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RESEARCH ARTICLE

Biological Evaluation of Selected 1,2,3-triazole Derivatives as Antibacterial and Antibiofilm Agents

Lialyz Soares Pereira André[1,2,#], Renata Freire Alves Pereira[1,3,#], Felipe Ramos Pinheiro[1,2], Aislan Cristina Rheder Fagundes Pascoal[4], Vitor Francisco Ferreira[5], Fernando de Carvalho da Silva[6], Daniel Tadeu Gomes Gonzaga[7], Dora Cristina Silva Costa[6], Tainara Ribeiro[8], Daniela Sachs[8], and Fábio Aguiar-Alves[1,2,4,*]

[1]Universidade Fluminense Federal, Molecular Epidemiology and Biotechnology Laboratory (LEMB), Rodolpho Albino University Laboratory (LURA), Niterói-RJ, Brazil; [2]Universidade Federal Fluminense, Graduate Program in Pathology and Graduate Program in Applied Microbiology and Parasitology, Niterói-RJ, Brazil; [3]Universidade Federal Fluminense, Graduate Program in Biotechnology, Niterói-RJ, Brazil; [4]Universidade Federal Fluminense, Department of Basic Sciences, Nova Friburgo Health Institute, Nova Friburgo-RJ, Brazil; [5]Universidade Federal Fluminense, Department of Pharmaceutical Technology, Pharmacy School, Niterói-RJ, Brazil; [6]Universidade Federal Fluminense, Instituto de Química, Departamento de Química Orgânica, Niterói-RJ, Brazil; [7]Universidade Estadual da Zona Oeste, Rio de Janeiro-RJ, Brazil; [8]Universidade Federal de Itajubá, Department of Physics and Chemistry, Institute of Exact Sciences, Itajubá-MG, Brazil

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Abstract: ***Background*:** Resistance to antimicrobial agents is a major public health problem, being *Staphylococcus aureus* prevalent in infections in hospital and community environments and, admittedly, related to biofilm formation in biotic and abiotic surfaces. Biofilms form a complex and structured community of microorganisms surrounded by an extracellular matrix adhering to each other and to a surface that gives them even more protection from and resistance against the action of antimicrobial agents, as well as against host defenses.

***Methods*:** Aiming to control and solve these problems, our study sought to evaluate the action of 1,2,3-triazoles against a *Staphylococcus aureus* isolate in planktonic and in the biofilm form, evaluating the activity of this triazole through Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests. We have also performed cytotoxic evaluation and Scanning Electron Microscopy (SEM) of the biofilms under the treatment of the compound. The 1,2,3-triazole **DAN 49** showed bacteriostatic and bactericidal activity (MIC and MBC 128 μg/mL). In addition, its presence interfered with the biofilm formation stage (1/2 MIC, $p < 0.000001$) and demonstrated an effect on young preformed biofilm (2 MICs, $p < 0.05$).

***Results*:** Scanning Electron Microscopy images showed a reduction in the cell population and the appearance of deformations on the surface of some bacteria in the biofilm under treatment with the compound.

***Conclusion*:** Therefore, it was possible to conclude the promising anti-biofilm potential of 1,2,3-triazole, demonstrating the importance of the synthesis of new compounds with biological activity.

Keywords: Heterocycles, Azoles, MRSA, Biofilm. Antibacterial, Antibiofilm agent.

1. INTRODUCTION

Resistance to antimicrobial agents is one of the major public health challenges of our time [1]. *Staphylococci*, with the leading species *Staphylococcus aureus* and *Staphylococcus epidermidis* are prevalent in infections in hospital and community settings and admittedly biofilm-forming agents [2].

Biofilms form a complex and structured community of microorganisms surrounded by an extracellular matrix of polysaccharides adhering to each other and to a surface [3]. Høiby, Costerton and their collaborators were the first to suspect a direct correlation between persistent infections and the development of biofilms, with *Pseudomonas aeruginosa* being the cause of chronic lung infection in patients with

*Address correspondence to this author at the Universidade Fluminense Federal, Molecular Epidemiology and Biotechnology Laboratory (LEMB), Rodolpho Albino University Laboratory (LURA), Niterói-RJ, Brazil; Universidade Federal Fluminense, Graduate Program in Pathology and Graduate Program in Applied Microbiology and Parasitology, Niterói-RJ, Brazil and Universidade Federal Fluminense, Department of Basic Sciences, Nova Friburgo Health Institute, Nova Friburgo-RJ, Brazil;
Tel: +55 (21) 985251891. E-mail: faalves@gmail.com
[#]*Contributed equally to the publication*

cystic fibrosis (CF) [4, 5]. A case report in 1982 presented the first evidence of involvement of biofilms in device-related infection by an electron microscopic study of a pacemaker electrode in a patient with recurrent infection of the bloodstream by *Staphylococcus aureus* (BSI) [6]. Over the last decades, many types of human infection have been found to progress with the involvement of biofilms or originate from biofilm-associated primary infections [7, 8].

These trends have emphasized the pressing need for new, more effective and safe antibacterial agents, especially those with novel mechanisms of action [9]. At present, the role of heterocyclic compounds has become increasingly important in the planning of new classes of structural molecules for biological activity [10]. Among the heterocycles, 1,2,3-triazoles and their derivatives have attracted considerable attention because of their interesting biological activities. The triazole ring is an important pharmacophoric group found in first-choice drugs used to treat mycoses. They have antiviral, antitumorigenic [11-13], analgesic [14-16], anti-inflammatory [17], antimalarial [18], antituberculosis [19], antidepressant, and anticonvulsant [20] properties.

Therefore, this study aims to investigate the antibacterial and antibiofilm properties of three 1,2,3-triazole derivatives against an *S. aureus* clinical isolate strain.

2. MATERIAL AND METHODS

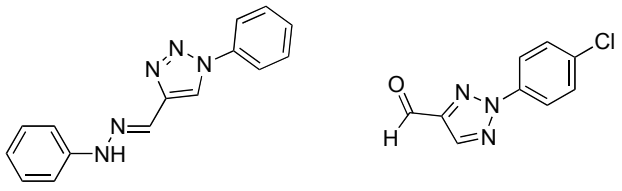
2.1. Biological Assays

2.1.1. Bacterial Strain

S. aureus (HU25) clinical isolate used in this study was described as a methicillin-resistant *S. aureus* (MRSA). Isolated from the Hospital Universitário Clementino Fraga Filho (HUCFF), it has been described in the literature as a carrier of *mec*A gene and susceptible to vancomycin [21].

2.1.2. 1,2,3-Triazole Derivatives

In this work, two compounds (Fig. **1**) were found promising in the preliminary antibacterial study from the DAN series, viz., **DAN 06** (1*H*-1,2,3-triazole) and **DAN 49** (2*H*-1,2,3-triazole) that were synthesized in the Applied Organic Synthesis Laboratory of the Universidade Federal Fluminense [22].



DAN 06

(*E*)-1-phenyl-4-((2-phenylhydrazineylidene)methyl)-1*H*-1,2,3-triazole

DAN 49

2-(4-chlorophenyl)-2*H*-1,2,3-triazole-4-carbaldehyde

Fig. (1). Molecular structure of selected 1,2,3-triazole derivatives. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

2.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Derived triazoles were diluted in a 96-well microplate (KASVI) with 100 µL of growth medium in geometrically decreasing concentrations (512 µg/mL to 4 µg/mL). Then, 100 µL of bacterial suspension (0.5 McFarland) in saline was added to each well. The concentration of DMSO used in this system was 2.5 %, which had no effect on bacterial growth. Vancomycin was used as positive control against the Gram-positive strain. After 24 hours of incubation at 37 °C, the result of the Resazurin dye was analyzed. Experiments were performed in triplicate and MIC was the lowest concentration of the substance that prevented visible bacterial growth (pink indicates bacterial growth and blue represents the absence of growth) [23]. TSA media incubation plates divided into nine fields, received 10 µL of the respective dilution in each field, following incubation at 37 ºC/24 h. After this interval, the smallest concentration able to eliminate 99.9 % of the inoculum (MBC) was defined, the field in which there was no bacterial growth.

2.2.1. Microtiter Dish Biofilm Formation Assay

In order to study the early stages in biofilm formation as well as their mature form, microtiter dish assays were performed. *Staphylococcus aureus* HU25 was inoculated into Brain Heart Infusion (BHI) medium supplemented with 1 % glucose and incubated in a shaker (250 rpm) at 37 °C for 20 h. The bacterial culture was then diluted (1:100) in the same medium containing sub-minimal concentrations (1/2xMIC, 1/4xMIC and 1/8xMIC) of the different derivatives. A volume of 200 µL of this mixture was added to each well of a 96-well inert polystyrene microtiter plate (Nunc; Nunclon). After incubation at 37 °C for 24 h, the supernatants were removed and the biofilms were rinsed once with ultrapure water. The microplate was dried and fixed at 65 °C for 1 h. Finally, the biofilms were stained with crystal violet solution (1 %), washed with water, and read at 570 nm using the BMG FLUOstar® OPTIMA. Vancomycin (1/2xMIC, 1/4xMIC and 1/8xMIC) was also used for comparative purposes and 2.5 % DMSO was used as a negative control.

The influence of active derivatives on preformed biofilms was initially analyzed against biofilm formation by *S. aureus* HU25, as described above, without the derivatives. After 6 h (young biofilm) and 24 h (mature biofilm) of incubation, the medium was carefully removed and 1/2 - 2xMIC of the derivatives were added to each well of the microtiter plate. After 24 h of incubation, the assay was analyzed as described above. All experiments were performed in triplicates.

2.2.2. Analysis of the Cytotoxic Effect

3T3 cells were cultured in RPMI medium with 10% Fetal Bovine Serum in a stove at 37 °C with 5 % CO_2. The cells were seeded in a 96-well plate, 3x10 4 cells / ml and after 24 h, they were treated with the compounds dissolved in RPMI medium with 1 % DMSO. Plates were incubated in the oven at 37 °C with 5% CO_2. After 24 hours of treatment, the medium was withdrawn and the MTT solution added and the plate was kept in the oven for 4 hours, after which, the MTT solution was withdrawn and the formazan crystals were dissolved with DMSO. Cells treated with vehicle alone were considered for 100 % cell viability.

2.2.3. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to comparatively illustrate the biofilms formed on the glass coverslips by HU25 with and without the influence of the triazole C (DAN 49). The coverslips were previously placed in 24-well plates with 2 mL of BHI broth supplemented with 5 % sucrose and inoculated with 0.1 mL of the bacterial suspension (1.5×10^8 CFU/mL). The samples were incubated at 37 ºC / 48 h, and the medium was changed after 24 h. After this period, the disks were fixed for 1 h in 2.0 % glutaraldehyde and dehydrated in several washes with ethanol (10, 25, 50, 75 and 90 % for 20 minutes and absolute alcohol for 1 h). The samples were dried for 24 hours at 37 °C and subsequently were coated with gold in a low-pressure atmosphere with an ion spray coating [24]. The surface topographies of the biofilms were visualized in a scanning electron microscope (Carl Zeiss® Evo MA 15).

2.2.4. Statistical Analysis

The experiments were independently performed in triplicate, and the results are shown as mean ± SD. Statistical significance was determined by unpaired t-test using the GraphPad software (GraphPad Prism 8.0.2). P values <0.05 were of statistical significance.

3. RESULTS AND DISCUSSION

The *S aureus* HU25 clinical isolate was classified as a strong biofilm former. The search for new alternatives for the treatment of infections caused by biofilm-forming bacteria is a major challenge, with actions aimed at prevention, destabilization of the polymeric matrix, weakening of the bacterial communication system (quorum detection) or even the death of the microbial community [25]. The use of new synthetic compounds has been an alternative for the control of infections caused by biofilm-producing strains. Based on recent publications, some triazoles and their derivatives have exhibited a wide range of biological activities [26-28]. Studies on the 1,2,3-triazoles have shown that hydrogen bonds and dipole interactions of the triazole nucleus may favor their bond for biomolecular targets and improve their solubility [29]. The triazole **DAN 06** presented MIC and MBC >512 μg/mL, while **DAN 49** presented bacteriostatic and bactericidal activity (MIC and MBC 128 μg/mL) for the *S. aureus* HU25 clinical strain. After the screening phase, the derivative that obtained activity in the MIC assay was investigated for its effect against biofilm formation in subinhibitory concentrations (MIC from 1/8 to 1/2) after 24 hours of incubation. The compound **DAN 49** presented an inhibitory effect over the initial processes of the biofilm formation by HU25 at 1/2 MIC (p value <0.000001) in comparison to vancomycin activity and the negative control (Fig. **2A**). Results observed in (Fig. **2A**) show that there was a significant reduction in the production of biofilm after treatment with the synthetic 1,2,3-triazole derivative **DAN 49**. The tested substances (**DAN 49** and vancomycin) were able to reduce the production of *S aureus* HU25 biofilm in the concentra-

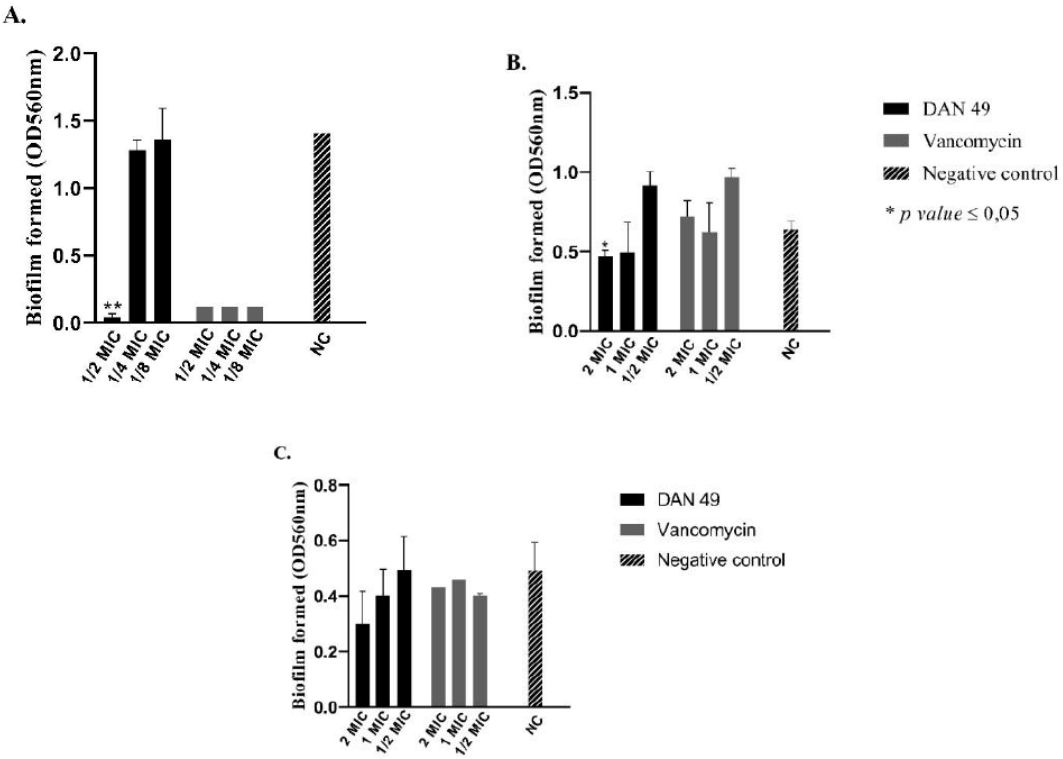


Fig. (2). Graphics of HU25 biofilm formation, young and mature biofilm under the influence of 1,2,3-triazole **DAN 49**. A). Effect of the triazole derivative 1,2,3-triazole **DAN 49** against the initial biofilm formation process by *Staphylococcus aureus* HU25. The vertical columns and bars represent the mean ± SD, *p <0.05. B) Effects of the 1,2,3-triazole **DAN 49** against young preformed biofilm by *Staphylococcus aureus* HU25. C) Effects of the 1,2,3-triazole **DAN 49** against the HU25 mature preformed biofilm. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

A.

Sample concentration (µg/mL)	% of viable cells
I 125	51,90
I 250	31,69
I 500	30,66
I 1000	65,46
I 2500	62,25

B.

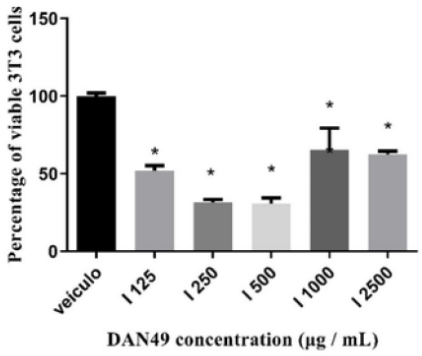


Fig. (3). Cytotoxic effect of triazole C against 3T3 Cells. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

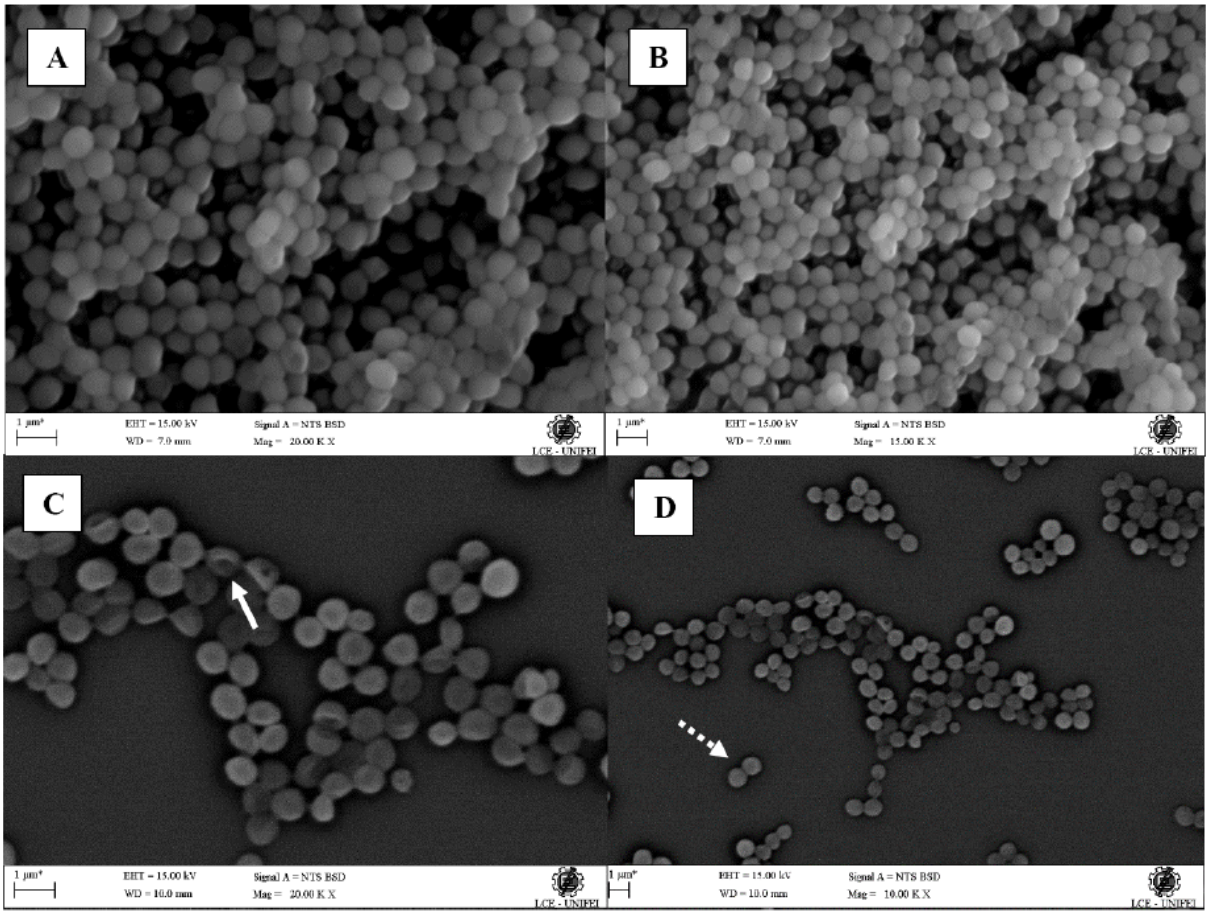


Fig. (4). SEM analysis of the *S. aureus* Biofilm. A and B) Biofilm formed by HU25 without the presence of 1,2,3-triazole **DAN 49**. Mag = 20.00 KX; and Mag = 15.00 KX, respectively. B and C) Biofilm formed by HU25 in the presence of 1,2,3-triazole **DAN 49**, detailing the presence of bacterial cells with deformed structure (white arrow) and the appearance of isolated cells (dotted arrow), when compared to the normal biofilm formed by HU25. Mag = 20.00 KV; and Mag = 10.00 KV, respectively. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

tion of 1/2 MIC (Fig. **2A**). Vancomycin, despite demonstrating a significant anti-biofilm action in the early stages of biofilm formation, is potentially nephrotoxic, often prolonging hospitalization, and in some circumstances, dialysis treatment thereby increasing medical costs and mortality [30]. The activity of **DAN 49** derivative against the preformed young and mature HU25 biofilm was tested using higher concentrations (1/2 MIC to 2 MIC). At a concentration of 2 MICs, the substance has shown to significantly reduce (p-value <0.05) the preformed young biofilm (Fig. **2B**). However, in a more advanced stage of the biofilm formation (24 hours of incubation), the substance did not show a significant reduction comparing to the controls (p-value >0.05) (Fig. **2C**).

Cytotoxicity tests performed with 3T3 cells revealed that, even at the concentration of 2,500 µg/mL of C, 62.25 % of the cells remained viable (Fig. **3**). This concentration was approximately 19 times the concentration that inhibited bacterial growth, demonstrating that in MIC concentrations, the substance has no cytotoxic effect and can be considered a safe substance to be used as an antimicrobial agent to treat humans.

To access the biological activity of these substances, we also performed Scanning Electron Microscopy. The images show a dense and uniform cell mass for HU25 biofilm under normal conditions (without the influence of 1,2,3-triazole **DAN 49**) (Figs. **4A** and **4B**). There was, apparently, an interference in the biofilm production in the presence of the tested 1,2,3-triazole **DAN 49** (Figs. **4C** and **4D**). In (Fig. **4C**), a process of destabilizing the formation of biofilm by strain HU25 under influence of the 1,2,3-triazole **DAN 49** has been seen. The cells, which were previously clustered in groups of bacteria were now dispersed. We also observed a reduction in the population of bacterial cells when compared to the biofilm formed without the presence of the 1,2,3-triazole. This suggests that 1,2,3-triazole **DAN 49** may act in the mechanism of bacterial adhesion. In addition, (Fig. **4C**) demonstrates the presence of deformations on the surface of the bacterial cell (white arrow), which may indicate that triazole has caused damage to the bacterial cell wall during the treatment. Isolated cells were also observed (Fig. **4D**), suggesting a disintegration of the biofilm caused by 1,2,3-triazole **DAN 49** (dotted arrow).

S. aureus resistance has been increasing in both hospital and community infections, especially among MRSA strains. The ability to form biofilm can cause even more serious complications, involving endocarditis, toxic shock syndrome and pneumonia [31-33]. Our results with the 1,2,3-triazole **DAN 49** are a promising alternative to be explored as a treatment against the life-threatening Methicillin-resistant *S. aureus* strains.

CONCLUSION

The triazole derivative C demonstrated important biological activity against *S. aureus* HU25, with bacteriostatic and bactericidal activities in the MIC and MBC tests. In addition, it was able to inhibit bacterial growth and reduce the biofilm production of this deadly *S. aureus*-multiresistant-clinical isolate, as seen in SEM images. These results suggest that 1,2,3-triazole **DAN 49** may be effective against both forms of the bacteria, even interfering with the adhesion phase of the biofilm.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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