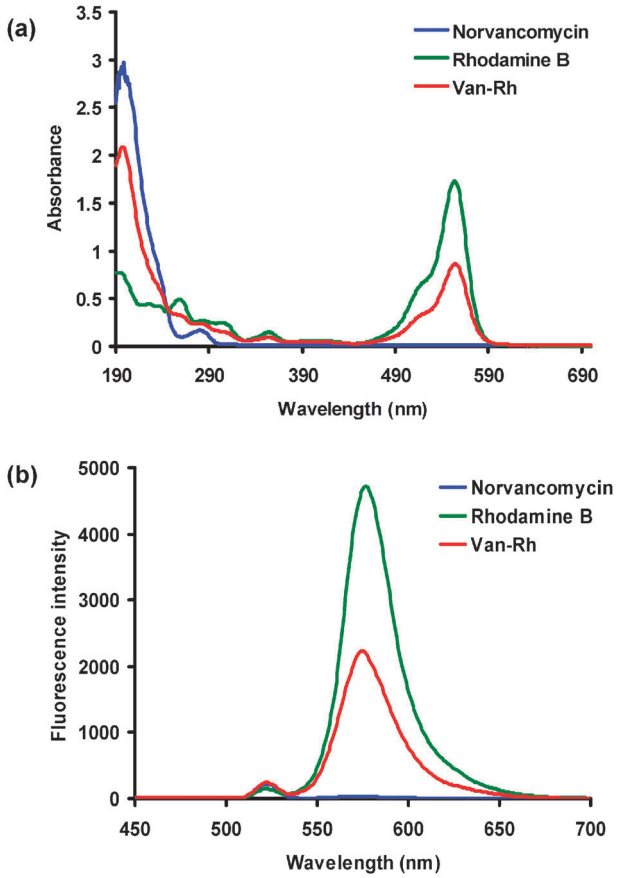

Fig. 2 Absorption (a) and fluorescence (b) spectra of norvancomycin, rhodamine B and Van–Rh in 10 mM PBS (pH 7.5) at room temperature (λ_{ex} = 556 nm). The concentration of each compound was 10 μM.

method.[18] The collected MIC data are listed in Table 1. The results indicated that rhodamine B had no antibacterial activity for all tested bacterial strains (>128 mg L^{-1}), and norvancomycin exhibited effective activity against *B. subtilis* (2 mg L^{-1}). However, the combination of the rhodamine B moiety led to norvancomycin almost losing its antimicrobial activity. The MIC value of Van–Rh towards *B. subtilis* was 64 mg L^{-1}, which increased 32-fold as compared to that of norvancomycin (2 mg L^{-1}). Van–Rh showed no effective activity against VRE strains (>128 mg L^{-1}).

The reason for the low antimicrobial activity of Van–Rh is unclear at this moment, while similar results were obtained in previous studies.[19,20] Xing *et al.* reported that the MIC values of monovalent and divalent vancomycin–porphyrin conjugates towards *B. subtilis* were 6 and 7 mg L^{-1}, respectively, this higher antibacterial activity may due to the toxicity of porphyrin, which has a MIC value of 1.5 mg L^{-1}.[19]

Table 1 The antibacterial activities (MIC) of rhodamine B, norvancomycin and Van–Rh[a]

	MIC (mg L^{-1})		
Compounds	*B. subtilis* (ATCC 6633 vancomycin-sensitive)	*E. faecalis* (ATCC 51299, Van B genotype)	*E. faecalis* (clinical isolates of VRE)
Norvancomycin	2	128	128
Rhodamine B	>128	>128	>128
Van–Rh	64	>128	>128

[a] All these experiments were conducted in the dark.

2.4 Fluorescent imaging tests

The binding affinity of Van–Rh towards various bacterial strains was investigated by a fluorescent imaging test according to a previously reported method.[19] The results showed that rhodamine B could not stain *B. subtilis* (Fig. 3a) or VRE strains (data not shown). In contrast *B. subtilis* treated with Van–Rh showed obvious fluorescent signals (Fig. 3b), suggesting the higher binding association of Van–Rh to the surface of the Gram-positive strain. In the case of VRE strains, incubation with a low concentration of Van–Rh (5 μM) did not lead to obvious fluorescence (data not shown). When a higher concentration (20 μM) of Van–Rh was used, several spots of VRE strains with fluorescence were observed (Fig. 3c and d), revealing the lower binding affinity of Van–Rh to the vancomycin-resistant bacterial strains. In addition, Fig. 3d shows stronger fluorescent signals compared to Fig. 3c, suggesting the lower affinity of Van–Rh to the Van B strain. Also, we tried to label the Gram-negative strain *E. coli* (ATCC 25922), but no obvious fluorescent signal was observed.

The rhodamine dyes show specific fluorescence staining of mitochondria and other cell organelles in eukaryotic cells,[21,22] but could not stain bacteria (Fig. 3a). Upon the specific targeting of the norvancomycin affinity ligand, Van–Rh enabled obvious fluorescent imaging of *B. subtilis*, revealing that

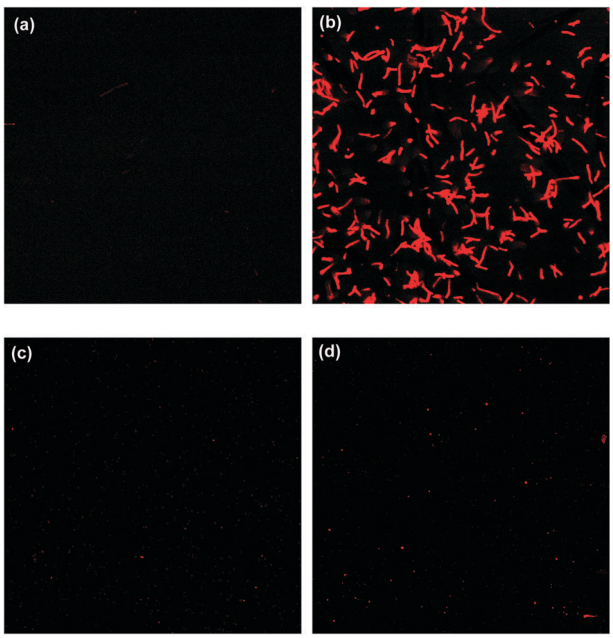

Fig. 3 Fluorescent imaging of bacterial strains with rhodamine B and Van–Rh. (a) *B. subtilis* (ATCC 6633) with 5 μM of rhodamine B; (b) *B. subtilis* (ATCC 6633) with 5 μM of Van–Rh; (c) *E. faecalis* (ATCC 51299, Van B) with 20 μM of Van–Rh; (d) clinical isolates of VRE with 20 μM of Van–Rh.

norvancomycin acted as an efficient affinity ligand, and led the rhodamine B moiety onto the cell walls of Gram-positive strains. In the case of VRE strains, several spots of VRE strains with fluorescent signals were observed only at a higher concentration of Van–Rh (Fig. 3c and d), which may be due to the low affinity of Van–Rh to the D-Ala-D-Lac end of the peptidoglycan pentapeptide of VRE strains. A Gram-negative strain of *E. coli* was tested for labeling, and no obvious fluorescent signal was observed, indicating the selectivity of Van–Rh to Gram-positive bacteria. The peptidoglycan layers surrounding Gram-positive bacterial cells play an important role in determining the cell shape. Compared with the reported vancomycin–porphyrin conjugate,[19] Van–Rh exhibited stronger fluorescent signal in the case of *B. subtilis* because of the high quantum yield of rhodamine B and lower antibacterial activity, suggesting that Van–Rh can be used for noninvasive imaging studies of living bacterial strains because it yields clear staining patterns at concentrations below its MIC value.

2.5 Photodynamic inactivation evaluation

The photodynamic inactivation by Van–Rh of various bacterial strains was evaluated using a previously published method.[23] As shown in Fig. 4, the photo-treated Van–Rh effectively inactivated *B. subtilis*, clinical isolates of VRE and *E. faecalis* with inactivation rates of 71, 54 and 47% at 9 μM upon 3 min of light exposure, respectively. With the increase of Van–Rh concentration, the bacterial lethality increased (Fig. 4a, c and e). As positive controls, the bacteria treated with different concentrations of Van–Rh but without light irradiation did not show obvious inactivation. Also, the bacterial lethality was dependent on the irradiation time; with the increase in irradiation time, the bacterial lethality increased (Fig. 4b, d and f). As positive controls, the strains treated with light irradiation but without any compound did not display obvious lethality. Among the tested strains, Van–Rh displayed the highest photoinactivation effect against *B. subtilis* (Fig. 4e), which may be due to the higher binding affinity of Van–Rh to the cell walls of the vancomycin-sensitive strain. No bacterial lethality was observed for norvancomycin or rhodamine B incubated with these strains upon light irradiation, demonstrating that norvancomycin and rhodamine B did not have a PDT effect. In order to investigate the lethality of Van–Rh against Gram-negative strains and eukaryotic cells, *E. coli* (ATCC 25922) and a human fetal lung fibroblast cell line (MRC-5 Line) were treated with 9 μM Van–Rh and 5 min of light exposure. Almost no lethality was observed in *E. coli* and the MRC-5 cell line.

The rhodamine dyes are weak PSs because their singlet excited state does not undergo intersystem crossing to the triplet excited state efficiently.[24] To overcome this limitation, (1) the chemical structures of rhodamine dyes can be modified by substituting hydrogen atoms with heavy atoms, such as Br or other halides;[25,26] and (2) the membrane binding and cell uptake can be increased by modulating the amphiphilicity.[27] Gaboury and co-workers synthesized rhodamine B *n*-butyl-ester (RBBE) to modulate the hydrophobicity, which was shown to be the most important factor influencing the cell uptake *in vitro*.[11] RBBE exhibited higher tumor cell killing efficiency compared to rhodamine B. Compared to rhodamine B, Van–Rh displayed effective photoinactivation against the vancomycin-sensitive and VRE bacterial strains, indicating that the phototoxicity of rhodamine B was enhanced by employing norvancomycin as the affinity ligand to accumulate it on the cell walls of Gram-positive bacterial strains. Although the inactivation rates were not particularly satisfactory, rhodamine analogs, such as rose bengal and erythrosine, which have been proved to be better PSs, will be used in our subsequent research.

3. Conclusions

In summary, based on the specificity of vancomycin binding to the D-Ala-D-Ala end of the peptidoglycan pentapeptide of Gram-positive bacterial strains, a novel and simple conjugate Van–Rh was synthesized and characterized. This Van–Rh effectively inactivated *B. subtilis*, VRE and *E. faecalis* upon white light exposure and enabled the fluorescent imaging of the strains. Norvancomycin acted as an excellent affinity ligand to direct rhodamine B to the cell walls of Gram-positive bacterial strains, thus enhancing its phototoxicity and binding affinity. The combination of the rhodamine B moiety led to norvancomycin almost losing its antimicrobial activity. The information gained in this work is useful to guide construction of a wide variety of fluorescent conjugates for photoinactivation of the antibiotic resistant bacteria.

4. Experimental

4.1 Bacterial strains

Bacillus subtilis (ATCC 6633, vancomycin sensitive strain), *Enterococcus faecalis* (ATCC 51299, genotype Van B) and clinical isolates of VRE were from Clinical Laboratory, Xijing Hospital, The Fourth Military Medical University, China. Single colonies of the strains on Luria–Bertani (LB) agar plates were transferred to 5 mL of LB medium and grown overnight at 37 °C. The bacterial strains were collected by centrifuging (4000 rpm for 10 min) followed by washing three times with 0.01 M sterile phosphate-buffered saline (PBS) buffer, pH 7.5. The supernatant was discarded and the remaining cells were re-suspended and diluted to an OD_{600} of 0.5 with the PBS buffer.

4.2 Synthesis of *N*-hydroxysuccinimidyl rhodamine B ester (RB-S)

RB-S was synthesized according to a previously reported method.[28] 1.2 g rhodamine B and 0.3 g *N*-hydroxysuccinimide were dissolved in 50 mL acetonitrile and heated to 45 °C. With agitation, a solution of 0.6 g dicyclohexylcarbodiimide (DCC) in 25 mL acetonitrile was added slowly with stirring under dry conditions. After continued stirring for 20 h at 45 °C and then stirring for 1 h at room temperature, the resulting white precipitate was removed by filtration, the filtrate was distilled and the crude product was re-crystallized with absolute ethanol to give RB-S as dark green crystals.

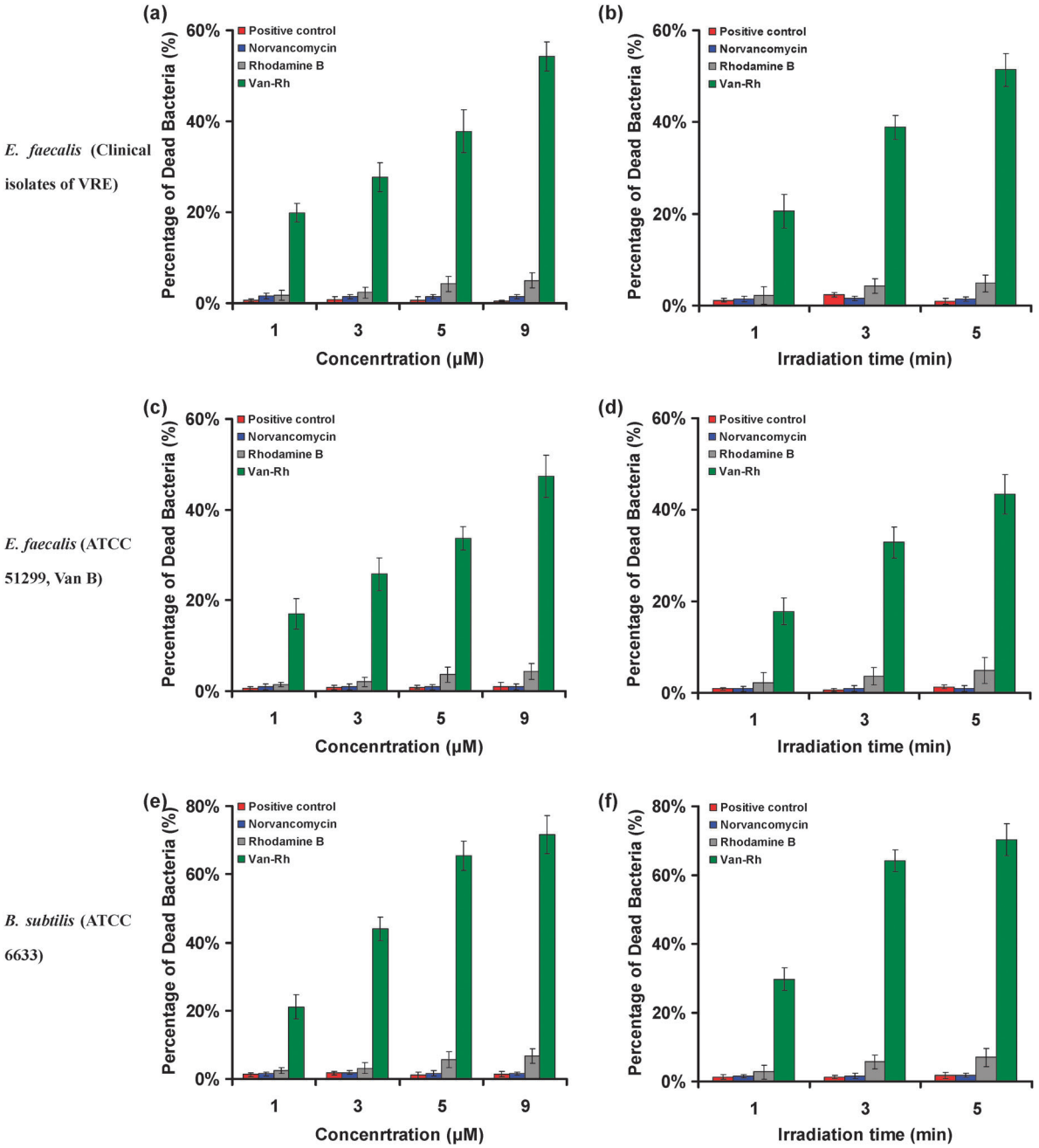

Fig. 4 Photodynamic inactivation of bacterial strains at different concentrations of compounds and irradiation time. (a), (c) and (e): the irradiation time was 3 min. Bacteria treated with Van–Rh but no light irradiation as positive controls of the three groups. (b), (d) and (f): the concentrations of the compounds were 5 μM. Bacteria treated with light irradiation but without any compound as positive controls of the three groups. The treatment of Van–Rh is statistically significantly different from the others ($P < 0.05$).

The purified RB-S was characterized by ^{1}H NMR, (400 MHz, $CDCl_3$) δ: 8.43 (d, J = 7.9 Hz, 1H), 7.99 (t, J = 7.5 Hz, 1H), 7.84 (t, J = 7.7 Hz, 1H), 7.48 (d, J = 7.5 Hz, 1H), 7.08 (d, J = 9.5 Hz, 2H), 6.87 (d, J = 8.4 Hz, 4H), 3.65 (dd, J = 13.9, 6.9 Hz, 8H), 2.78 (s, 4H), 1.34 (t, J = 6.9 Hz, 12H).

4.3 Synthesis of norvancomycin–rhodamine B

Norvancomycin hydrochloride (74 mg) was dissolved in 5 mL 0.1 M PBS buffer, pH 8.3, and RB-S (29 mg, 1.0 equiv.) dissolved in 200 μL of dry *N*,*N*-dimethylformamide (DMF) was added dropwise. The mixture was adjusted to pH 9.5 with 2 N NaOH and stirred for 18 h at 4 °C. The reaction mixture was concentrated by freeze-drying and loaded onto a short silica gel column. Based on the information from TLC, the column was first eluted with methanol to remove the starting materials, and the product was eluted with methanol–water (v/v 1 : 1). After removing the solvent by reduced-pressure distillation and freeze-drying, the resulting solid was added to 2 mL DMF, and the residual silica gel was removed by filtration. The filtrate was

loaded onto a Sephadex G-15 column (10 × 700 mm) and eluted with water. Subsequently, the solvent was removed by freeze-drying to offer Van–Rh as a deep purple solid.

4.4 Spectroscopic characterization

2 mM stock solution of norvancomycin hydrochloride, rhodamine B and Van–Rh in 0.01 M PBS buffer (pH 7.5) was diluted to 10 μM with the same buffer, respectively. 1 mL solution of each compound was added into a 1 cm path quartz cell and the UV-Vis spectra were recorded on an Agilent 8453 UV-Vis spectrometer at room temperature. Fluorescence spectroscopic studies were performed on a Hitachi F-4500 Fluorescence Spectrophotometer at room temperature.

4.5 MIC determination and imaging tests

The MIC values of the tested samples were determined by the Clinical and Laboratory Standards Institute (CLSI) macrodilution (tube) broth method.[18] Imaging tests were carried out as previously described.[19] Briefly, the bacterial strains were washed with PBS buffer, incubated with 5 μM or 20 μM of rhodamine B or Van–Rh for 1 h at 37 °C with shaking in the dark, respectively. The bacterial cells were washed three times with PBS buffer, spotted on glass slides and immobilized using a coverslip. The cell imaging tests were performed on an Olympus FluoView FV1000 Confocal Microscope.

4.6 Photodynamic inactivation evaluation

Photodynamic inactivation of compounds against the bacterial strains was evaluated according to the methods previously described.[23] Briefly, the bacterial strains were washed with PBS buffer, incubated with the tested compounds in the dark at 37 °C for 15 min with shaking, and illuminated by light with a wavelength of 400–800 nm (350 mW cm^{-2}), which was produced by a Xenon lamp and isolated by means of the optical filters and a heat-dissipating water bath. The time of illumination was adjusted from 0 to 5 min, corresponding to the total light doses from 0 to 105 J cm^{-2}. After irradiation, bacterial suspensions were centrifuged (4000 rpm for 10 min) and the supernatant was removed directly. The bacterial pellet was re-suspended and serially diluted 10^4-fold with PBS buffer. A 20 μL portion of the diluted bacterial cells was spread on three LB agar plates and incubated for 16 h at 37 °C. The colonies formed were counted. The percentage of dead bacteria was quantitated by dividing the number of colony-forming units between the photo-treated samples incubated with the tested compounds and the negative controls without any compound and light exposure treatment. All the experiments were repeated three times.

Acknowledgements

We are grateful for assistance in the photodynamic studies from Dr Dan Sun at the Laboratory of Photonics and Photon Technique in Northwest University. This work was supported by the National Natural Science Foundation of China (20972127, 21272186), National Science Foundation of Shaanxi Province (2009JM2002) and Shaanxi Provincial Bureau of Foreign Experts Fund (SLZ2008013).

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