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In vitro antimicrobial activity of nanoconjugated vancomycin against drug resistant *Staphylococcus aureus*

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ABSTRACT

The mounting problem of antibiotic resistance of *Staphylococcus aureus* has prompted renewed efforts toward the discovery of novel antimicrobial agents. The present study was aimed to evaluate the *in vitro* antimicrobial activity of nanoconjugated vancomycin against vancomycin sensitive and resistant *S. aureus* strains. Folic acid tagged chitosan nanoparticles are used as Trojan horse to deliver vancomycin into bacterial cells. *In vitro* antimicrobial activity of nanoconjugated vancomycin against VSSA and VRSA strains was determined by minimum inhibitory concentration, minimum bactericidal concentration, tolerance and disc agar diffusion test. Cell viability and biofilm formation was assessed as indicators of pathogenicity. To establish the possible antimicrobial mechanism of nanoconjugated vancomycin, the cell wall thickness was studied by TEM study. The result of the present study reveals that nano-sized vehicles enhance the transport of vancomycin across epithelial surfaces, and exhibits its efficient drug-action which has been understood from studies of MIC, MBC, DAD of chitosan derivative nanoparticle loaded with vancomycin. Tolerance values distinctly showed that vancomycin loaded into nano-conjugate is very effective and has strong bactericidal effect on VRSA. These findings strongly enhanced our understanding of the molecular mechanism of nanoconjugated vancomycin and provide additional rationale for application of antimicrobial therapeutic approaches for treatment of staphylococcal pathogenesis.

1. Introduction

At present there is great concern about the emergence of multi-drug resistant bacterial strains, and continuous research efforts are being devoted for the development of new and effective antimicrobial agents. *Staphylococcus aureus* produces many toxins and many extracellular products that may act as virulence factors (Archer and Climo, 2001). More than 90% of *Staphylococcus* strains are resistant to penicillin, methicillin, aminoglycosides, macrolides and lincosamide (Chambers, 2001; Levin et al., 2005; Schmitz et al., 2002). Resistance of *S. aureus* strains to penicillin is mediated by penicillinase (a form of β-lactamase) production, an enzyme which breaks down the β-lactam ring of the penicillin molecule. In 1961 *S. aureus* developed resistance to methicillin, invalidating almost all antibiotics including the most potent β-lactams (Jevons, 1961). The first report of a Japanese patient harboring MRSA intermediately resistant to vancomycin appeared in 1996 (Hiramatsu et al., 1997). Fully vancomycin-resistant *S. aureus* was first appeared in the USA in 2002 (Chang et al., 2003). Treatment of vancomycin-resistant *S. aureus* is a serious problem in globe. Vancomycin acts against Gram-positive bacteria by inhibiting the steps in murien (peptidoglycan) bio-synthesis and assembly of NAM-NAG-polypeptide into the growing peptidoglycan (PG) chain. It inhibits this process by

Abbreviations: AFM, atomic force microscopy; BHI, brain heart infusion; CFU, colony formation unit; CMC, carboxymethyl chitosan; CMC-EDBE-FA, carboxymethyl chitosan-2,2′-ethylenedioxy bis ethylamine-folate; CS, chitosan; DAD, disc agar diffusion; D-Ala-D-Ala, D-alanine-D-alanine; D-Ala-D-Lac, D-alanine-D-lactate; DLS, dynamic light scattering; DMSO, dimethyl sulphoxide; EDBE, 2,2′-ethylenedioxy bis-ethylamine; EDC, 1-[3-dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride; FA, folic acid; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; FTIR, Fourier transform infrared spectroscopy; LB, luria broth; MBC, minimum bactericidal concentration; MHA, Mueller–Hinton agar; MHB, Mueller–Hinton broth; MIC, minimum inhibitory concentration; MRSA, methicillin resistant *Staphylococcus aureus*; MSSA, methicillin sensitive *Staphylococcus aureus*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NA, nutrient agar; Nacl, sodium chloride; NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; NCCLS, National Committee for Clinical Laboratory Standards; NHS, N-hydroxysuccinimide; NV, nanoconjugated vancomycin; PBP, penicillin binding protein; PBS, phosphate buffer saline; PG, peptidoglycan; PRSA, penicillin resistant *Staphylococcus aureus*; Rh123, rhodamine 123; *S. aureus*, *Staphylococcus aureus*; SD, standard deviation; S.E.M., standard error of mean; TEM, transmission electron microscopy; Van, vancomycin; VISA, vancomycin intermediate *Staphylococcus aureus*; VRE, vancomycin resistant enterococci; VRSA, vancomycin resistant *Staphylococcus aureus*; VSSA, vancomycin sensitive *Staphylococcus aureus*.

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reacting with D-Ala-D-Ala, which consequently blocks the release of terminal D-Ala and intra-chain bond formation (Saha et al., 2008a).

Chitosan is a well-known biopolymer that possesses antibacterial activity against Gram-positive and Gram-negative bacteria, which has been exploited in a number of studies (Li et al., 2008; Rabea et al., 2003). Chitosan is a mucoadhesive polymer that is able to open tight junctions and allow the paracellular transport of molecules across mucosal delivery of vaccines (Van der Lubben et al., 2001). Some hypotheses indicate that polycationic chitosan could interact with anionic groups on the cell surface thereby causing an increase in membrane permeability and probably its disruption and subsequent leakage of cellular proteins (Qi et al., 2004). Another mechanism suggested involves the formation of chitosan chelates with trace elements or essential nutrients resulting in the inhibition of the activity of enzymes (Rabea et al., 2003). A generally accepted idea is that the antimicrobial activity of chitosan strongly depends on several factors including its molecular weight, degree of deacetylation, pH, etc., and that high molecular weight chitosan exhibits higher toxicity against Gram-positive bacteria compared to Gram-negative ones (Eaton et al., 2008). Due to its excellent biocompatibility, biodegradability and nontoxicity (Kean and Thanou, 2010), chitosan has been successfully used in nanomedicine for delivering therapeutic drugs (De Campos et al., 2004), proteins (Garcia-Fuentes et al., 2007) and genes (Roy et al., 1999). Chitosan nanoparticles had also been employed as a gene carrier to enhance gene transfer efficiency in cells (Mao et al., 2001; Kim et al., 2004). Chitosan microspheres have been used for gastric drug delivery and controlled release of active antimicrobial agents, such as amoxycillin and metronidazole in the gastric cavity (Portero et al., 2002). The unique character of nanoparticles for their small size and quantum size effect could make chitosan nanoparticles exhibit superior activities. Solubility in neutral and basic solutions can be achieved by further modification to the structure of chitosan, such as in carboxymethyl chitosan (CMC). This chitosan modification is synthesized by carboxylation of the hydroxyl and amine groups (Liu et al., 2001). The degree of carboxymethylation, which is controlled by reaction temperature and duration, strongly affects the solubility of CMC (Chen and Park, 2003). CMC is reported to have a higher sorption of metal ions than chitosan (Varma et al., 2004). It has been proposed that the higher sorption capacity is due to increased chain flexibility and higher concentrations of chelating groups (Guibal, 2004; Ngah and Liang, 1999).

In our previous laboratory report, CMC-EDBE-FA nanoparticles was prepared based on CMC tagged with folic acid (FA) by covalently linkage through 2,2′-(ethylenedioxy) bis-(ethylamine) (EDBE). Physicochemical characteristics of this nanoparticles were examined by FTIR spectroscopy, DLS and TEM study; and it was also observed that the nanoparticles has no antimicrobial and toxic effect (Chakraborty et al., 2010, 2011a, 2012). Hence the present study was aimed to prepare nanoconjugated vancomycin by loading of vancomycin on CMC-EDBE-FA nanoparticles through physical adsorption and observes its antimicrobial activity against vancomycin sensitive and resistant *S. aureus* strains.

2. Materials and methods

2.1. Culture media and chemicals

Mueller–Hinton broth, nutrient agar, luria broth, tryptic soy broth, agar powder, vancomycin salt, vancomycin disc, crystal violet, alanine, lactate, lysostaphin, sucrose, RPMI 1640 were purchased from Himedia, India. Tris–HCl, Tris buffer, sodium chloride, sucrose, potassium dihydrogen phosphate (KH_2PO_4), di potassium hydrogen phosphate (K_2HPO_4), EDTA, sodium dodecyl sulphate, NaOH, uranyl acetate, potassium chloride, ethanol, cell culture grade DMSO, magnesium sulphate, magnesium chloride, ouabain were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. Rhodamine 123 (Rh123), folic acid (FA), chitosan, dicyclohexyl carbodiimide (DCC), EDBE, di-tert-butyldicarbonate (BoC_2O), N-hydroxysuccinimide (NHS) and 1-[3-dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (EDC), DNase, ATP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), α-toxin antibody, fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co., USA. All other the chemicals, reagents, were purchased from Himedia, India; SRL Pvt. Ltd., Mumbai, India and were of the highest grade available.

2.2. Bacterial strains

Eight vancomycin sensitive *S. aureus* (VSSA) [MMC-1, MMC-2, MMC-3, MMC-5, MMC-6, MMC-7, MMC-8, MMC-15] and vancomycin resistant *S. aureus* (VRSA) [MMC-4, MMC-9, MMC-12, MMC-16, MMC-17, MMC-18, MMC-19, MMC-20] strains were selected for this study. All these strains were isolated from human post operative pus sample in our laboratory (Chakraborty et al., 2011b).

2.3. Preparation of nanoconjugated vancomycin

2.3.1. Loading of vancomycin on nanoparticle

Vancomycin loading onto CMC-EDBE-FA nanoparticles was performed by the method of Cevher et al. (2006). Vancomycin loaded CMC-EDBE-FA nanoparticle (VNP1-VNP3) was prepared with polymer:drug ratios (w/w) of 1:1, 2:1 and 1:2 (Table 1). 10 mg, 20 mg and 10 mg CMC-EDBE-FA was dissolved in 1.0 ml PBS (pH 7.4) separately by sonication at 100 W for 1 min (10S working and 10S rest) in an ice water bath to which 10 mg, 10 mg and 20 mg vancomycin was dissolved respectively to obtain the nanoparticle: drug ratio of 1:1 (VNP1), 2:1 (VNP2) and 1:2 (VNP3). Each suspension was then emulsified by mechanical shaking at 200 ± 1 rpm for 36 h at 37 ± 0.1 °C (Orbitek shaker incubator) to prevent aggregation. 10 mg, 20 mg and 10 mg CMC-EDBE-FA nanoparticle without vancomycin was parallely checked as negative control.

2.3.2. Actual drug content and encapsulation efficiency

Actual drug content and encapsulation efficiency was measured by the method of Cevher et al. (2006). After 36 h of shaking, the mixtures were centrifuged at 3500 × *g* for 10 min to get vancomycin loaded CMC-EDBE-FA as pallet. Drug content was determined by analyzing the CMC-EDBE-FA solution and pallet (dissolve in 1.0 ml PBS pH 7.4) using Hitachi U2001 UV/vis spectrophotometer at a wavelength of 282 nm with PBS as reference. Drug content was determined by comparing with the standard curve of vancomycin which was achieved from vancomycin solution in PBS (pH 7.4) with concentration between 0.001 and 0.1 mg/ml. Actual drug content (AC) and encapsulation efficiency (EE) were calculated (Eqs. (1) and (2)). All analyses were carried out in triplicate. Results are expressed as the mean percentage (w/w) ± SD of three formulations:

$$\mathrm{AC}\,(\%) = \frac{M_{\mathrm{act}}}{M_{\mathrm{ms}}} \times 100 \tag{1}$$

$$\mathrm{EE}\ (\%) = \frac{M_{\mathrm{act}}}{M_{\mathrm{the}}} \times 100 \tag{2}$$

where M_{act} is the actual vancomycin content in weighed quantity of CMC-EDBE-FA, M_{ms} is the weighed quantity of powder of CMC-EDBE-FA and M_{the} is the theoretical amount of vancomycin in CMC-EDBE-FA calculated from the quantity added in the process.