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RESEARCH ARTICLE

Anti-*Staphylococcus aureus* Methicillin-Resistant (MRSA) Activity of a Novel 3-Chalcogenyl Indole

Atividade Anti-Staphylococcus aureus Meticilina Resistente (MRSA) de um novo composto 3-Calcogenil Indol

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Abstract

Objective: the development of new drugs against Methicillin-resistant *Staphylococcus aureus* is a priority to the World Health Organization. So, the objective of this study was to evaluate the antibacterial activity and toxicity of 5-bromo-3-((4-methoxyphenyl) sulfenyl)-*1H*-indole (3b) against MRSA.

Methods: minimum inhibitory concentration (MIC) of 3b was determined against *S. aureus* ATCC 29213 and 43 clinical isolates. The time-kill assay was performed for 9 isolates. Analysis of variance followed by the *post hoc* Bonferroni test was used for the statistical tests.

Results and conclusions: the MIC_{50} and MIC_{90} of 3b were 4 µg.mL-1 and 16 µg.mL^{-1} respectively. In time-kill assay, the 3b showed bactericidal activity to all evaluated isolates at concentrations of 1xMIC and 2xMIC and the re-growth effect was not observed. About the toxicity tests, 3b has not presented cytotoxicity, mutagenicity, or allergenicity. 3b had particularly good activity against MRSA demonstrating high potential for the development of new antimicrobials products.

Keywords: anti-MRSA, chalcogenyl-indoles, new antimicrobials, time-kill, *Staphylococcus aureus*.

Resumo

Objetivo: o desenvolvimento de novos antimicrobianos contra *Staphylococcus aureus* resistentes à meticilina (MRSA) é uma prioridade para a Organização Mundial da Saúde. Então, o objetivo desse estudo foi avaliar a atividade antibacteriana e a toxicidade do 5-bromo-3-((4-metoxifenil) sulfenil)-*1H*-indol (3b) contra MRSA.

Métodos: a concentração inibitória minima de 3b foi determinada contra *S. aureus* ATCC 29213 e 43 isolados clínicos. O ensaio de curva de morte foi realizado para nove isolados. Análise de variância seguida pelo teste *post hoc* Bonferroni foi usada para testes estatísticos.

Resultados e conclusões: a MIC_{50} e MIC_{90} do 3b foi 4 µg.mL^{-1} e 16 µg.mL^{-1}, respectivamente. No ensaio de curva de morte, o 3b demonstrou atividade bactericida contra todos os isolados avaliados na concentração de 1xMIC e 2xMIC e o recrescimento não foi observado. Em relação aos testes de toxicidade, 3b não apresentou citotoxicidade, mutagenicidade ou alergenicidade. 3b apresentou atividade particularmente interessante contra MRSA, demonstrando alto potencial para o desenvolvimento de novos produtos antimicrobianos.

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Palavras-chave: anti-MRSA, chalcogenil-indóis, novos antimicrobianos, ensaios de curva de morte, *Staphylococcus aureus*.

ABBREVIATIONS: 3b, 5-bromo-3-((4-methoxyphenyl) sulfenyl)-*1H-indole*; ATCC, american type culture collection; CFU, colony forming unit; dimethylsulfoxide; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*.

Introduction

The excessive use of antimicrobials in human and veterinary medicine has increased the prevalence of resistant microorganisms, leading to therapeutic failure and high mortality rates. In this context, the World Health Organization has published a list of bacteria with epidemiological importance. This list included Methicillin-Resistant *Staphylococcus aureus* (MRSA) as a high-priority issue (1).

Meanwhile, the discovery of new classes of antimicrobials has undergone a gradual decline in recent decades due to difficulties to identify new natural or synthetic drugs with low toxicity and a relevant spectrum of antimicrobial properties, along with economic and legal concerns (2). One interesting approach in the discovery of new drugs is studying the "privileged scaffolds", which are structures that can interact with different molecular targets due to their appropriate molecular size. Substituents can be built into these structures and it allows the construction of libraries of highly diverse compounds[3]. Indoles are a well-known example of privileged scaffolds and several commercial drugs (including indomethacin, ondansetron, tadalafil, delavirdine among others) are indole derivatives. These drugs interact with a myriad of molecular targets (3).

Within our ongoing research program seeking new molecules with antimicrobial properties(4) for future *in vivo* studies, a library of 14 3-sulfenyl- and 3-selenyl-indoles were screened for antibacterial activity. The 5-bromo-3-((4-methoxyphenyl) sulfenyl)-*1H*-indole, here called 3b (**Figure 1**) presented the most promising results in screening tests, especially against *Staphylococcus* spp. That is the reason why this study aimed to deeply evaluate the activity of 3b against *S. aureus*, mainly MRSA strains.

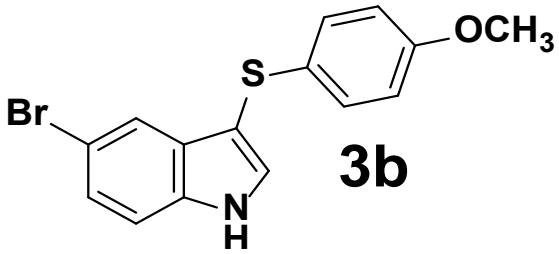

Figure 1 – Chemical structure of 5-bromo-3-((4-methoxyphenyl)sulfenyl)-*1H*-indole (3b).

Matherial and Methods

Study area

The study was developed at Applied Microbiology Laboratory (Federal University of Rio Grande do Sul) from March 2016 to March 2018.

Experiments

The preparation of 3b was performed as previously described by Azeredo *et al*. (5). The crude product was purified by column chromatography by using a mixture of ethyl acetate/hexanes (20:80) as eluent. The compound was characterized based on the melting point (when solid) and infrared and ^{1}H and ^{13}C NMR spectra (5).

The microdilution broth was performed to determine the Minimal Inhibitory Concentration (MIC), according to the Clinical and Laboratory Standards Institute (6). The 3b was prepared in 2% dimethylsulfoxide and diluted in Mueller-Hinton broth. The compound was tested in concentration ranged from 0.125 to 32 $\mu g.ml^{-1}$ against *S. aureus* ATCC 29213 and 43 clinical isolates of *S. aureus* (including 25 MRSA) from the library of Laboratory of Research in Bacterial Resistance (LABRESIS). The isolates were previously identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using the equipment of Bruker in its ion positive mode. The software BioTyper (Bruker, version 3.4) was used to analyze results.

Nine clinical isolates of *S. aureus* were selected according to their susceptibility profile for time-kill assay, which was performed as described by Isenberg (7) and following recommendations of Clinical and Laboratory Standards Institute (6). Bacterial inoculum

was incubated at concentrations corresponding to 0.5x, 1x, and 2x the MIC of 3b. Aliquots of 50 µL were taken at 0, 1, 2, 4, 6, 12, and 24 h. After serial dilutions, 20 µL of each dilution was plated in mannitol salt agar and incubated for 18 to 24h at 35°C for CFU counting. The assay was performed in duplicate.

The human leukocyte cultures were prepared according to the methodology described by Dos Santos *et al*. (8) to evaluate the genotoxicity and mutagenicity (9,10). Phosphate buffered saline (PBS) pH 7.4 was used as the negative control and 3 µM bleomycin as the positive control. 3b was evaluated in a concentration of 32 µg ml^{-1}. Cell cultures were incubated (5% CO_2, incubator model MCO19AIC, Sanyo) for 72 h at 37 °C. Cell viability was assessed with 0.2% trypan blue (Sigma-Aldrich), according to Burow et al (9). The Hen´s Egg Test on the Chorio-allantoic Membrane was used to allergenicity test (11).

Analysis of variance followed by the *post hoc* Bonferroni test was performed in the statistical analysis of toxicity test results. Results with $p < 0.05$ were considered significant. Data were analyzed using the GraphPad PRISM version 5.02 software program. The assays were performed in duplicate.

Results

The most of the clinical isolates selected to time-kill assay were resistant to -lactams (MIC90: oxacillin 64 $\mu g.mL^{-1}$; Piperacillin + Tazobactam 16 $\mu g.mL^{-1}$; Ceftriaxone 64 $\mu g.mL^{-1}$; Meropenem 16 $\mu g.mL^{-1}$) and all of them were susceptible to levofloxacin, tigecycline, and vancomycin. The MIC of 3b was 4 $\mu g.mL^{-1}$ for 4 isolates (including ATCC strain) and 8 $\mu g.mL^{-1}$ for the remaining 6 isolates.

Figure 2 presents the results of the time-kill assay. The 3b demonstrated bactericidal activity (LogΔ CFU ml^{-1} > 3) for all isolates and ATCC 29213 strain at concentrations of 1xMIC and 2xMIC. For control strain, *S. aureus* ATCC29213 the experiment was performed with oxacillin (Figure 2A) and with the 3b (Figure 2B). Figures 2C-K represent, the results of time-kill using 3b for isolates 442, 444, 459, 460, 463, 467, 469, 480 and 487. Each figure shows the results of experiments with 1/2x (blue line), 1x (red line), and 2x (green line) the MIC of 3b for the isolate tested.

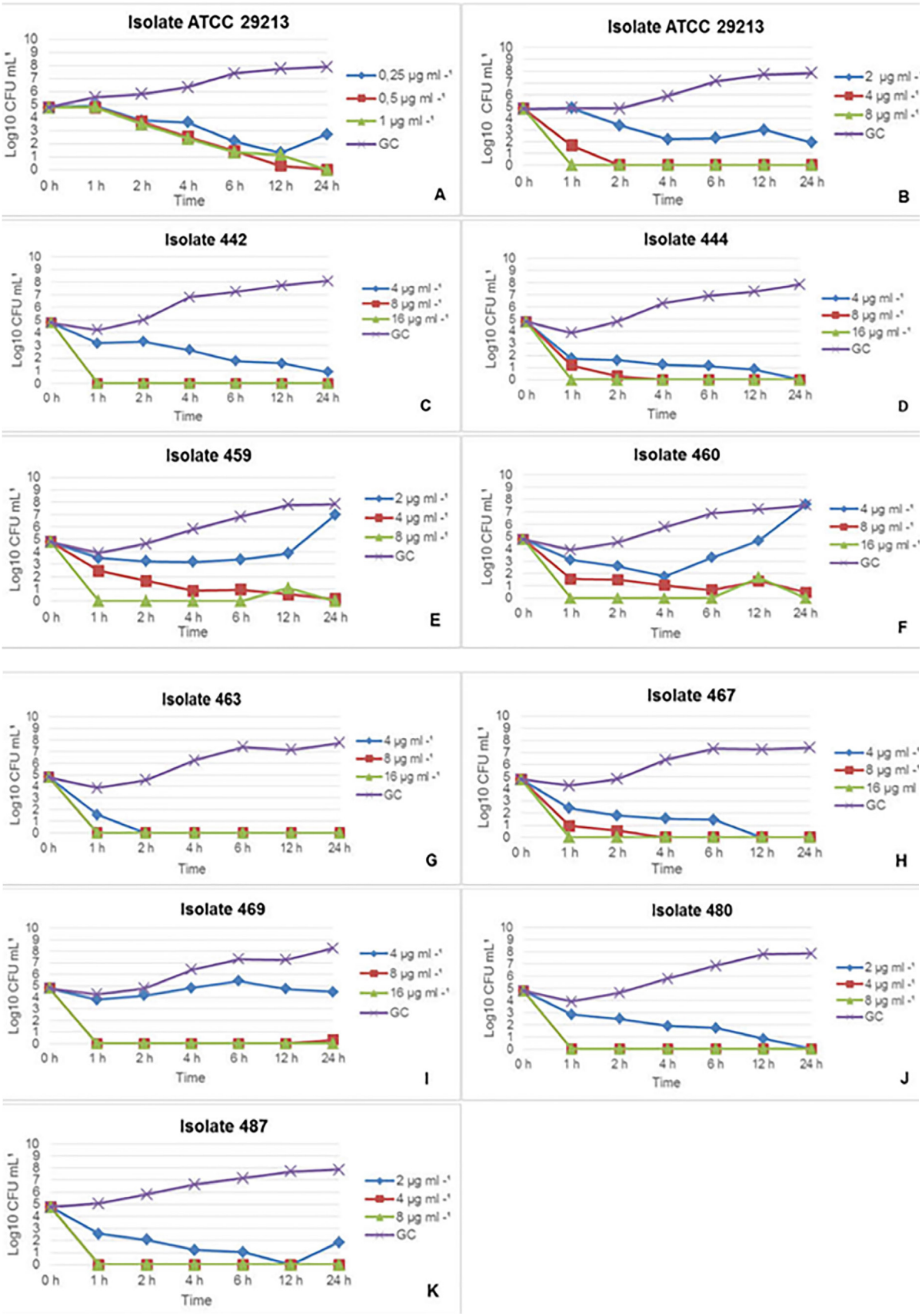

Figure 2 – Time-kill curves of oxacillin and compound 3b for 9 clinical isolates and *S. aureus* (ATCC 29213). A: oxacilin. B-K: compound 3b. dots: 0.5x MIC; square: 1xMIC; triangle: 2xMIC; X: GC - growth control (growth of bacteria without any antibiotic at the medium).

The bactericidal activity (LogΔ CFU ml^{-1} > 3) against isolates 442, 463, 469, 480, and 487 at 1xMIC was observed in the first hour of incubation (Figure 2 C, G, I, and K), while for the isolates 444 and 467 the same effect was demonstrated after 4h (Figure 2, D and H). Compound 3b presented bactericidal activity toward the whole bacterial panel at 2xMIC after 1h (Figure 2 B-K).

At subinhibitory concentrations (0.5xMIC), 3b was bactericidal against isolate 444 (after 24h), 467 (after 12h), 480 (after 24h), and 487 (after 12h) (Figure 2 D, H, J, and K). However, a re-growth of 487 was observed after 24h of the experiment (Figure 2 K).

When the bactericidal activities of oxacillin and 3b were compared, it was observed that the oxacillin colony count reduced to zero after 12h and 24h for *S. aureus* ATCC 29213 strain at 1xMIC and 2xMIC, respectively (Figure 2 A) while compound 3b had the same effect after 4h at 1xMIC and 1h at 2xMIC (Figure 2 A and B). Besides, re-growth of the ATCC strain in the presence of oxacillin at 0.5xMIC was observed after 12h. This behavior was not observed in the presence of 3b (Figure 2 A *vs* Figure 2 B).

The viability of human leukocytes was approximately 98% in the presence of 3b and 100% with PBS (negative control). The 3b (32 $\mu g.ml^{-1}$) caused an increase in DNA strand breaks, which were 173±8.02% ($p < 0.05$) higher than PBS (negative control) but did not cause cell apoptosis or necrosis. Results obtained from the micronucleus test (mutagenicity) were evaluated using Fenech's cytotoxicity index, also known as the nuclear division index. The 3b showed an nuclear division index of 0.03±0.02 ($p < 0.05$), which is similar to the value obtained for PBS (negative control). The nuclear division index for the 3 mM bleomycin (positive control) was 0.31±0.03 ($p < 0.05$). In the allergenicity evaluation, the irritancy score (IS) was 3.47, while 0.1M sodium hydroxide (positive control) showed an irritancy score of 19.9. Thus, 3b was non-irritant, non-mutagenic, and non-cytotoxic according to our assays.

Discussion

Indole compounds are interesting privileged scaffolds molecules explored for different activities in human medicine. Cruz-Muñiz and co-workers demonstrated that several compounds as 3-methoxyphenyl-5-bromo-indole that present anti-cancer activity also has antimicrobial potential (12). Therefore, our group previously evaluated a library of 14 3-chalcogenyl indoles against ATCC strains, including Gram-positive cocci and Gram-negative bacilli. The most promising results were observed for the compound 3b, which encouraged us to increment antibacterial activity evaluation. The 3b has a substituent at the *para* position of the phenyl ring, which seems to be important for the Gram-positive spectrum of action since other indole derivatives without this characteristic did not show activity against Gram-positive at all.

Many studies have been published that showed the antimicrobial activity of the indole derivates. Most of them reported activity against gram-positive bacteria (13-15) including MRSA. ***On the other hand, some authors*** identifying analogs with enhanced antibacterial activity towards gram-negative bacteria and fungi (13,15,16) However, although all of the compounds are indole derivatives, there are variations in the molecules that can alter their activity (16,17).

To the best of our knowledge, this is the first study that evaluated the antibacterial activity of 3b against MRSA. The 3b caused full growth inhibition of several clinical isolates at 1xMIC and 2xMIC. This compound also had a bactericidal effect, which was maintained throughout the experiment (Figure 2). Interestingly, 3b was more active than oxacillin against *S. aureus* ATCC 29213 and showed a bactericidal effect against all clinical MRSA isolates. Daly *et al* (14). have found similar results when they have evaluated the 2,3 Disubstituted indoles against MRSA. Compound 3b demonstrated no evidence of cytotoxicity, mutagenicity, or mucous irritancy, but seems to present low genotoxicity. Nevertheless, the 3b did not cause cell apoptosis or necrosis as have been reported for other indole derivates. Although there can be restrictions on the use of 3b in oral formulations due to its genotoxicity, the application as an antiseptic, disinfectant and antibiofilm can be promising since medical devices are susceptible to *S. aureus* colonization.

Over the last decade, there has been a considerable focus on the prevention of health-associated infections, including those caused by MRSA. Most clinical isolates included in this study were characterized as MRSA and presented resistance to at least 3 different classes of antimicrobials analyzed, which defines them as multidrug-resistant. This scenario is quite similar to what is commonly found in many health institutions in Brazil and around the world (18).

Several authors have reported that preventive measures, such as the use of antiseptic agents for hand hygiene, besides the use of mupirocin and fusidic acid, combined or not with oral vancomycin for skin decolonization before invasive procedures, can reduce the risk of MRSA infections (19). However, bacterial resistance to these topical antibiotics highlights that the discovery and development of new antibacterial agents are urgently required.

Conclusion

In this study, we demonstrated that 3b had a very good antibacterial activity against MRSA without re-growth *in vitro*. Besides that, it was not cytotoxic, mutagenic, and irritant. Therefore, the results obtained in this study suggested that 3-chalcogenyl indoles are promising candidates that merit further study.

Notes

Acknowledgments: Laboratory of Research in Bacterial Resistance (LABRESIS) of the Hospital de Clínicas of Porto Alegre.

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Conflicts of interest disclosure

The authors declare no competing interests relevant to the content of this study.

Authors' contributions

All the authors declare to have made substantial contributions to the conception, or design, or acquisition, or analysis, or interpretation of data; and drafting the work or revising it critically for important intellectual content; and to approve the version to be published.

Availability of data and responsibility for the results

All the authors declare to have had full access to the available data and they assume full responsibility for the integrity of these results.

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