In Vitro Antibacterial Activity of 4-Phenyl-1-(2-phenyl-allyl)pyridinium bromide: A Novel Class of Pyridinium Based Antibacterial Compounds

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Abstract The continuing increase in the incidence of multi drug resistant pathogenic bacteria and shortage of new antimicrobial agents are the prime driver in efforts to identify the novel antimicrobial classes. In vitro antibacterial activity of 4-phenyl-1-(2-phenylallyl) pyridinium bromide was tested against Gram positive *Staphylococcus aureus*, *Streptococcus* species, *Bacillus subtilis*, and Gram negative *Klebsiella aerogenes* and *Escherichia coli* using disk diffusion method. Among them *S. aureus* showed strong antibacterial activity (21.99 ± 0.03 mm) while *E. coli* showed very little activity (8.97 ± 0.06 mm) towards the compound. The MIC of 4-phenyl-1-(2-phenyl-allyl)-pyridinium bromide for 90% *S. aureus* was ≤ 20 $\mu\text{g/ml}$ and was compared with phenoxymethylpenicillin, cloxacillin, erythromycin and vancomycin. When 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide showed MIC at ≤ 20 $\mu\text{g/ml}$, all others showed MIC at ≤ 100 $\mu\text{g/ml}$. Strong antibacterial activity of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide against *S. aureus* indicates that there is a possibility to use it as an effective antibacterial agent.

Keywords MIC · Antibiotics · 4-Phenyl-1-(2-phenyl-allyl)pyridinium bromide · Zone of inhibition · *Staphylococcus aureus*

Introduction

A wide variety of approaches are being used in the search for novel antibacterial agents. Most often natural products

and synthetic chemicals are being used to screen for identifying compounds having antibacterial activity. As antibacterial agents are heavily used and misused, the bacterial strains rapidly become resistance to the antimicrobial agents [1, 2]. Therefore it is vital to design and synthesize new antibacterial agents to support for the battle against pathogenic bacteria. Pyridinium salts which are unsaturated heterocyclic compounds having different functional groups present either on pyridine ring or at nitrogen atom are being used in various applications in industrially and domestically for a longer period of time [3]. Some pyridinium salts are effective against number of microorganisms such as *Staphylococcus* sp., *Streptococcus* sp. and therefore used as antimicrobial agents. Antibacterial properties of pyridinium salts with various chain lengths and functional groups were previously reported and further they were cited that pyridinium compounds act on the cell wall and have a direct or indirect lethal effect on bacterial cell wall biosynthesis. [3–9].

4-Phenyl-1-(2-phenyl-allyl)pyridinium bromide (Fig. 1) has been identified as one of the most effective vesicular monoamine transporter inhibitors [10]. In the present study, we investigate the in vitro antibacterial activity of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide as a novel type of pyridinium derivative against Gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus* sp.) and Gram positive bacteria (*Klebsiella aerogenes* and *Escherichia coli*).

Materials and Methods

Synthesis of 4-Phenyl-1-(2-phenyl-allyl)pyridinium bromide

Synthesis was carried out according to previously published method by Wimalasena et al. [10]. Reagents and solvents

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Fig. 1 Structure of 4-phenyl-1-(2-phenylallyl)pyridinium bromide

used in the synthesis were obtained from various commercial sources with the highest purity available and did not subject to further purification. ^1H and ^{13}C NMR spectra of the final product were recorded on a Varian Oxford NMR YH 300 MHz spectrometer, and mass spectrum was obtained from Varian 310-MS TQ Mass Spectrometer (Varian, USA) with electron spray ionization (ESI). Melting point was uncorrected and determined using STUART scientific melting point apparatuses. Synthesized compound was subjected to antibacterial susceptibility testing after conformation of structure and purity using above data.

Bacterial Strains and Antibiotics

The bacterial strains selected for the present study were collected from Department of Plant Science, University of Colombo, Sri Lanka. Identification of each culture was done by conventional method. A total of five bacteria namely *S. aureus*, *B. subtilis* *Streptococcus* sp. (Gram positive) and *E. coli* and *K. aerogenes* (Gram negative) were screened for present investigation. Among them only *S. aureus* and *E. coli* showed significant inhibition and to further investigation we have carried out with ATCC strain of both *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922). The bacterial cultures were maintained in nutrient agar slants at 37°C . Each of the microorganisms was reactivated prior to susceptibility testing by transferring them into a separate test tube containing nutrient broth and incubated overnight at 37°C . Then they were suspended in saline solution 0.85% NaCl and adjusted to yield approximately 1.0×10^8 – 1.0×10^9 cfu/ml by using spectrophotometer (25% transmittance at 530 nm). All the media were purchased from Hi Media, Mumbai, India and chemicals used were of analytical grade. All antibiotics used in experiment were purchased from State Pharmaceutical Co. Ltd, Sri Lanka.

Antibacterial Assay

In vitro antibacterial activity of 4-phenyl-1-(2-phenylallyl)pyridinium bromide was determined by standard disk

diffusion method [11]. A portion (200 μl) of adjusted bacterial diluents were aseptically transferred on to the five separate sterilized Mueller–Hinton Agar (MHA) plates and uniformly spreaded over the solid surface using a glass spreader until the agar plates absorbed the broth culture to ensure the confluent growth of the organisms. Plates were kept for 30 min to allow broth cultures to absorb. Sterilized Whatman filter paper disks of 6 mm diameter were taken and a portion (20 μl) of 2,500 $\mu\text{g}/\text{ml}$ 4-phenyl-1-(2-phenylallyl)pyridinium bromide stock solution was aseptically introduced to each sterile filter paper disk. The filter paper disks were kept for few minutes in order to allow them to absorb the solution and were placed on the surface of the inoculated MHA plates. A filter paper disk impregnated with sterile distilled water (20 μl) was placed on the center of the agar plate as a negative control. Finally plates were incubated at 37°C for overnight. The experiment was performed in four replicates under strict aseptic conditions and the antibacterial activity of the compound was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by the compound at the end of incubation period.

Determination of Minimum Inhibitory Concentration (MIC)

Determination of MIC for 4-phenyl-1-(2-phenylallyl)pyridinium bromide was carried out against *S. aureus* using disk diffusion method. 20, 4, and 0.8 $\mu\text{g}/\text{ml}$ of test material was prepared by using double distilled water and 6 mm filter paper disk were prepared using each concentration as described above. After that disk diffusion experiment was carried out according to previously mentioned protocol in four replicates for each concentration level. Following incubation, the MIC value of 4-phenyl-1-(2-phenylallyl)pyridinium bromide was calculated according to free diffusion and dissipative diffusion models as described by Bonev et al. [12]. The MIC was defined as the lowest drug concentration that inhibited visible growth. The MIC of 4-phenyl-1-(2-phenylallyl)pyridinium bromide was compared with the antibiotics phenoxymethylpenicillin, cloxacillin, erythromycin, and vancomycin which are already being used as effective antibiotics against *S. aureus*.

Free Diffusion Model

According to the free diffusion model, value of MIC was determined using a plot where squared size of inhibition zone, x , plotted against the natural logarithm of the antibiotic concentration, c :

$$\ln(\text{MIC}) = \ln(c) - \frac{x^2}{4Dt}$$

Here, D is the diffusion coefficient, presumed to be independent of concentration, and t the time of antibiotic diffusion.

Dissipative Diffusion Model

According to the dissipative diffusion model, value of MIC was determined using a plot where size of inhibition zone, x , plotted against the natural logarithm of the antibiotic concentration, c :

$$\ln(\text{MIC}) = \ln(c) - \left(V \pm \sqrt{V^2 - 4D} \right) x$$

Here, D is the diffusion coefficient, presumed to be independent of concentration, and V is a coefficient characterizing the dissipation rate.

Statistical Analysis

The data of each parameter in experiments were performed in four replicates were statistically analyzed and expresses as mean $\pm$ standard deviation.

Results and Discussion

In vitro antibacterial activity analysis of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide showed that it was active against *S. aureus* (Gram positive) 21.99 ± 0.03 mm and *E. coli* (Gram negative) 8.97 ± 0.06 mm (Fig. 2). It was unable to inhibit the growth of *Streptococcus* sp., *Bacillus subtilis* (Gram positive bacteria) and *K. aerogenes* (Gram negative bacteria). According to the results *S. aureus* was more susceptible than *E. coli*. This is possibility due to the

Fig. 2 Zone of inhibition exhibited by 4-phenyl-1-(2-phenylallyl)pyridinium bromide (2,500 $\mu\text{g/ml}$) against *S. aureus*

differences in chemical composition and structure of cell wall of both types of microorganisms.

As *S. aureus* showed strong activity against the compound, it was selected to determine MIC of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide. MIC of the compound was compared with the available antibiotics standards which show susceptibility against *S. aureus*. Table 1 shows the antibacterial activity in terms of zone of inhibition (in mm diameter) for 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide and other standard antibiotic used.

For many infections, the results of sensitivity testing are important in the choice of antibiotic. These results are commonly reported as the MIC, which is defined as the lowest concentration of drug that inhibits the growth of the organism [13]. *S. aureus* is a highly pathogenic bacteria and responsible for number of diseases. It is interesting to notice that 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide possess significant inhibitory effects against medically important *S. aureus*. The MIC of 4-phenyl-1-(2-phenyl-allyl)-pyridinium bromide for *S. aureus* was found to be <20 $\mu\text{g/ml}$ and corresponding inhibition zone diameter was 11.46 ± 0.07 mm. Interpretation of results from disk diffusion method relies on model-dependent analysis [12]. Two models that have been previously described were used to interpret the results obtained from disk diffusion method in order to find the MIC of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide for *S. aureus*.

Free diffusion model is based on the assumption that antibiotics diffuse freely in the solid nutrient medium [12]. Figure 3 shows that the plot of squared size of inhibition zone x , against the natural logarithm of the 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide concentration, c . According to the graph MIC of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide for *S. aureus* was approximately 4.89 $\mu\text{g/ml}$ and R^2 value of the plot is 0.968. R^2 value of 0.968 shows the better linearity of the plot.

In many cases, assumption of free diffusion of antibiotics may be incorrect, which leads to significant deviations of the predicted behavior from the experiment and to inaccurate assessment of bacterial susceptibility to antibiotics [12]. Therefore this model describes the agar diffusion assay that takes into consideration loss of antibiotic during diffusion and provides higher accuracy of the MIC determined from the assay. Figure 4 shows the plot of size of inhibition zone x , against the natural logarithm of the 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide concentration, c . According to the graph MIC of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide for *S. aureus* was approximately 0.15 $\mu\text{g/ml}$. R^2 value for the plot was 0.989 and this indicates a better linearity than the free diffusion model. Therefore, diffusion of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide in the solid agar medium can be considered as a dissipative process and dissipative

Table 1 Diameter of effective zone of inhibition (mm) for 4-phenyl-1-(2-phenylallyl)pyridinium bromide and other antibacterial standards against *S. aureus*

Compound	Diameter of effective zone of inhibition (mm)					
	2,500 (μg/ml)	500 (μg/ml)	100 (μg/ml)	20 (μg/ml)	4 (μg/ml)	0.8 (μg/ml)
PPAPB ^a	21.98 ± 0.06	17.98 ± 0.04	13.98 ± 0.06	11.46 ± 0.07	–	–
Cloxacillin	26.97 ± 0.05	25.02 ± 0.05	10.02 ± 0.05	–	–	–
Erythromycin	22.02 ± 0.05	18.02 ± 0.05	13.95 ± 0.1	–	–	–
Vanomycin	18.02 ± 0.05	13.05 ± 0.06	08.02 ± 0.05	–	–	–
Penicillin	11.02 ± 0.05	08.95 ± 0.1	07.02 ± 0.05	–	–	–

Results are the means of diameter values ± standard deviation, – no activity

^a 4-Phenyl-1-(2-phenylallyl)pyridinium bromide

diffusion model is the best suit model for the determination of MIC of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide for *Staphylococcus aureus*.

Comparison test results obtained from disk diffusion method (Fig. 5) revealed that concentrations at 500 μg/ml, both penicillin and Vancomycin showed lower inhibitory effects than 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide. Both erythromycin and 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide showed similar level of inhibition. Interestingly among all the standard antibiotic tested, 4-phenyl-1-(2-

phenyl-allyl)pyridinium bromide was the compound that could show antibacterial effect at very low concentration 20 μg/ml (Table 1). It showed high efficacy towards medically important *S. aureus* and it is a one of the bacterial strain that rapidly growing resistant towards antibiotics which are use to cure *S. aureus* infections [11, 13]. More than 90% of *S. aureus* strains contain plasmids that encode beta lactamase, the enzyme that degrade many, but not all, penicillins. Some strains of *S. aureus* are resistant to the beta lactamase resistant penicillins, such as methicillin and

Fig. 3 Free diffusion model of 4-phenyl-1-(2-phenylallyl)pyridinium bromide against *S. aureus*; squared values of inhibition zone radii (mm) x^2 are plotted against the logarithm of concentration. Residuals are shown above the plot **Fig. 4** Dissipative diffusion model of 4-phenyl-1-(2-phenylallyl)pyridinium bromide against *S. aureus*; inhibition zone radii (mm) x are plotted against the logarithm of concentration. Residuals are shown above the plot

Fig. 5 Comparison of antibacterial activities of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide (PPAPB) with other four selected antibacterial compounds

nafcillin, by virtue of changes in the penicillin binding protein in their cell membrane. These strains are commonly known as methicillin resistant *S. aureus* (MRSA) or nafcillin resistant *S. aureus* (NRSA). Rare strains called Vancomycin in terminated *S. aureus* (VISA), with reduced sensitivity to Vancomycin, have emerged [11, 13, 14].

Penicillin, cloxacillin, erythromycin and vancomycin are some of the antibiotics which are used to treat *S. aureus* infections [13–15]. Therefore, it was important to compare the antibacterial activity of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide with above antibiotics.

According to the comparison results given in the Fig. 3 penicillin shows least efficacy towards *S. aureus* and also it was reported that penicillin not active against the beta lactamase producing bacteria such as *S. aureus* [11, 13–15]. Even though Vancomycin is used against methicillin resistance *S. aureus*, in this experiment 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide showed high efficacy than Vancomycin towards *S. aureus* strains (Fig. 5). Therefore, this compound can be considered as more effective antibacterial agent against *S. aureus* than Vancomycin. The results showed similar level of antibacterial activity for Erythromycin and 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide against *S. aureus*. Erythromycin is insoluble in water and 4-phenyl-1-

(2-phenyl-allyl)pyridinium bromide is freely soluble in water. Therefore, it is an advantage of 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide to use as an effective antibacterial agent. Taking together our results showed 4-phenyl-1-(2-phenyl-allyl)pyridinium bromide is a potential antibacterial compound against *S. aureus*.

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