A bis(pyrazolyl)methane derivative against clinical Staphylococcus aureus strains isolated from otitis externa

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Abstract

Objective: The purpose of this study was to evaluate the in vitro antibacterial effects of a p-Cymene-based bis(pyrazolyl)methane derivative (SC-19) to advance in developing alternative therapeutic compounds to fight against bacterial isolates from patients with otitis externa (OE).

Methods: Eighteen swab specimens were collected from patients aged over 18 years diagnosed with OE within at least 7 days of symptom onset, contaminated by only one bacterium type: Pseudomonas aeruginosa (n = 5); Staphylococcus aureus (n = 8); Klebsiella aerogenes (n = 2); Serratia marcescens (n = 1); Morganella morganii (n = 2).

To appraise antibacterial activity, minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), minimum biofilm inhibitory concentration (MBIC), and minimum biofilm eradication concentration (MBEC) assays were run at different SC-19 concentrations.

Results: When using SC-19, S. aureus strains showed less bacterial growth, but no bactericidal effect was observed. The MIC and MBC of SC-19 were 62.5 and 2000 μg/ml against S. aureus and were >2000 μg/ml against the other isolates obtained from OE, respectively. In addition, the MBICs and MBECs of SC-19 against S. aureus were 125 and >2000 μg/ml, respectively.

Conclusion: Nowadays the acquired antibiotic resistance phenomenon has stimulated research into novel and more efficient therapeutic agents. Hence, we report that, helped by the structural diversity fostered herein by a range of bis(pyrazolyl)methane derivatives, SC-19 can be a promising alternative therapeutic option for treating OE caused by S. aureus given the observed effects on both planktonic state and biofilm.
1 | INTRODUCTION

Otitis externa (OE) is a condition involving the inflammation or infection of the external auditory canal, which is the tube between the outer ear and the eardrum. Common treatments reduce symptoms and patients usually recover within a few days. Only in certain cases does the pathology last several months or longer. Infectious agents, including bacteria, fungi and viruses, are frequently responsible for OE. The commonest causative agents of an acute form of OE are Pseudomonas aeruginosa and Staphylococcus aureus, but other microorganisms like Proteus mirabilis, Klebsiella aerogenes, Escherichia coli, Streptococcus pyogenes, Serratia marcescens, Morganella morganii, Klebsiella pneumoniae, and Staphylococcus epidermidis have also been isolated in OE samples. Topical antimicrobials or antibiotics, such as quinolones, aminoglycosides, neomycin, or their combination, are applied using ear drops to treat OE. Thus only oral administration is prescribed when rapidly progressing infection is identified. In line with this, the acquired antibiotic resistance phenomenon is becoming one of the main concerns for treating many pathologies, including OE, and Gram-negative bacteria are found, such as E. coli, K. pneumoniae, and P. aeruginosa, as are Gram-positive bacteria like S. aureus. To date, there is no antimicrobial, antibiotic, or a combination of them, that is clinically superior to another to treat OE and, therefore, endogenous non-antibiotic antimicrobial agents are currently being investigated. N-chlorotaurine has shown excellent tolerability as long broad-spectrum activity for treating OE while N-acetylcysteine seems to inhibit common pathogens of both Gram-positive and Gram-negative bacteria. Moreover, other novel antimicrobial substances used to treat a variety of human infections have sparked a surge of interest in pharmacological chemicals. Accordingly, we very recently reported a study to screen bis(triazolyl)methane and bis(pyrazolyl)methane derivatives as compounds with antibacterial activity against both planktonic and biofilm states. After considering all the obtained results, a nitrogen-based compound was identified with anti-Gram-positive activity and low toxicity to eukaryotic cells. The structure of this compound (SC-19) includes p-Cymene [1-methyl-4-(1-methylethyl)-benzene], an alkyl aromatic monoterpene naturally found in over 100 plant species, and used in medicine and food chemistry for its antioxidant, anti-inflammatory, anti-nociceptive, anxiolytic, anticancer, and antimicrobial properties. This last property has been widely investigated given the urgent need for new substances with antimicrobial properties to be used to treat communicable diseases, whose diffusion in developed countries has been facilitated by globalization and the evolution of antimicrobial resistance.

Following our previous results, the aim of this study was to investigate the efficacy of SC-19, a p-Cymene-based compound, in treating OE by studying the susceptibility of bacterial strains isolated from human otical infection cases in both planktonic and biofilm states.

2 | MATERIALS AND METHODS

2.1 | Bis(pyrazolyl)methane derivative (SC19) synthesis

The Bis(pyrazolyl)methane derivative (SC-19) was prepared according to procedures reported in the literature. Briefly, bis(3,5-dimethylpyrazol-1-yl)methane was dissolved in dry THF and cooled to −78°C. Then BuLi was added, and the mixture was transferred to a solution of 4-isopropylbenzyl bromide (p-Cymene) in THF. The product was hydrolyzed with saturated aqueous NH4Cl, the organic layer was extracted, dried over MgSO4, and filtered, and the solvent was removed in vacuum to give rise to the product as orange oil. The final product was obtained after purification by silica gel column chromatography.

2.2 | Patients and sample collection

The patients in this study were selected and examined during routine clinical visits as part of a diagnostic workup. Eighteen different bacterial strains were isolated from the patients who visited the Albacete University Hospital for OE. The inclusion criteria were (1) being 18 years of age or older; (2) being diagnosed with OE by an otologist within at least 7 days of symptom onset; (3) their samples were contaminated by only one type of bacterial strain. The exclusion criteria were (1) tympanic membrane perforation exceeding 3 mm; (2) otomycosis. No treatment decisions were made based on the clinical examination results.

All the superficial zones were sanitized using sterile saline solution before sample collection. Then fresh exudates were easily obtained by applying light pressure to lesion areas. The collected samples were cultivated on blood agar (ThermoFisher Scientific, USA) and incubated aerobically at 37°C for 18–24 h to follow further assays.

2.3 | Ethics approval

All the methods were conducted in accordance with relevant guidelines and regulations. The study began after receiving permission from the local Ethics Committee (Date: December 22, 2020; No: 2020/10/114). Written informed consent was obtained from all the study participants.
2.4 | Bacterial strains

Five strains of *P. aeruginosa*, eight of *S. aureus*, two of *K. aerogenes*, one of *Serratia marcescens*, and two of *M. morganii* were isolated from the patients with OE. Strains were identified by Matrix-Assisted Laser Desorption/Ionization (MALDI)-Time-Of-Flight (TOF) VITEK® MS (bioMérieux, St. Louis, MO). All the procedures were performed in accordance with manufacturers’ recommendations.

2.5 | Antibiotic efficacy tests

The antibiotic susceptibilities of the strains included in this study were carried out by the standard Kirby–Bauer disk diffusion method. Briefly, a disk diffusion analysis was performed on Mueller–Hinton agar enriched with 5% defibrinated sheep blood and was incubated at 37°C in a microaerophilic atmosphere with 5% CO2.

Nineteen antibiotics were used to determine antibiotic susceptibility, including: 25 μg of Amoxicillin (AMOXY), 10 μg of Ampicillin (AMP), 30 μg of Cefotiofur (CFT), 30 μg of Ciprofloxacin (CIP), 5 μg of Levofloxacin (LEV), 10 μg of Colistin sulfate (CT), 30 μg of Doxycycline (DOXIC), 5 μg of Enrofloxacin (ENR), 10 μg of Gentamicin (CN), 15 μg of Lincomycin (MY), 10 μg of Neomycin (N), 30 μg of Oxytetracycline (OT), 10 μg of Penicillin G (P), 100 μg of Spectinomycin (SH), 1.25/23.75 μg of Trimethoprim-Sulphamethoxazole (TSX), 30 μg of Tetracyclin (TE), 15 μg of Erythromycin (ERY), 2 μg of Clindamycin (CL), and 30 μg of Tylosin (TY). The resistance break points were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards, as previously reported.

2.6 | Minimal inhibitory concentration and minimal bactericidal concentration

Minimum inhibitory concentrations (MICs) were determined by the previously reported broth microdilution method. Briefly, a series of SC-19 concentrations starting from 2000 to 1.96 μg/ml at the twofold dilution was added to the cation-adjusted Müller-Hinton broth (Sigma Aldrich, USA) (CAMHB) at a final volume of 100 μl per well. One hundred microliters of bacterial suspension in CAMHB containing approximately 1.6 × 10^6 CFU/ml were added to a Costar 96-well round-bottom polypropylene plate (Corning Inc., USA), followed by static incubation at 37°C and 5% CO2 for at least 20 h. After incubation, MICs were determined by measuring absorbance at 600 nm. A series of SC-19 concentrations at the twofold dilution without bacteria was used as a negative control of absorbance.

Minimum bactericidal concentrations (MBCs) were determined by the flash microbiocidal method described elsewhere. Briefly after 24 h incubation, 10 μl of each well were mixed with 190 μl of tryptic soya broth (Biomérieux, France) in a new 96-well plate, which was incubated statically at 37°C and 5% CO2 for 24 h. After incubation, MBCs were determined by measuring absorbance at 600 nm. These experiments were performed four times.

2.7 | Minimal biofilm inhibitory concentration and minimal biofilm eradication concentration

Minimal biofilm inhibitory concentrations (MBICs) and minimal biofilm eradication concentrations were determined by the previously described methodology. The MBIC is the minimum concentration required to inhibit the visible growth of a bacterial biofilm. For the MBEC, biofilm formation at the bottom of a MicroWell™ 96-well flat-bottom plate (Thermo Fisher Scientific, USA) was induced by inoculating 100 μl of CAMHB containing 10^5 CFU/ml of bacteria per well. The plate was incubated at 37°C and 5% CO2 for 24 h. After incubation, the supernatant was aspirated, 200 μl per well with the different concentrations were deposited, and the plate was incubated at 37°C and 5% CO2 for at least 20 h. After incubation, the MBIC was determined by measuring absorbance at 600 nm.

The minimal biofilm eradication concentration (MBEC) is the minimum concentration required to kill a bacterial biofilm. For the MBEC, the biofilm grown at the bottom of each well was scraped and mixed with the supernatant. Thereafter, 20 μl of each well were mixed with 180 μl of tryptic soy broth in a new 96-well plate, which was incubated statically at 37°C and 5% CO2 for 24 h. After incubation, the MBEC was determined by measuring absorbance using a wavelength of 600 nm. In these assays, a series of SC-19 concentrations at the twofold dilution without bacteria was also used as a negative control of absorbance. These experiments were performed four times.

No statistical test was used in this study. The present study aimed to screen the antimicrobial activities of SC-19 to determine whether it had any potential antimicrobial effect or not. So, as we did not obtain quantitative results from many different clinical isolates, we were unable to compare our results to a statistical method.

3 | RESULTS

3.1 | Synthesis and characterization of the bis(pyrazolyl)methane derivative (SC19)

SC-19 was synthesized following a previously published procedure. The SC-19 chemical structure is shown in Figure 1, along with the 1H-NMR as a representative assay for its characterization. The one-pot reaction of bis(3,5-dimethylpyrazol-1-yl)methane with t-BuLi, followed by the addition of 1-methyl-4-(1-methylethyl)benzene (p-Cymene) bromide to THF at 0°C, gave SC-19, which was isolated as an orange solid after the appropriate workup. The presence of signals at 1.12 and 2.70 ppm within the 1H-NMR spectrum of SC-19 confirmed the presence of p-Cymene moiety in the chemical structure.

3.2 | Bacterial strains and antibiotic efficacy tests

Eighteen bacterial strains, isolated from patients with OE, were used to determine antibiotic susceptibilities, and mainly the antibacterial
effects of SC-19 on both planktonic and biofilm states. The distribution of positive isolates in the 18 patients was \( P. \) aeruginosa \((n = 5)\), \( S. \) aureus \((n = 8)\), \( K. \) aerogenes \((n = 2)\), \( S. \) marcescens \((n = 1)\), and \( M. \) morganii \((n = 2)\). They were all confirmed by molecular methods. The susceptibility rate of the tested \( S. \) aureus strains was in the following order: penicillin G \((75.00\%; n = 6)\), clindamycin \((62.50\%; n = 5)\),

![FIGURE 1](image)

The synthetic procedure, chemical structure, and \(^1\)H NMR spectrum of SC-19

### TABLE 1  Antibiotic susceptibility profile of strains isolated from clinical otitis externa infections \((n = 18)\)

| Antibiotics                          | Antibiotics susceptibility test results | \( Pseudomonas \) aeruginosa | \( Staphylococcus \) aureus | \( Klebsiella \) aerogenes | \( Serratia \) marcescens | \( Morganella \) morganii |
|--------------------------------------|----------------------------------------|-------------------------------|-----------------------------|-----------------------------|---------------------------|---------------------------|
|                                      | No. %                                  | No. %                         | No. %                       | No. %                       | No. %                     | No. %                     |
| Ciprofloxacin \((30 \mu g)\)         | S                                      | 4 80                          | 8 100                       | 2 100                       | 1 100                     | 1 50                      |
|                                      | R                                      | 1 20                          | 0 0                         | 0 0                         | 0 0                       | 1 50                      |
| Levofoxacin \((5 \mu g)\)            | S                                      | 5 100                         | 5 62.5                      | 2 100                       | 1 100                     | 2 100                     |
|                                      | R                                      | 0 0                           | 3 37.5                      | 0 0                         | 0 0                       | 0 0                       |
| Penicillin G \((10 \mu g)\)          | S                                      | 5 100                         | 6 75                        | 0 0                         | 0 0                       | 0 0                       |
|                                      | R                                      | 0 0                           | 2 25                        | 2 100                       | 1 100                     | 2 100                     |
| Erythromycin \((15 \mu g)\)          | S                                      | 5 100                         | 5 62.5                      | 2 100                       | 1 100                     | 2 100                     |
|                                      | R                                      | 0 0                           | 3 37.5                      | 0 0                         | 0 0                       | 0 0                       |
| Clindamycin \((2 \mu g)\)            | S                                      | 5 100                         | 5 62.5                      | 2 100                       | 1 100                     | 2 100                     |
|                                      | R                                      | 0 0                           | 3 37.5                      | 0 0                         | 0 0                       | 0 0                       |
| Trimethoprim/sulfamethoxazole \((1.25/23.75 \mu g)\) | S                                      | 5 100                         | 7 87.5                      | 2 100                       | 1 100                     | 0 0                       |
|                                      | R                                      | 0 0                           | 1 12.5                      | 0 0                         | 0 0                       | 2 100                     |

Abbreviations: S, susceptible; R, resistant.
erythromycin (62.50%; n = 5), levofloxacin (62.50%; n = 5), and trimethoprim-sulfamethoxazole (87.50%; n = 7), with 100.00%; n = 8 for the other tested antibiotics. The S. aureus strains susceptible to all the tested antibiotics included three isolates (37.50%). The results of the antimicrobial susceptibility tests performed on the 18 clinical strains isolated from OE are presented in Table 1.

Fifty percent of S. aureus from OE were resistant to multiple antimicrobial classes, including the synthetic antimicrobial agents used mainly in human medicine. In line with this, significant variability of the susceptibility profiles was observed among these S. aureus isolates, as detailed in Table 2. Moreover, all the isolates identified for K. aerogenes, S. marcescens, and M. morganii were also resistant to multiple antimicrobial classes. The P. aeruginosa strains from OE were susceptible to most of the tested antibiotics, and only one (20%) strain was resistant to ciprofloxacin.

### 3.3 Antibacterial assessments

The antibacterial effect of SC-19 was evaluated by studying both MICs and MBCs. For this purpose, SC-19 was studied against the clinical isolates of P. aeruginosa, S. aureus, K. aerogenes, S. marcescens, and M. morganii at different concentrations. Except for S. aureus, SC-19 did not show any activity against the bacteria isolated from OE lesions. The MIC and MBC of SC-19 were respectively 62.5 and 2000 μg/ml against S. aureus for all the tested strains. The MICs and MBCs of SC-19 against P. aeruginosa, K. aerogenes, S. marcescens, and M. morganii were >2000 μg/ml for them all.

In addition, the antibiofilm effect of SC-19 was evaluated by studying both MBICs and MBECs. For this purpose, SC-19 was tested at different concentrations against the biofilms formed by clinical strains P. aeruginosa, S. aureus, K. aerogenes, S. marcescens, and M. morganii. As observed against the planktonic bacterial strains, SC-19 only displayed an inhibitory effect on those biofilms formed by S. aureus. Thus, the MBICs and MBECs of SC-19 against S. aureus were 125 and > 2000 μg/ml, respectively, for all the tested strains. The MBICs and MBECs of SC-19 against P. aeruginosa, K. aerogenes, S. marcescens, and M. morganii were >2000 μg/ml for them all.

### 4 DISCUSSION

OE is the acute or chronic inflammation of the external ear. As a secondary cause of otitis, bacterial infection is a common complication in OE with primary causes (e.g., adverse food reaction, atopic dermatitis) that mostly require lifelong treatment. The principal microorganisms responsible for OE are P. aeruginosa and S. aureus, although other microorganisms can also be found in patients’ OE lesions. Recently, antimicrobials’ efficacy has become limited due to resistance and biofilm formation. In addition, overusing antibiotic prescriptions is a major burden on the healthcare economy, and antibiotic resistance is a major problem in many countries. In the present study, we report S. aureus isolate resistance rates of: 37.5% against clindamycin, 30.0% against tetracycline, 20.0% against ciprofloxacin, and 12.5% against linezolid.
levofoxacin and erythromycin; 25% against penicillin; 12.5% against trimethoprim-sulphamethoxazole. We also report that S. aureus isolates were susceptible to the other tested antibiotics. Resistance to clindamycin, levofloxacin, erythromycin, penicillin G and trimethoprim-sulfamethoxazole was 27.77%, 16.67%, 16.67%, 33.33%, and 11.11%, respectively, in the overall evaluation of all the isolates.

There are many reports about compounds with effects against the human bacterial pathogens involved in OE,22–25 while research into new molecules that may be effective against infectious agents continues worldwide.26,27 Thus p-Cymene [1-methyl-4-(1-methylethyl)-benzene] is an alkyl-substituted aromatic hydrocarbon found in nature whose benzene ring features the substitution of a methyl and an isopropyl group. It is found in more than 100 aromatic plants, such as species belonging to the Thymus and Origanum genera.28 This compound shows a variety of biological activities, including antioxidant, anticonceptive, anti-inflammatory, anxiolytic, anticancer, and antimicrobial activities.29–34 In relation to the last activity, a large body of evidence suggests that this monoterpene possesses antibacterial, antiviral and antifungal activities,13 and is “generally recognized as safe” (GRAS) by the US Food and Drug Administration.13

Unfortunately, one of the major limitations of p-Cymene for pharmaceutical applications is its short half-life.35 For example, Martins et al.37 successfully encapsulated thymol and p-Cymene in poly lactide microparticles to protect these active agents and to provide controlled release. In a previous study, our group studied the antimicrobial activity of a library of bis(triazolyl)methane and bis(pyrazolyl)methane nitrogen-based derivatives. Notably, the p-Cymene derivative (SC-19) showed a significant effect on Gram-positive bacterial strains,12 and SC-19 displayed no significant reduction in viability in both human liver carcinoma HepG2 and human colorectal adenocarcinoma Caco-2 cell lines compared to untreated control cells.

Currently, the most effective treatment for clinical OE is the topical application of a range of antimicrobials and anti-inflammatory agents. However, there is no clinical evidence to position one of these treatments as the most effective one, which is likely due to the antibiotic resistance displayed by many patients. Bearing in mind p-Cymene properties, with the present study we aimed to test the antibacterial abilities of SC-19 on both the planktonic and biofilm states of bacterial isolates obtained from patients with OE symptoms. As previously reported, SC-19 may act as an agent against collection strains of Gram-positive bacteria by showing a significant antibacterial effect at non-toxic doses for eukaryotic cell cultures.12 Thus SC-19 toxicity depends on its concentration, which is higher than 500 μg/ml for eukaryotic cells.12 We observed in the present study that SC-19 induces cytotoxicity on eukaryotic cells at higher concentrations than those able to inhibit S. aureus clinical isolate growth. However, our study has several limitations. First, eight strains are not enough to signify S. aureus bacterial species. Therefore, more studies with a higher number of strains are needed. Second, surface conditions are quite different from OE in vivo conditions in a clinical setting. Finally, our study confirmed the inhibition of the bacterial growth biofilm for 24 h, but not its eradication. Third, the synergy between SC-19 and other antimicrobials should be evaluated in further studies because SC-19 might be an ideal adjuvant for current topical OE treatments. In this regard, other compounds of natural origin such as saponins and isoflavonoids have recently shown a synergistic anti-staphylococcal effect in combination with some antibiotics.38,39

According to our results, Hashemi et al.40 tested essential oils whose major component was p-Cymene and showed a potent antibacterial effect on S. aureus. Different studies that have investigated the antimicrobial effects of other compounds rich in p-Cymene have also shown an inhibitory effect on the growth of S. aureus strains among other Gram-positive and Gram-negative bacteria.41–43 Regarding antibiofilm activity, it has been previously demonstrated that substances composed of p-Cymene are able to inhibit the growth of S. aureus preformed biofilms, and to even inhibit biofilm formation during planktonic growth.43,44 In addition to SC-19’s mechanisms of action proposed in our previous study,12 other authors have also found potential mechanisms based on the existence of p-Cymene moiety, which can be attributable to SC-19 given its structure and composition. Ultee et al.45 reported that p-Cymene influences liposmal membrane expansion by looking into its influence on membrane potential, changes in intracellular pH, influences on the amount of ATP, and effects on the growth of another Gram-positive bacterium, B. cereus. Other studies have suggested that p-Cymene’s antimicrobial effects could be due to a perturbation of lipids in the bacterial membrane,46 as well as a rise in ROS species.13,34

SC-19’s antibacterial effects were tested in both the planktonic and biofilm states of the clinical isolates of P. aeruginosa, S. aureus, K. aerogenes, S. marcescens, and M. morganii. Remarkably, SC-19 showed selectivity against S. aureus with very similar behavior in the eight isolated S. aureus clinical strains, with MIC, MBC, MBIC and MBEC of 62.5, 125, 200, and >2000 μg/ml against S. aureus, respectively. It would seem the p-Cymene moiety plays a significant role in the selectivity observed for this derivative. The mechanism of action is unknown, even though many proposals have been reported (REF). Finally, and as previously reported, the MIC and MBIC values of SC-19 were very low to produce cytotoxicity in both the human liver carcinoma HepG2 and human colorectal adenocarcinoma Caco-2 cell lines at 48 h.12

5 | CONCLUSION

Due to increased bacterial resistance to the antibiotics used in daily practice, SC-19 appears to be a promising alternative or adjuvant for treating OE caused by S. aureus. The results herein reported provide information that could translate into potential new OE treatment options. SC-19 actively inhibits S. aureus growth, a clinically relevant pathogen that is usually resistant to most current antibiotics. Additional studies are necessary to determine the in vivo toxicological effects of SC-19, and to standardize their usage doses for patients. Further studies on combinations of SC-19 with antibiotics could also
provide valuable information about the clinical potential of this new antimicrobial agent.

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CONFLICT OF INTERESTS

The authors have no funding, financial relationships, or conflicts of interest to disclose.

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