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The effects of D-Tyrosine combined with amikacin on the biofilms of *Pseudomonas aeruginosa*

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ABSTRACT

The biofilm formation of microorganisms causes persistent tissue infections resistant to treatment with antimicrobial agents. *Pseudomonas aeruginosa* is commonly isolated from the airways of patients with chronic fibrosis (CF) and often forms biofilms, which are extremely hard to eradicate and a major cause of mortality and morbidity. Recent studies have shown that D-amino acids (D-AAs) inhibited and disrupted biofilm formation by causing the release of the protein component of the polymeric matrix. However, the effects of D-AAs combined with common antibiotics on biofilms have rarely been studied. The current study first determined whether D-AAs disrupted the biofilms of PAO1 and the clinical airway isolates of *P. aeruginosa.* It was then determined whether combinations of D-Tyr (the most effective one) and the antibiotic amikacin (AMK) enhanced the activity against these biofilms. The results of the current study showed that D-Tyr is the most effective among those that disassemble the D-amino acids (D-leucine, D-methionine, D-Tyrptophan, and D-tryptophan), and D-Tyr at concentrations higher than 5 mM significantly reduced the biofilm biomass of *P. aeruginosa* ($p < 0.05$) without influencing bacterial growth. It was also revealed that D-Tyr improved the efficacy of AMK to combat *P. aeruginosa* biofilms, as indicated by a reduction in the minimal biofilm-inhibiting concentration ($MBIC_{50}$ and $MBIC_{90}$) without a change in the minimal inhibitory concentration (MIC) of planktonic bacteria. Thus, the findings indicated that D-Tyr supplementation overcame the resistance of *P. aeruginosa* biofilms to AMK, which might be helpful for preventing AMK overuse when this specific D-Tyr is recommended for combatting these biofilms. Also, toxicity of the liver and kidney from AMK could be potentially mitigated by co-delivery with D-Tyr.

1. Introduction

Bacteria are able to live either as independent planktonic cells or as members of organized surface-attached microbial communities called biofilms, which are composed of microorganisms and the extracellular matrix-forming polymers they produce [1]. In contrast to their planktonic counterparts, biofilm-derived bacteria have a distinctive phenotype regarding metabolic activity and gene expression, conferring an inherent resistance to antimicrobial agents as well as mechanisms of host clearance and making the treatment of biofilm-associated infections extremely difficult [2]. Biofilms persist and are hard to eradicate because of mechanisms that involve the restricted penetration of antimicrobials, differential physiological activity, and the presence of phenotypic variants and persisters, efflux systems, and enhanced repair systems [3]. Resistance is often genetically inherited and therefore transmitted progeny of bacterial colonies, or it can be acquired through horizontal gene transfer [4].

Pseudomonas aeruginosa is commonly isolated from the airways of patients with cystic fibrosis (CF). Colonization can occur both in the paranasal sinuses and lungs, where it most often establishes chronic infections that usually persist for the rest of the patients' lives [5], eventually leading to respiratory failure and lung transplantation or death [6]. Despite improvement in therapies and a considerable increase in longevity over the past decades, the prevalence of *P. aeruginosa* in respiratory cultures increases with age from approximately 30% at ages 0–5 years to 80% at 18 years and older [7,8], so it still represents a therapeutic challenge. Clinical data suggest that *P. aeruginosa* forms antibiotic-resistant biofilms in the CF lung, which hinders the efficacy of currently available antibiotics and precludes the eradication of *P. aeruginosa* [9]. Chronic CF is characterized by the continuous growth of *P. aeruginosa* in airway

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secretions and by the development of *P. aeruginosa*-specific antibodies. It is also correlated with a higher degree of inflammation, a higher number of neutrophils, and a greater amount of released serine proteinases than are found in intermittently colonized individuals. Together, these factors cause increased lung obstruction and destruction [10,5]. The continuous presence of *P. aeruginosa* in the chronically-infected lung leads to immune complex-mediated chronic inflammation, which is dominated by PMNs and represents a major cause of lung tissue damage and decreased lung function along with the damage actively caused by the bacteria [11].

Since D-amino acids are synthesized and released by many bacterial species, including the opportunistic human pathogen *P. aeruginosa*, and have been shown to lack significant toxicity, the idea of using them to combat biofilm-associated infections is highly attractive [3]. Peptidoglycan (PG) is the major component of the bacterial cell wall and the most commonly cited source of D-amino acids in bacteria. PG is comprised of long glycan chains linked by short peptide stems. It is a plastic structure that provides a protective barrier for the cell, enabling cells to survive under variable physicochemical conditions [12]. Stems from adjacent glycan strands can be linked either directly or by an interpeptide bridge, which is itself comprised of amino acids. D-amino acids can be incorporated into both the peptide stem and the interpeptide bridge [13]. Incorporation of a D-amino acid into PG was also first reported to induce biofilm disassembly in *Bacillus subtilis* (*B. subtilis*) [14,15]. In addition to their incorporation into PG, free D-amino acids may directly modulate the activity of periplasmic transpeptidase enzymes [16], and certain D-amino acids may serve as signaling compounds to mediate biofilm dispersal.

On the basis of these observations, we hypothesized that combining dispersal agents with antimicrobials may be an effective therapeutic strategy for biofilms, functionally restoring the susceptibility of biofilms to antimicrobials through the release of bacteria from the biofilm. The dispersal activity of D-AAs on the biofilms of standard strain (PAO1) and clinical isolates was evaluated, and it was also investigated whether the combination of D-Tyr with antibiotics enhanced the activity against biofilm-producing bacteria *in vitro*.

2. Materials and methods

2.1. Strains and growth conditions

P. aeruginosa PAO1 Strain (ATCC 15692) was used in this study. Six biofilm-forming clinical isolates, (designated stains JYK-0001, JYK-0002, JYK-0016, JYK-0004, JYK-0047 and JYK-0071) were chosen from a collection of 72 clinical isolates of *P. aeruginosa* during Jan 2014 to Dec 2014, from the Third Xiangya Hospital of Central South University, Changsha, China. No special ethical permit was required for this study according to the Chinese law. Cultures were stored at −80 °C, and all isolates were routinely grown in Luria–Bertani broth (LB) (10 g tryptone per liter, 5 g yeast extract per liter, 5 g NaCl per liter) at 37 °C with constant shaking (160 rpm).

2.2. Antibiotics and D-AAs

Amikacin (AMK) was purchased from Sigma–Aldrich (St. Louis, MO). D-AAs were purchased from Sigma–Aldrich and prepared as a concentrated stock solution in distilled water or 1.0 N HCl, followed by filter sterilization. From the prepared stock solutions, D-AAs was diluted into Mueller-Hinton (M–H) broth to a final concentration of 50 mM and neutralized when necessary with NaOH (1 M) (pH 7 to 7.4). All subsequent working concentrations of D-AAs were prepared by diluting the neutralized 50 mM stock into M–H broth to yield a final working concentration. Because D-Tyr is the most effective among those D-AAs (D-leucine, D-methionine, D-Tyrptophan, and D-Tryptophan) that disassemble the biofilms (Fig. 2), it was selected for the following investigation.

2.3. Biofilm development of PAO1

To determine the kinetics of biofilm formation, an overnight culture was diluted at a ratio of 1:200 with fresh media, and 200 μl was deposited in a 6-well plate with 4 ml LB broth and a 9 × 9 mm glass slide inside. The plate was incubated at 37 °C without agitation, and the biofilm formation was determined by crystal violet (CV) staining after removing the culture and washing the slides vigorously with 1 × PBS every 6 h for a total of 24 h. The biofilm was observed using an optical microscope at 10 × 100 magnification.

2.4. Minimal inhibitory concentration (MIC) and minimal biofilm-inhibiting concentration (MBIC)

MICs were determined by a microtitre broth dilution method, as recommended by the Clinical and Laboratory Standards Institute (CLSI). The MIC was defined as the lowest antibiotic concentration that yielded no visible growth. The test medium was M–H broth, and the density of bacteria was 5×10^5 colony-forming units (CFU)/mL. Cell suspensions (100 μL) were inoculated into the wells of 96-well microtitre plates in the presence of AMK with different final concentrations (0, 1, 2, 4, 6, 8, 16, 32, 64, 128, 256, 512, and 1024 μg/ml). The inoculated microplates were incubated at 37 °C for 24 h before being read. Antimicrobial susceptibility assays were performed in triplicate.

The MBICs was calculated as the concentration of the antibiotic in which biofilms were eradicated (i.e. killed and/or dispersed) to a level ≥50% for MBIC_{50} or ≥90% for MBIC_{90} in comparison to control wells only treated with LB broth for 24 h [17].

2.5. Assessment of the effects of D-Try alone or D-Tyr combined with AMK on biofilms in 96-well plates

The semi-quantitative determination of biofilm formation was performed in 96-well sterile tissue culture plates (Corning, NY, America) based on the modified method reported by Christensen et al. [18]. Briefly, 1:200 diluted overnight bacterial culture was added to a 96-well plate, and bacteria were grown at 37 °C in LB medium. After 24 h of growth, the plates were washed with 1 × phosphate-buffered saline (PBS), and 200 μl of media without or supplemented with D-Tyr or D-Tyr combined with AMK at the designated concentrations in M–H broth was added to each well for an additional 8, 16, or 24 h, respectively. Following exposure, the cells were washed as described above, and the biomass was determined by measuring the $A_{490\mathrm{nm}}$ of 0.5% (w/v) crystal violet solubilized in ethanol. Experimental assays were performed in triplicate.

2.6. Time–kill assay

The 24 h biofilms formed in 96-well plates were washed with 1 × PBS to remove the planktonic cells. After 8, 16, and 24 h of incubation in AMK alone or combined with D-Tyr at the designated concentrations, the cells that adhered to the wells were mixed with the planktonic cells by thoroughly rubbing the surfaces with two moistened swabs, then mixing vigorously with a 1 ml pipette. The mixture was serially diluted (10^{-1}–10^{-5}) in saline, and aliquots of 100 μL were plated onto sterile NA plates. The plates were incubated for 24 h at 37 °C. After the incubation period, the number of viable cells were counted, and the results were expressed in Log10 cfu/ml.