ORIGINAL ARTICLE

Antimicrobial activity of a quinuclidine-based FtsZ inhibitor and its synergistic potential with β -lactam antibiotics

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Filamenting temperature-sensitive mutant Z (FtsZ) is an essential cell division protein that cooperates in the formation of the cytokinetic Z-ring in most bacteria and has thus been recognized as a promising antimicrobial drug target. We have recently used a structure-based virtual screening approach to identify pyrimidine-linked quinuclidines as a novel class of FtsZ inhibitors. In this study, we further investigated the antibacterial properties of one of the most potent compounds (quinuclidine 1) and its synergistic activity with β -lactam antibiotics. Susceptibility results showed that quinuclidine 1 was active against multiple antibiotic-resistant bacterial strains including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* with minimal inhibitory concentrations of $24 \mu\text{g ml}^{-1}$. When quinuclidine 1 was combined with β -lactam antibiotics, synergistic antimicrobial activities against antibiotic-resistant strains of *S. aureus* were found. Further *in vitro* studies suggest that prevention of FtsZ protofilament formation by quinuclidine 1 impairs the formation of Z-ring, and thus inhibits bacterial division. These findings open a new approach for development of quinuclidine-based FtsZ inhibitors into potent antimicrobial agents.

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INTRODUCTION

The treatment of bacterial infections has been a clinical challenge worldwide because of the limited number of potent antibacterial therapies against antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium*.¹ Therefore, new antimicrobial agents with innovative mechanisms of action against antibiotic-resistant bacteria are urgently needed.^{2,3} Combining new compounds with existing antibacterial drugs is an alternative approach to treat bacterial infections. Combination therapies exploring the synergistic effects of compounds with different modes of action have been widely used to treat infectious diseases.⁴ For example, β -lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam have been successfully combined with β -lactam antibiotics to treat Gram-negative drug-resistant bacterial infections.⁵

Filamenting temperature-sensitive mutant Z (FtsZ), a prokaryotic homolog of mammalian β -tubulin,⁶ is a self-activating guanosine triphosphatase (GTPase) that polymerizes into a highly dynamic cytoskeleton Z-ring at the site of septum formation.^{7,8} Given the essential role of FtsZ in the recruitment of downstream proteins for completion of bacterial cell division, FtsZ is an attractive target for the development of new antibacterial agents that has yet to be developed clinically.^{9–12} A number of FtsZ inhibitors have been reported to impair the bacterial cell division, such as the small-molecule

PC190723 that exhibits *in vivo* activity in a murine infection model system and restores the susceptibility of MRSA to β -lactams.^{9–17} A recent report indicated that genetic depletion of FtsZ can cause the delocalization of penicillin-binding protein 2, the target of β -lactam antibiotics, from the septum in *S. aureus*, resulting in the impairment of the assembly of bacterial cell wall.¹⁸

Our previous results from virtual screening combined with biological evaluations led to the identification of a new class of quinuclidine-based FtsZ inhibitors. One of the most potent compounds, (5-((((2R,4S,5R)-5-(6-(pentan-3-yl)-2-(pyridin-4-yl)pyrimidin-4-yl)quinuclidin-2-yl)methyl)amino)methyl)furan-2-yl)methanol (quinuclidine 1, Figure 1), inhibited the proliferation of bacterial strains *Escherichia coli* and *S. aureus* by inhibiting the GTPase activity of FtsZ.¹⁴ The purposes of this study were to investigate the underlying mechanisms of antibacterial activity of quinuclidine 1 and to explore its synergistic effect in combination with β -lactam antibiotics against antibiotic-resistant strains of *S. aureus*.

MATERIALS AND METHODS

Reagents and strains

Quinuclidine 1 was purchased from AnalytiCon Discovery GmbH (Potsdam, Germany). The stock solution was prepared in dimethyl sulfoxide (DMSO). The final percentage of DMSO in the assays was 1% (v/v) for all experiments. All other chemicals and reagents were purchased from Sigma-Aldrich

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Quinuclidine 1

Figure 1 Structure of quinuclidine 1.

(Shanghai, China), unless otherwise specified. The bacterial strain *Bacillus subtilis* 168 was available in our laboratory collection. Other bacterial strains were obtained from the American Type Culture Collection (Manassas, VA, USA). *E. coli* JM109 WM647 was kindly provided by Dr W. Margolin (University of Texas-Houston Medical School, TX, USA).

Determination of MICs and synergistic activity

Minimum inhibitory concentrations (MICs) for the tested compound and β -lactam antibiotics were determined using a broth microdilution method in cation-adjusted Mueller–Hinton broth for antibiotic-susceptible strain *S. aureus* ATCC 29213, ampicillin-resistant *S. aureus* ATCC 29247, methicillin-resistant *S. aureus* ATCC BAA-41 and multidrug-resistant *S. aureus* ATCC BAA-44 or Mueller–Hinton broth for antibiotic-susceptible strains *B. subtilis* 168, *E. faecalis* ATCC 29212, *E. faecium* ATCC 49624 and *E. coli* ATCC 25922, and vancomycin-resistant *E. faecium* ATCC 700221 in accordance with the Clinical and Laboratory Standards Institute standard.¹⁹ A serial dilution of the compounds were added to the test bacteria (5×10^5 CFU ml⁻¹) in Mueller–Hinton broth. After 18 h incubation at 37 °C, the absorbance at 600 nm (A_{600}) was recorded to calculate the percentage of bacterial cell inhibition with respect to the vehicle (1% DMSO) using a microplate reader (Bio-Rad Laboratory, Hertfordshire, UK). The MIC values were calculated as the lowest concentration of compounds at which the growth of bacteria was inhibited by $\geq 90\%$. Each assay was performed in triplicates.

The synergistic activity of quinuclidine 1 and β -lactam antibiotics against ampicillin-resistant *S. aureus* ATCC 29247 and MRSA ATCC BAA-41 were assessed using a checkerboard broth microdilution method.²⁰ The MICs of the tested compound and β -lactam antibiotics were determined as described above. The checkerboard test was performed in a 96-well microplate containing Mueller–Hinton broth in which a two-dimensional array of serial concentrations of quinuclidine 1 and β -lactam antibiotic is used. For example, synergy between quinuclidine 1 and ampicillin was tested against ampicillin-resistant *S. aureus* in the concentration range of 1.5–24 $\mu\text{g ml}^{-1}$ ($1/8 \times \text{MIC}$ to $1 \times \text{MIC}$). For the synergistic tests against MRSA, the concentration ranges of 3–24 $\mu\text{g ml}^{-1}$ of quinuclidine 1 and 4.5–36 $\mu\text{g ml}^{-1}$ of ampicillin, 16–256 $\mu\text{g ml}^{-1}$ of oxacillin, 128–1024 $\mu\text{g ml}^{-1}$ of methicillin, 2–16 $\mu\text{g ml}^{-1}$ of imipenem, 16–256 $\mu\text{g ml}^{-1}$ of cefoxitin and 2–32 $\mu\text{g ml}^{-1}$ of ceftazidime were used.

The synergistic activity of quinuclidine 1 in combination with β -lactam antibiotics were then evaluated using a fractional inhibitory concentration index (FICI). The FICI was calculated using the following formula:²¹

$$\begin{aligned} \text{FICI} &= \text{FIC}_{\text{cpd A}} + \text{FIC}_{\text{cpd B}} \\ &= (\text{MIC}_{\text{cpd A in combination}} / \text{MIC}_{\text{cpd A alone}}) \\ &\quad + (\text{MIC}_{\text{cpd B in combination}} / \text{MIC}_{\text{cpd B alone}}). \end{aligned}$$

A FICI ≤ 0.5 was defined as synergistic; a FICI between 0.5 and 1 was defined as partial synergistic; a FICI between 1 and 4 was defined as additive effect or indifferent; and a FICI > 4 was defined as antagonistic. Experiments from the synergy tests were performed in triplicates.

Determination of frequency of resistance (FOR)

To determine the FOR for quinuclidine 1 alone and in combination with the β -lactam antibiotic imipenem, MRSA ATCC BAA-41 cells were grown to late-exponential phase ($\sim 1 \times 10^9$ CFU ml⁻¹) and spread on brain-heart infusion agar plates containing quinuclidine 1 at twofold (48 $\mu\text{g ml}^{-1}$), fourfold

(96 $\mu\text{g ml}^{-1}$) and eightfold (192 $\mu\text{g ml}^{-1}$) of the MIC level, or a combination of quinuclidine 1 at twofold the MIC level plus imipenem at the clinical break point MIC (4 $\mu\text{g ml}^{-1}$). To determine the number of viable cells in the starting inoculum, dilutions of the culture were plated on compound-free brain-heart infusion agar plates. Compound plates were incubated for 24 h to allow resistant mutants to grow. The spontaneous FOR was calculated as the number of resistant colonies on these compound plates divided by the number of CFUs originally plated. Each assay was performed in triplicates.

Expression and purification of *S. aureus* FtsZ

E. coli BL21(DE3) cells were transformed with the pRSET-A-S vector carrying *S. aureus* FtsZ with a 6-histidine tag attached at its N terminus under the control of a T7 promoter.¹⁴ The transformed *E. coli* strain was streaked on a nutrient agar plate containing 50 $\mu\text{g ml}^{-1}$ ampicillin and the agar plate was incubated at 37 °C overnight. A single colony was inoculated into 5 ml of Luria–Bertani (LB) medium in the presence of 50 $\mu\text{g ml}^{-1}$ ampicillin, which was then incubated at 37 °C, with shaking at 250 r.p.m. for 16 h. The overnight culture was transferred into a fresh $2 \times \text{TY}$ medium (16 g bactotryptone, 10 g yeast extract, 5 g NaCl per litre of water) in a dilution ratio of 1:100 and 50 $\mu\text{g ml}^{-1}$ ampicillin was then added, followed by incubation at 37 °C with shaking at 250 r.p.m. When the OD of the culture at 600 nm (OD_{600}) reached 0.8, protein expression was induced with 0.4 mM isopropyl- β -D-thiogalactopyranoside for 4 h. Cells were harvested by centrifugation at 9000 r.p.m. for 20 min at 4 °C. The cell pellet was resuspended in 20 ml of solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM PMSF and 1 mM EDTA, pH 7.4) and then lysed with 1 $\mu\text{g ml}^{-1}$ of lysozyme. The mixture was incubated for 1 h on ice. The cells were disrupted by sonication and the crude lysate obtained was centrifuged at 13 000 r.p.m. for 1 h at 4 °C. The supernatant containing 6-histidine-tagged *S. aureus* FtsZ was collected and loaded onto a nickel charged HiTrap chelating column pre-equilibrated with starting buffer (20 mM sodium phosphate buffer, 0.5 M NaCl, pH 7.4). The column was then washed with eight column volumes of the starting buffer to remove the unbound proteins, and the histidine-tagged enzyme was eluted by a linear gradient of 0–0.2 M imidazole. Fractions containing *S. aureus* FtsZ were pooled, buffer-exchanged with 20 mM NH_4HCO_3 (pH 8.0) at 4 °C, lyophilized and stored at -20 °C. A stock solution of *S. aureus* FtsZ for the subsequent bioassay was prepared from the lyophilized powder.

Light-scattering assay

S. aureus FtsZ (12 μM) in 50 mM MOPS buffer (pH 6.5) was incubated with vehicle (1% DMSO) or different concentrations of the tested compound for 10 min at 25 °C. Then 50 mM KCl, 10 mM MgCl_2 and 1 mM GTP were added to the reaction mixture. The rate and extent of polymerization were measured by 90° light scattering in a thermostatically (37 °C) controlled LS 50B spectrofluorimeter (Perkin Elmer, Waltham, MA, USA) for 1500 s using excitation and emission wavelength of 600 nm with slit width of 2 nm.²² Appropriate blanks were subtracted from all experimental data. Reported results are the average of three independent experiments.

Transmission electron microscopy

S. aureus FtsZ (12 μM) was incubated in the absence and in the presence of the tested compound (50–100 μM) in 50 mM MOPS (pH 6.5) buffer at 25 °C. After 10 min, 50 mM KCl, 5 mM MgCl_2 and 1 mM GTP were added to the reaction mixtures at 37 °C for 15 min. Then, 10 μl of the sample mixtures were placed on a glow-discharged Formvar carbon-coated copper grid (400 mesh) for 10 min. The grids were subsequently subjected to negative staining using 10 μl of 0.5% phosphotungstic acid for 30 s, air-dried and digital images of the specimen were observed with a transmission electron microscope (JEOL model JEM 2010, Tokyo, Japan) operated at 200 kV and equipped with a Gatan MSC 794 CCD camera.¹⁷ Three independent experiments were performed for each test.

Bacterial morphology and membrane staining studies

Visualization of the cell morphology and staining of the cell membrane were performed as previously described.²³ In brief, the *E. coli* cells were grown in LB medium. The cultures at an OD_{600} of 0.01 from an overnight culture were

grown in the absence or in the presence of 50 μM of quinuclidine **1** for 4 h at 37 °C. The bacterial cells were then harvested and resuspended in phosphate-buffered saline buffer (PBS, pH 7.4) containing 0.25% agarose. For membrane staining, *E. coli* cells were stained with a red fluorescent dye FM4-64 (1.6 μM , Invitrogen, Eugene, OR, USA) for 15 min at 37 °C without shaking before harvested and resuspended in 100 μl PBS buffer containing 0.25% agarose. Ten microliter of sample mixtures were placed on a microscopic slide pre-treated with 0.1% (w/v) poly-L-lysine. The bacterial cell morphology was observed under a light phase-contrast microscope Leica DMRB (Leica Microsystems, Wetzlar, Germany) at $\times 40$ magnification. The bacterial cell membrane was visualized using a fluorescence microscope Leica DMRB (Leica Microsystems, Germany) equipped with a $\times 100$ oil immersion magnification with a standard fluorescein isothiocyanate filter set. The images were captured using a Nikon camera. The length of bacterial cells was measured using pre-installed Nikon software. Experiments were performed in triplicates.

Z-ring visualization in *E. coli* cells

A culture of *E. coli* JM109 WM647 containing the IPTG-inducible plasmid for the overexpression of green fluorescent protein-tagged FtsZ was grown in LB medium supplemented with 30 $\mu\text{g ml}^{-1}$ of chloramphenicol. After overnight incubation, a sample of the culture was diluted to 1% in the LB medium containing quinuclidine **1** (50 μM) and 40 μM of IPTG. After incubation at 37 °C for 4 h, the *E. coli* cells were fixed, harvested and resuspended in PBS buffer containing 0.25% of agarose. Ten microliter of sample mixture were added to a pre-treated microscopic slide with 0.1% (w/v) poly-L-lysine and visualized using a fluorescence microscope at $\times 100$ oil immersion magnification with a standard fluorescein isothiocyanate filter set. One hundred cells were counted to calculate the frequency of occurrence of Z-rings. Experiments were performed in triplicates.

Tubulin polymerization assay

The effects of quinuclidine **1** on bovine brain tubulin polymerization were tested using the Tubulin Polymerization Assay Kit (Cytoskeleton, Denver, CO, USA). Bovine brain tubulin was used at a final concentration of 2 $\mu\text{g ml}^{-1}$. Quinuclidine **1** (600 μM) was tested in a concentration > 10 times its IC_{50} value for *S. aureus* FtsZ, which has been previously reported.¹⁴ Paclitaxel (20 μM) and vinblastine (3 μM) were also included as reference compounds. Fluorescence was measured using a PolarStar Optima microplate reader (BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 360 and 450 nm, respectively. Reported results are the average of three independent experiments.

RESULTS

Quinuclidine **1** (Figure 1) has recently been reported as a new FtsZ inhibitor with antibacterial activity against the Gram-negative bacterial strain *E. coli* ATCC 25922 and the Gram-positive bacterial strain *S. aureus* ATCC 29213.¹⁴ This study aims to further investigate its spectrum of antibacterial activity, as well as the underlying mechanism of action.

Spectrum of antibacterial activity and synergistic effects

Quinuclidine **1** was tested against an extended panel of clinically relevant bacterial strains. The results are summarized in Table 1. The compound inhibits the growth of antibiotic-susceptible strains (*B. subtilis* 168, *E. faecalis* ATCC 29212 and *E. faecium* ATCC 49624) and antibiotic-resistant strains (ampicillin-resistant *S. aureus* ATCC 29247, methicillin-resistant *S. aureus* ATCC BAA-41, multidrug-resistant *S. aureus* ATCC BAA-44 and vancomycin-resistant *E. faecium* ATCC 700221) with MIC values of 24 $\mu\text{g ml}^{-1}$. This indicates that quinuclidine **1** is equally potent against antibiotic-susceptible and antibiotic-resistant strains.

To assess the antibacterial activity of quinuclidine **1** in combination with the β -lactam antibiotics against ampicillin-resistant *S. aureus* and MRSA strains, a broth microdilution checkerboard experiment was

performed. As shown in Table 2, either compound **1** or ampicillin alone showed moderate antibacterial activities against ampicillin-resistant *S. aureus* with MIC values of 24 $\mu\text{g ml}^{-1}$. On the opposite, treating the bacteria with a combination of quinuclidine **1** and ampicillin apparently improved the antibacterial activities, resulting in a synergistic effect of FICI 0.50. Similar synergistic effects were observed on MRSA by combining quinuclidine **1** with methicillin or imipenem. Combining quinuclidine **1** with other β -lactam antibiotics showed partial synergistic activity with FICIs of 0.63–0.75 against MRSA. In these assays, quinuclidine **1** could improve the antibacterial activity of β -lactam antibiotics against MRSA by reducing the MIC values of ampicillin and oxacillin by fourfold (from 36 to 9 $\mu\text{g ml}^{-1}$) and eightfold (from 256 to 32 $\mu\text{g ml}^{-1}$), respectively. Improvement in antibacterial activity of cefoxitin (from 256 to 32 $\mu\text{g ml}^{-1}$) and ceftazidime (from 32 to 4 $\mu\text{g ml}^{-1}$) were also observed in the presence of quinuclidine **1**.

Table 1 Minimum inhibitory concentration (in $\mu\text{g ml}^{-1}$) of quinuclidine **1** against a panel of bacterial strains

Organisms ^a	MIC
<i>B. subtilis</i> 168	24
<i>E. coli</i> ATCC 25922	36 ^b
<i>E. faecium</i> ATCC 49624	24
<i>E. faecium</i> ATCC 700221	24
<i>E. faecalis</i> ATCC 29212	24
<i>S. aureus</i> ATCC 29213	24 ^b
<i>S. aureus</i> ATCC 29247	24
<i>S. aureus</i> ATCC BAA-41	24
<i>S. aureus</i> ATCC BAA-44	24

^a*E. faecium* ATCC 49624 and *E. faecalis* ATCC 29212 are antibiotic-susceptible, *E. faecium* ATCC 700221 is vancomycin-resistant, *S. aureus* ATCC 29247 is ampicillin-resistant, *S. aureus* ATCC BAA-41 is methicillin-resistant and *S. aureus* ATCC BAA-44 is multidrug-resistant.

^bMIC values reported in our previous study and shown here for comparison.¹⁴

Table 2 MIC values (in $\mu\text{g ml}^{-1}$) of quinuclidine **1** combined with β -lactam antibiotics against antibiotic-resistant strains of *S. aureus*

Bacterial strain ^a	Compound	Single compound	Combination of 1 and β -lactam	FICI ^b
		MIC	MIC	
ARSA	1	24	6	0.50
	Ampicillin	24	6	
MRSA	1	24	12	0.75
	Ampicillin	36	9	
MRSA	1	24	12	0.63
	Oxacillin	256	32	
MRSA	1	24	6	0.50
	Methicillin	1024	256	
MRSA	1	24	6	0.50
	Imipenem	16	4	
MRSA	1	24	12	0.63
	Cefoxitin	256	32	
MRSA	1	24	12	0.63
	Ceftazidime	32	4	

^aARSA is ampicillin-resistant *S. aureus* ATCC 29247 and MRSA is methicillin-resistant *S. aureus* ATCC BAA-41.

^bThe sum of fractional inhibitory concentration index (FICI) of the two compounds in combination.

Frequency of resistance

To assess possible drug resistance induced by quinuclidine **1**, MRSA cells were plated on various concentrations of the compound. No single resistant colony was obtained on agar containing quinuclidine **1** at twofold, fourfold and eightfold the MIC (48 , 96 and $192\ \mu\text{g ml}^{-1}$, respectively), implying a spontaneous FOR of $<1 \times 10^{-9}$. Similar results were observed in the presence of quinuclidine **1** combined with imipenem.

Inhibiting the assembly and bundling of FtsZ protofilaments *in vitro*

Perturbing the polymerization of FtsZ would result in inappropriate FtsZ assembly and disruption of bacterial cell division. The effect of **1** on FtsZ polymerization was monitored by measuring the change of light-scattering signal in the presence of the compound.²² The light-scattering results showed that the presence of **1** significantly slowed down the assembly of FtsZ monomers (Figure 2a). Moreover, quinuclidine **1** strongly inhibited the polymerization of *S. aureus* FtsZ in a dose-dependent manner. Compared with the vehicle (1% DMSO), the polymerization of FtsZ was inhibited by 30% and 45% in the presence of $50\ \mu\text{M}$ and $100\ \mu\text{M}$ of **1**, respectively. The inhibition of FtsZ polymerization could also be observed under a transmission electron microscope. Quinuclidine **1** was found to drastically reduce the size and thickness of the FtsZ polymers and the bundling of the FtsZ protofilaments in a concentration-dependent manner (Figure 2b). At $100\ \mu\text{M}$, quinuclidine **1** reduced the thickness of FtsZ protofilaments from 118 ± 21 to 60 ± 12 nm. Only a few straight, short and thin FtsZ filaments were observed in the presence of quinuclidine **1**.

Inducing filamentation in *E. coli* cells

The influence of quinuclidine **1** on the bacterial cell morphology was observed under the microscope to further investigate the underlying

Figure 2 Inhibition of FtsZ assembly. (a) Effect of quinuclidine **1** on the kinetics of *S. aureus* FtsZ polymerization. The experiments were performed in triplicates with symbols representing the mean value ($N=3$). (b) Electron micrographs of *S. aureus* FtsZ in the absence (vehicle—1% DMSO) and in the presence of quinuclidine **1**. Scale bar = 2000 nm. The experiments were performed in triplicates.

mechanism of its antibacterial activity. In the absence of **1**, the average length of *E. coli* cells was found to be $2.8 \pm 0.5\ \mu\text{m}$. On the other hand, quinuclidine-treated cells were remarkably elongated with lengths in the range of 10 – $50\ \mu\text{m}$ (Figure 3a). As the perturbation of membrane structure may also lead to bacterial cell lysis and death, the effect of quinuclidine **1** on the bacterial cell membrane was assessed by staining the bacterial cell membrane with the red fluorescent dye FM4–64. In spite of an increased length of the *E. coli* cells, compound **1** did not induce any detectable perturbation of the cell membrane, as compared with the untreated cells (Figure 3b).

Perturbing the Z-ring formation in *E. coli* cells

The effect of quinuclidine **1** on cytokinetic Z-ring formation at mid-cell during bacterial cell division was investigated by fluorescence microscopy (Figure 4). In the absence of quinuclidine **1**, *E. coli* cells had integral Z-rings that consist of green fluorescent protein-tagged FtsZ. On the contrary, green fluorescent protein-tagged FtsZ were found to disperse randomly throughout the elongated *E. coli* cells in the presence of quinuclidine **1**. Furthermore, treatment with quinuclidine **1** markedly reduced the percentage of cells having Z-rings from 93% ($0\ \mu\text{M}$) to 24% ($50\ \mu\text{M}$). The frequency of Z-ring occurrence per unit cell length of *E. coli* cells was found to be 0.38 ± 0.01 and 0.05 ± 0.01 in the absence (vehicle—1% DMSO) and in the presence of quinuclidine **1**, respectively.

Effects on eukaryotic tubulin polymerization

The effects of quinuclidine **1** on bovine brain tubulin polymerization were monitored by fluorescence microscopy after incorporation of a fluorescent reporter 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) into the microtubules. A known tubulin polymerization enhancer (paclitaxel) and a known inhibitor of tubulin-dependent GTP hydrolysis (vinblastine) were tested as reference compounds in the same assay conditions (Figure 5). In the presence of vinblastine

Figure 3 Effects of quinuclidine **1** on the cell morphology and membrane structure of *E. coli*. (a) *E. coli* cells were grown in the absence (vehicle—1% DMSO) or in the presence of quinuclidine **1**. (b) Observations after membrane staining with the red fluorescent dye FM 4–64 are shown in the absence (vehicle—1% DMSO) or in the presence of quinuclidine **1**. Scale bar = $10\ \mu\text{m}$. The images are representative of three independent experiments ($N=3$).

Figure 4 Perturbation of the cytokinetic Z-ring in *E. coli* cells. Scale bar = 10 μ m. The images are representative of three independent experiments ($N=3$).

Figure 5 Polymerization of mammalian tubulin. The experiments were performed in triplicates with symbols representing the mean value ($N=3$).

(30 μ M), the polymerization of mammalian tubulin was completely inhibited. On the opposite, the fluorescence intensity was significantly increased in the presence of paclitaxel (20 μ M), confirming that the rate of polymerization was significantly enhanced. Quinclidine **1** (600 μ M) and a mixture of **1** (600 μ M) and ampicillin (600 μ M) showed similar results to a control experiment with untreated mammalian tubulin, indicating that **1** is not an inhibitor of tubulin polymerization.

DISCUSSION

The results of this study confirm that quinclidine **1** has broad-spectrum antibacterial activity against both Gram-positive and Gram-negative strains through a FtsZ inhibition mechanism. It is well known that the amino-acid sequence of FtsZ is highly conserved in a wide range of bacteria.²⁴ Alignment of the GTP-binding sites of FtsZ from various bacterial strains shows differences with pairwise root-mean-square deviation <0.8 Å.¹⁴ The broad-spectrum antibacterial activity of quinclidine **1** may be attributed to the highly conserved GTP-binding site of FtsZ. The susceptibility antibacterial test also showed that there is no difference in the antibacterial potency of quinclidine **1** against antibiotic-susceptible and antibiotic-resistant strains of *E. faecium* and *S. aureus*, thus confirming that the activity of the compound is not affected by common mechanisms of antibiotic resistance. To evaluate the potential use of quinclidine **1** in combination with clinically used β -lactam drugs, the compound was

tested in combination with several β -lactam antibiotics. This study showed that the activities of two β -lactams (methicillin and imipenem) against MRSA were greatly enhanced by quinclidine **1**, while the activity of quinclidine **1** itself was also improved. A previous report by Tan *et al.* on a small-molecule FtsZ inhibitor from a different class of compounds, PC190723, showed similar synergistic effects with β -lactam antibiotics against MRSA strains.¹⁵ However, PC190723 displayed no synergistic activity with non- β -lactam antibiotics. They hypothesized that the interdependent network of functional interactions between FtsZ and the cell wall biosynthetic proteins (such as the penicillin-binding proteins) might be responsible for the synergy between PC190723 and β -lactams. Possibly, PC190723-mediated inhibition causes delocalization of FtsZ, which in turn leads to PBP2 delocalization. Consequently, a significantly reduced concentration of the β -lactam is needed to inhibit the residual functional and correctly localized penicillin-binding protein 2. The synergistic effects of quinclidine **1** with β -lactams probably work through a similar mechanism. However, PC190723 binds to the less conserved C-terminal T7 loop of FtsZ and only works against Gram-positive drug-resistant bacteria,²⁵ whereas quinclidine **1** binds to the GTP-binding site and exhibits antibacterial activities against both Gram-positive and Gram-negative bacteria.

No spontaneous resistant mutant of MRSA was found in the presence of quinclidine **1** alone or in combination with imipenem. This is different from PC190723 in which a high spontaneous FOR to MRSA has been reported.¹⁵ As quinclidine **1** is supposed to bind to the GTP-binding site of FtsZ, which is highly conserved compared with the terminal T7 loop. Any mutations of the GTP-binding site in FtsZ might change the protein structure and the GTPase activity, which in turn would seriously affect the function of the protein and therefore could not be compromised. Thus, the failure to identify high-level resistance to quinclidine **1** could be related to the intolerance of structural changes or mutations in the GTPase binding site of FtsZ. Undetectable or very low frequency of spontaneous resistance have also been reported for other GTP-binding site FtsZ inhibitors such as trisubstituted benzimidazole SB-P17G-A20 and PC58538.^{26,27}

Quinclidine **1** was found to reduce the light-scattering signal of FtsZ assembly and the bundling of FtsZ protofilaments in a dose-dependent manner. The compound impaired the localization of FtsZ into the cytokinetic Z-ring by disruption of the assembly of FtsZ protein. Consequently, a cytokinesis defect was observed in the elongated bacterial cells. Taken together, our findings suggest that quinclidine **1** inhibits bacterial proliferation by inducing bacterial cell filamentation through targeting the FtsZ protein. Our results are in good agreement with findings on FtsZ inhibitors of other chemotypes.^{17,23}

In conclusion, quinclidine **1** exhibits a broad spectrum of antibacterial activity, as well as synergistic effects with β -lactams against antibiotic-resistant *S. aureus* through the inhibition of FtsZ functional activity. Given the novel molecular scaffold and the encouraging results, quinclidine derivatives are a promising new class of compounds for further optimization into potent and selective FtsZ-based antibacterial agents.

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