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Tetracycline improved the efficiency of other antimicrobials against Gram-negative multidrug-resistant bacteria

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Summary Treatment of infectious diseases with antimicrobials constituted a great achievement in the history of medicine. Unfortunately, the emergence of resistant strains of bacteria to all classes of antimicrobials limited their efficacy. The present study was aimed at evaluating the effect of combinations of antibiotics on multi-drug resistant Gram-negative (MDRGN) bacteria.

A liquid micro-broth dilution method was used to evaluate the antibacterial activity of 10 different classes of antimicrobials on 20 bacterial strains belonging to six different species. The antimicrobials were associated with phenylalanine β-naphthylamide (PAβN), an efflux pump inhibitor, and with other antimicrobials at their sub-inhibitory concentrations. The effectiveness of each combination was monitored using the minimal inhibitory concentration (MIC) and the fractional inhibitory concentration (FIC).

Most of the antimicrobials tested showed low antibacterial activity with a MIC value of 128 mg/L on a majority of the bacterial strains, justifying their multidrug-resistant (MDR) profile. Synergistic effects were mostly observed (FIC $\leq$ 0.5) when ampicillin (AMP), cloxacillin (CLX), erythromycin (ERY), chloramphenicol (CHL), kanamycin (KAN) and streptomycin (STR) were combined with tetracycline (TET) at the sub-inhibitory concentration of MIC/5 or MIC/10.

The results of the present work suggest that the association of several antimicrobials with TET could improve the fight against MDRGN bacterial species.

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Introduction

In the twenty-first century, infectious diseases continue to ravage the human population, and they account for approximately half of the mortality rates in tropical countries. These alarming statistics indicate their devastating nature. Unfortunately, the worldwide dissemination of multi-drug-resistant bacteria has severely reduced the efficacy of antibacterial agents, thus increasing therapeutic failures [1]. One of the major antibiotic resistance mechanisms utilized by bacteria is active efflux. Efflux pumps (EPs) involved in this type of resistance are membrane-associated active transporters promoting the extrusion of toxic compounds, including antimicrobials. This extrusion decreases the intracellular concentration of antimicrobials and reduces the susceptibility of bacterial strains to these drugs. Therefore, antibiotic therapy has more than ever become a challenge for scientists, and new means of tackling resistant bacteria are urgently needed. The use of synergistic antibiotic association is an appealing way to optimize good results during antibiotherapy [2]. Although antibiotic combinations have long been used against multiple potential pathogens in the initial empirical treatment of critically ill patients, the selection of such drugs and their potential for providing increased activity in combination should be made with particular attention to minimize any negative interactions [3]. This work was aimed at evaluating the effect of antibiotic combinations against MDRGN bacteria.

Material and methods

Chemicals for antimicrobial assays

Tetracycline (TET), doxycycline (DOX), cefepime (FEP), streptomycin (STR), ciprofloxacin (CIP), norfloxacin (NOR), chloramphenicol (CHL), cloxacillin (CLX), ampicillin (AMP), erythromycin (ERY), and kanamycin (KAN) (Sigma-Aldrich, St Quentin Fallavier, France) were used as reference antibiotics. *p*-Iodonitrotetrazolium chloride (INT) and phenylalanine arginine β-naphthylamide (PAβN) were used as microbial growth indicators and efflux pump inhibitors (EPIs), respectively.

Bacterial strains and culture media

The studied microorganisms included reference (American Type Culture Collection) and clinical (laboratory collection) strains of *Providencia stuartii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter aerogenes* and *Enterobacter cloacae*. The bacterial strains and their characteristics were previously reported [4], as shown in Table S2. The preliminary treatment of these organisms and the culture media were prepared as described in a previous study [5].

Antibiotic susceptibility tests

Antibiotic susceptibility tests using single antimicrobials and in combination with PAβN

The MICs of each of the 10 antimicrobials used were determined for 20 bacterial strains using the rapid INT colorimetric assay [6,7]. One hundred microliters of prepared antimicrobial was added in the first well of each column of a micro titer plate containing 100 μl of broth in each well and were than serially diluted twofold. Next, 100 μl of the inoculum prepared in Mueller Hinton broth (MHB, Sigma-Aldrich) was added to have a final concentration of 10^6 UFC/ml. The final concentration of DMSO was 2.5% and did not affect the microbial growth. The plates were covered with a sterile plate sealer and were agitated to mix the content of the wells using a shaker. The MICs of the samples were determined after 18 h of incubation at 37 °C, following the addition (40 μl) of 0.2 mg/ml INT and incubation at 37 °C for 30 min [8,9]. The MIC was defined as the lowest concentration of antimicrobial that prevented color change of the medium and exhibited complete inhibition of microbial growth [6,8].

The role of efflux pumps in the susceptibility of Gram-negative bacteria to the antimicrobials used in this study

The antibacterial susceptibility test in the presence of the efflux pump inhibitor was carried out after the twofold serial dilution by adding 20 μl of PAβN (final concentration of PAβN 20 mg/L) to 100 μl of MHB in the wells of the micro titer plate. Then, 80 μl of the inoculum was introduced to obtain a final concentration of 10^6 UFC/ml. Wells containing MHB, 100 μl of inoculum, and DMSO at a final concentration of 2.5% served as negative controls (this internal control was systematically added). The total volume in each well was 200 μl. The MICs of the antimicrobials were obtained after 18 h of incubation at 37 °C, as mentioned above.

The inhibitory activity of the combined antimicrobials

The MICs of the two combined antimicrobials were monitored as follows: 100 μl of the antibiotic, A1,