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Effect of nitrofurans and NO generators on biofilm formation by *Pseudomonas aeruginosa* PAO1 and *Burkholderia cenocepacia* 370

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Abstract

Antibacterial drugs in the nitrofuran series, such as nitrofurazone, furazidin, nitrofurantoin and nifuroxazide, as well as the nitric oxide generators sodium nitroprusside and isosorbide mononitrate in concentrations that do not suppress bacterial growth, were shown to increase the capacity of pathogenic bacteria *Pseudomonas aeruginosa* PAO1 and *Burkholderia cenocepacia* 370 to form biofilms. At 25–100 μg/ml, nitrofurans 2–2.5-fold enhanced biofilm formation of *P. aeruginosa* PAO1, and NO donors 3–6-fold. For *B. cenocepacia* 370, the enhancement was 2–5-fold (nitrofurans) and 4.5-fold (sodium nitroprusside), respectively.

Keywords: Biofilms; Nitrofurans; NO; NO donors; *Pseudomonas aeruginosa*; *Burkholderia cenocepacia*

1. Introduction

In the natural environment, bacteria predominantly exist in matrix-enclosed multicellular communities, referred to as biofilms. Biofilms are complex assemblages of cells which exhibit channels that are presumed to permit the exchange of nutrients and wastes. Biofilm development is initiated by the attachment of individual cells to a surface, followed by their migration and reproduction to form microcolonies that later produce a mature biofilm. Bacteria growing as biofilms have significant morphological and biochemical differences from planktonic (non-adhering) cells. In particular, cells in biofilms are extremely resistant to treatment with antibiotics and other drugs, as well as to the immune response of the host organism. Therefore, the ability of bacteria to exist as biofilms causes serious difficulties in antimicrobial therapies. Formation of biofilms on medical devices like catheters, lenses etc. is the cause of serious problems associated with chronic infections. The capacity of bacteria to form biofilms is considered one of the important factors in pathogenicity of bacteria [3,4,8]. In this connection, special attention should be paid to the study of the effects of drugs used in medical practice on biofilm formation.

In this study, we investigated the influence of antibacterial drugs of the nitrofuran series on biofilm formation of Gram-negative bacteria. Nitrofurans are common therapeutic agents in human and veterinary medicine. Having a wide spectrum of action, these drugs are effective against Gram-negative and Gram-positive bacteria, including those resistant to other antibacterial agents. Nitrofurans are also active against protozoa and fungi [6,7,9]. The mechanisms of their action are still not completely understood. Nitrofurans are prodrugs, therapeutic agents which become toxic for microorganisms when activated by specific enzymes. The antibacterial activity of nitrofurans is derived from reductive metabolism of the nitro group, a process catalyzed by nitroreductase activities

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[9,13,18]. Nitrofurans can be transformed with release of nitric oxide, formation of toxic peroxynitrite and other oxidated derivatives [6,17]. Taking into account the possibility of nitric oxide formation from nitrofurans, as well as known antibacterial activity of a number of nitric oxide generators, it was suggested that the antibacterial effects of nitrofurans might be due to their NO donor activity [2,5,6].

Here we studied the effects of nitrofurazone, furazidin, nitrofurantoin, nifuroxazide and the well-known nitric oxide generators sodium nitroprusside and isosorbide mononitrate on biofilm formation of *Pseudomonas aeruginosa* PAO1 and *Burkholderia cenocepacia* 370. All compounds were found to increase the capacity of these pathogenic bacteria to form biofilms at concentrations not inhibiting bacterial growth under experimental conditions.

2. Materials and methods

2.1. Bacterial strains, growth conditions and materials

P. aeruginosa PAO1 and *B. cenocepacia* 370 strains were obtained from the collection of the Institute of Molecular Genetics, Russian Academy of Sciences, Moscow. The strains were grown in liquid or solid (1.5% wt/vol agar) Luria–Bertani broth (LB/LA) at 30 °C. Bacterial growth was monitored by absorbance at 600 nm. Nitrofurazone (the same as nitrofural or furacilin, Russia), furazidin (Sigma-Aldrich), nitrofurantoin (Sigma), nifuroxazide (Sigma), sodium nitroprusside (Sigma) and isosorbide mononitrate (Schwarz Pharma, Germany) were diluted in DMSO (1 mg/ml stock solution) and then in water.

2.2. Biofilm formation

Biofilm formation of *B. cenocepacia* 370 and *P. aeruginosa* PAO1 strains was analyzed as described [11]. Cells from fresh LA plates were inoculated into LB and incubated with aeration for 24 h at 30 °C. The cultures were then 100-fold-diluted in fresh LB supplemented with the tested substances. Inoculated cultures were grown in 96-well polystyrene microtiter plates for 24 h with aeration at 30 °C. The growth of planktonic (non-adhering) cells was evaluated by absorbance at 600 nm. Biofilm formation was measured by discarding the medium, rinsing the wells with distilled water three times, and staining attached cells with crystal violet. After staining, the liquid was discarded, wells were rinsed with distilled water three times, crystal violet was solubilized with ethanol and absorbance was measured at 600 nm. A microplate reader (Model 2550 Microplate Reader, Bio-Rad, USA) was used for measuring planktonic growth and biofilms. Bacterial growth for 24 h was found to be optimal for biofilm formation. At longer growth times, the level of biofilm formation did not change, or even decreased. There were 4–8 replicate wells in all experiments, and experiments were 4–5 times repeated with separately grown bacteria.

3. Results

We studied the effects of nitrofurans nitrofurazone, furazidin, nitrofurantoin and nifuroxazide on biofilm formation of two pathogenic bacteria, *P. aeruginosa* PAO1 and *B. cenocepacia* 370. Planktonic growth (unattached cells) of the *P. aeruginosa* PAO1 strain remained unaffected up to 25–50 μg/ml of nitrofurans and decreased at higher concentrations (Fig. 1). Nitrofurans were shown to enhance biofilm formation of *P. aeruginosa* PAO1 within the range of subinhibitory concentrations. An increase in the biofilm formation level under the effect of nifuroxazide and furazidin was also observed upon a slight decrease in strain planktonic growth. Nitrofurans tested increased the level of biofilm formation about 2.0–2.5-fold. As a result, the ratio of biofilms to planktonic cells increased upon increasing the concentration of the compounds tested to about 50–100 μg/ml.

Nitrofurazone, nitrofurantoin and furazidin were shown to enhance biofilm formation of *B. cenocepacia* 370 within the range of subinhibitory concentrations (Fig. 2). These compounds increased the level of biofilm formation up to 5-, 2.5- and 1.8-fold, respectively. Nifuroxazide lacked any pronounced stimulating effect on *B. cenocepacia* 370 biofilm formation.

As mentioned above, the antibacterial activity of nitrofurans is derived from reductive metabolism of the nitro group and is associated with formation of NO and its derivatives [9,13,18]. In this connection, we studied the action of two well-known NO generators (sodium nitroprusside and isosorbide mononitrate) on biofilm formation of *P. aeruginosa* PAO1 and *B. cenocepacia* 370 as well. Sodium nitroprusside and isosorbide mononitrate enhanced *P. aeruginosa* PAO1 biofilm formation within the range of subinhibitory concentrations up to 6- and 3-fold, respectively (Fig. 3). Sodium nitroprusside increased the level of biofilm formation of *B. cenocepacia* 370 at subinhibitory concentrations of 20–50 μg/ml up to 4.5 fold (Fig. 2).

4. Discussion

In recent years, screening of different compounds affecting biofilm formation have been actively conducted. Biofilm formation is an important factor promoting the development of bacterial infections. Since biofilm formation by pathogenic bacteria is a serious problem in antibacterial therapy, the study of possible effects of medical drugs on biofilm formation becomes a pivotal issue. Here we studied the effects of nitrofuran compounds nitrofurazone, furazidin, nitrofurantoin and nifuroxazide and two NO generators on biofilm formation by pathogenic bacteria *P. aeruginosa* PAO1 and *B. cenocepacia* 370. We showed that the tested compounds in concentrations which did not suppress bacterial growth enhanced biofilm formation by *P. aeruginosa* PAO1 and *B. cenocepacia* 370. The effect of the NO donors indicates that the action of nitrofurans on biofilm formation might be connected with nitric oxide or other derivatives formed as a result of nitro group and NO metabolism.