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SHORT COMMUNICATIONS

Potentiation of Antibiotic Activity by a Meldrum's Acid Arylamino Methylene Derivative against Multidrug-Resistant Bacterial Strains

Maria M. C. da Silva[1] · José B. de Araújo-Neto[1] · Ana C. J. de Araújo[1] · Priscilla R. Freitas[1] · Cícera D. de M. Oliveira-Tintino[1] · Iêda M. Begnini[3] · Ricardo A. Rebelo[3] · Luiz E. da Silva[2] · Sandro L. Mireski[3] · Michele C. Nasato[3] · Maria I. L. Krautler[3] · Jaime Ribeiro-Filho[4] · Henrique D. M. Coutinho[1] · Saulo R. Tintino[1]

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Abstract This study aimed to evaluate the intrinsic antibacterial activity and antibiotic-enhancing effect of an arylamino methylene derivative (MAD) in association with fluoroquinolones. The antibacterial activity against multiresistant *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* was analyzed by determining the minimum inhibitory concentration (MIC) using the broth micro dilution method. A reduction in the MIC of the fluoroquinolones against strains treated simultaneously with the MAD was interpreted as an enhanced antibiotic activity. While the MAD exhibited no clinically effective action (MIC $\geq$ 1.024 μg/mL), it was found to significantly potentiate the activity of norfloxacin, ofloxacin and lomefloxacin against all the strains, which may be related to structural similarities between the MAD and quinolones. Our findings suggest that Meldrum's acid arylamino derivatives may represent promising molecules in the elaboration of new drugs to reverse resistance to fluoroquinolones.

✉ Henrique D. M. Coutinho
hdmcoutinho@gmail.com

[1] Laboratory of Microbiology and Molecular Biology (LMBM), Department of Biological Chemistry, Regional University of Cariri—URCA, Rua Cel. Antonio Luiz, 1161. Pimenta, Crato, CE CEP: 63105-000, Brazil

[2] Postgraduate Program in Sustainable Territorial Development—Coastal Sector, Federal University of Paraná—UFPR, Curitiba, Brazil

[3] Department of Chemistry, Regional University of Blumenau—FURB, Blumenau, Brazil

[4] Laboratory of Investigation in Genetics and Translational Hematology, Gonçalo Moniz Institute, Oswaldo Cruz Foundation—IGM-FIOCRUZ/BA, Salvador, BA, Brazil

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Infections by resistant bacteria are important causes of mortality in Brazil and all over the world [1]. Despite the significant advances in drug development for the treatment of infectious diseases, the production of new antibiotics has not been enough to overcome the speed with which new resistance mechanisms are developed. Therefore, the search for compounds capable of modulating resistance to conventional antibiotics represents a promising strategy in antibacterial therapy [2]. In this context, evidence indicates that substances capable of potentiating the action of antibiotics in combination therapy have the potential for use in the treatment of infections caused by multiresistant bacterial strains [3].

The Meldrum's acid is a chemical compound widely used in the synthesis of heterocycles, among which the arylamino methylene derivatives stand out for having significant biological potential. Importantly, studies have shown that these compounds are capable of interfering with the biological mechanisms of sev

eral microorganisms [4]. However, the antibacterial properties of these compounds remain to be better characterized experimentally. Therefore, this work aimed to evaluate the intrinsic antibacterial activity and antibiotic-enhancing effect of the arylamino methylene derivative N-{6-[(2,2-Dimethyl-4,6-dioxo-[1,3]dioxane-5-ylidenomethyl)-amino]-pyridin-2-yl}-acetamide (MAD) in association with fluoroquinolones.

The Meldrum's acid derivative N-{6-[(2,2-Dimethyl-4,6-dioxo-[1,3]dioxane-5-ylidenomethyl)-amino]-pyridin-2-yl}-acetamide (MAD) (Fig. 1) was synthesized by Prof. Dr. Luiz E. da Silva from the Federal University of Paraná

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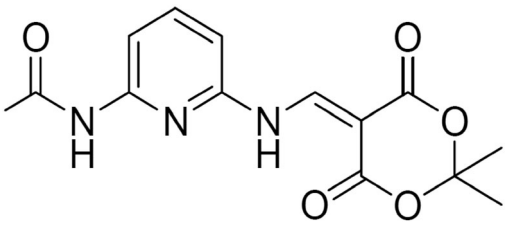

Fig. 1 Chemical Structure of N-{6-[(2,2-Dimethyl-4,6-dioxo-[1,3]-dioxane-5-ylidenomethyl)-amino]-pyridin-2-yl}-acetamide

(UFPR). The fluoroquinolone antibiotics norfloxacin, ofloxacin, and lomefloxacin (SIGMA Chemical Co.) and MAD were dissolved in 1 mL of DMSO and 8.765 µL of distilled water to 1.024 µg/mL [5] (Fig. 2).

This study used the MDR strains *Escherichia coli* 06, *Staphylococcus aureus* 10 and *Pseudomonas aeruginosa* 24. The bacteria were cultured on Heart Infusion Agar (HIA, Difco Laboratories) for 24 h before the experiments at 37 °C. For inoculum preparation, a sample of each bacterial culture was transferred to test tubes containing 5 mL of sterile saline (0.9% NaCl). After homogenization, the turbidity was compared to the McFarland scale, which corresponds to 10^5 CFU [6].

Following inoculum preparation, the MIC determination was performed according with the previously reported method [7]. Wells containing only the inoculum in BHI were used as growth control. Bacterial growth was analyzed by adding sodium resazurin to each well and incubating the plate at room temperature for 1 h. The MIC was defined as the lowest concentration capable of inhibiting bacterial growth, as observed by a change in the color of the solution from blue to pink. All experiments were performed in triplicate.

Antibiotic resistance modulation was analyzed following the method reported above. Each bacterial inoculum was prepared as previously described, and the compound was added at a concentration equivalent it's MIC/8. All tests were carried out in triplicate for all strains. Data were analyzed using GraphPad Prism software version 5.0 by analysis of variance (ANOVA), followed by Bonferroni's post-test ($p < 0.05$ considered significant).

The analysis of the antibacterial activity of the MAD found MIC values above 1.024 µg/mL against *E. coli* 06, *P. aeruginosa* 24 and *S. aureus* 10, indicating that this synthetic derivative has no clinically relevant antibacterial activity. However, the compound was found to enhance the activity of fluoroquinolone antibiotics against these MDR bacteria.

In this context, while in *E. coli* cultures norfloxacin, ofloxacin and lomefloxacin presented MIC values of 16, 32 and 16 µg/mL, respectively; when associated with the synthetic derivative, these values were reduced to 8, 4 and 8 µg/mL respectively. In cultures of *S. aureus* 10, norfloxacin, ofloxacin and lomefloxacin showed MIC values of 128, 16 and 64 µg/mL, respectively, which were reduced to 64, 8 and 16 µg/mL, respectively, in the presence of sub inhibitory concentrations of MAD. Finally, against *P. aeruginosa,* the association with MAD caused significant reduction in the MIC of these antibiotics. Together, these data indicate that MAD is capable of modulating antibiotic resistance in both Gram-positive and Gram-negative strains

The present study demonstrated the antibacterial properties of a Meldrum's acid derivative against MDR bacterial strains. The study demonstrated that the MAD had no clinically relevant antibacterial activity against strains of *S. aureus* 10, *P. aeruginosa* 24 and *E. coli* 06 [8]. However, this compound significantly modulated the resistance to antibiotics norfloxacin, ofloxacin and lomefloxacin in all tested strains. This finding can be related to the structural similarity between the MAD and fluoroquinolones,

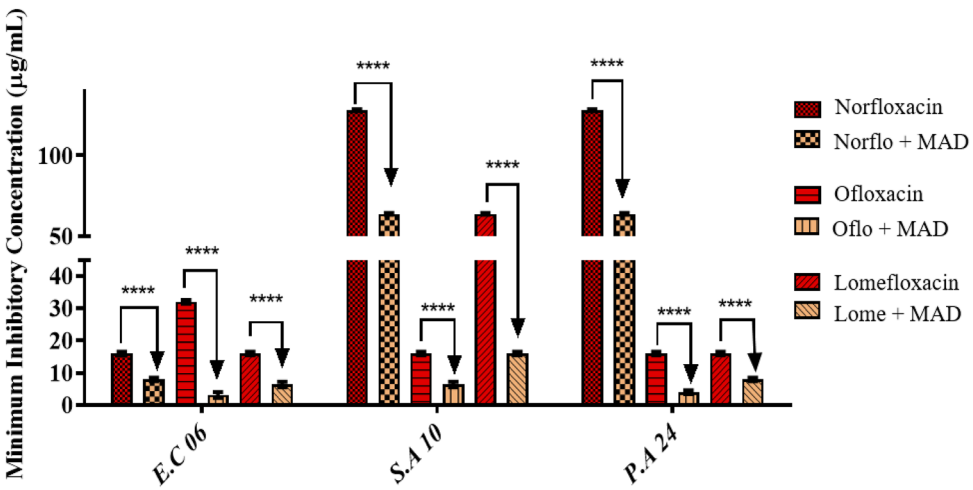

Fig. 2 Modulation of antibiotic resistance by N-{6-[(2,2-Dimethyl-4,6-dioxo-[1,3]dioxane-5-ylidenomethyl)-amino]-pyridin-2-yl}-acetamide (MAD) in association with norfloxacin, ofloxacin and lomefloxacin against MDR of *E. coli*, *S. aureus* and *P. aeruginosa*. **** $p < 0.0001$ indicates significant differences between groups

suggesting the existence of similar mechanisms of action and effects on bacterial growth [9].

The fluoroquinolones inhibit nucleic acid synthesis by dually targeting DNA gyrase and Topoisomerase type IV. Since these enzymes actively participate in the cleavage of the double-stranded DNA, their inhibition results in the disordered synthesis of proteins and messenger RNA, altering the replication of DNA and thus, harming bacterial growth. However, bacteria can develop resistance to the mechanism of action of fluoroquinolones through single or double mutations [10].

Vanillidene derivatives of Meldrum's acid presented clinically ineffective antibacterial activity against strains of *E. coli*, *S. aureus*, and *P. aeruginosa*. Similar effects were demonstrated by heterocyclic compounds derived from the Meldrum's acid against the same bacterial strains in studies using the disk diffusion method [11].

Fluoroquinolones have a high capacity of penetrating the cell wall of both Gram-positive and Gram-negative bacteria, altering their biological activities, through the inhibition of intracellular mechanisms. Thus, it is suggested that N-{6-[(2,2-Dimethyl-4,6-dioxo-[1, 3]dioxane-5-ylidenomethyl)-amino]-pyridin-2-yl}-acetamide could act reversing the mechanisms of resistance to fluoroquinolones at the intracellular level [12].

E. coli cultures showed that Meldrum's acid derivatives presented significantly lower MIC values compared to the fluoroquinolone ciprofloxacin. In addition, molecular docking analyses suggested that, like fluoroquinolones, these compounds could bind to topoisomerase II, which could favor the occurrence of synergistic effects between the MAD and fluoroquinolones. Accordingly, evidence suggests that structural changes in norfloxacin may result in potentiated antibiotic elevation, which corroborates the possibility that interactions between this drug and the Meldrum's acid derivatives may result in improved antibacterial action [13].

Meldrum's acid derivatives can also potentiate the antibacterial activity of aminoglycosides, a class of antibiotics that inhibits protein synthesis through a direct action on bacterial ribosomes [14]. Therefore, the possibility of interference with different mechanisms that lead to inhibition of protein synthesis reinforces the possibility that the MAD can modulate resistance to fluoroquinolones. In general, the presence of a lipid bilayer in Gram-negative bacteria hinders the intracellular action of most known antibacterial drugs. However, hydrophobic antibiotics such as quinolones can invade bacterial cells more easily. Moreover, evidence demonstrates that these antibiotics manage to cross the external barrier of Gram-negative bacteria such as *E. coli* through channels formed by porins [15].

The heterocyclic compound N-{6-[(2,2-Dimethyl-4,6-dioxo- [1, 3] dioxane-5-ylidenomethyl) -amino] -pyridin-2-yl) -acetamide showed no clinically antibacterial activity effective against MDR strains of *E. coli*, *S. aureus* and *P. aeruginosa*. However, this compound was found to significantly enhance the action of fluoroquinolones against all strains evaluated in this study. Our findings indicate that Meldrum's acid arylamino derivatives may represent promising molecules in the formulation of new drugs to reverse resistance to fluoroquinolones.

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