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7-(1-Methyl-3-Pyrrolyl-)-4,6-Dinitrobenzofuroxan Reduces the Frequency of Antibiotic Resistance Mutations Induced by Ciprofloxacin in Bacteria

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Abstract

The aim of the present study was to investigate biological properties of the novel nitrobenzoxadiazole derivative 7-(1-methyl-3-pyrrolyl-)-4,6-dinitrobenzofuroxan.

Materials and Methods: We used a bioluminescent test based on a set of lux-biosensors, which are genetically modified *E.coli* strains able to react on different types of factors that can induce an SOS-response with light emission. The spontaneous and induced mutation frequencies of antibiotic resistance in *E. coli* were determined by methods of classical genetics of microorganisms.

Results: 7-(1-methyl-3-pyrrolyl-)-4,6-dinitrobenzofuroxan demonstrated inhibition of SOS-response in a biosensor model system and significantly reduced the frequency of spontaneous mutations and mutations induced by ciprofloxacin of antibiotic resistance.

Conclusion: Based on our data, we can recommend using compound **1** as a starting point for the development of drugs that block mutagenesis associated with the emergence of antibiotic-resistant bacteria. (**Int J Biomed. 2016;6(3):228-232.)**.

Key Words: antibioitic resistance • fluoroquinolones • SOS-response • SOS-inhibitors • nitrobenzoxadiazole derivatives

Introduction

Resistance of microorganisms to fluoroquinolones is a big problem for international medicine, especially as regards nosocomial infections. The fastest resistance is formed by *Pseudomonas aeruginosa*. There are data an increase of resistance of pneumococci.[1] Currently, the natural and acquired mechanisms of antimicrobial resistance are well known.[2] In general, the mechanism of resistance is realized through modifying the target of action, inactivation of the antibiotic, actively removing it from the microbial cell, violation of the permeability of outer structures of the microbial cells (efflux), violation of the permeability of outer structures of the microbial cells, or the formation of a "metabolic shunt."

In the case of fluoroquinolones, the mechanism of target modification is implemented by modifying two bacterial enzymes: DNA gyrase and topoisomerase IV, which mediate conformational changes in the molecule of bacterial DNA, necessary for its normal replication.

Currently used anti-microbial agents often have the ability to induce mutations in microorganisms, thus causing the appearance of forms which are resistant to a range of antibiotics. A paper by Cirz et al.[3] describes the mechanisms of mutations of resistance to rifampicin and ciprofloxacin caused by the use of these drugs. It was shown that the main generator of resistance mutations is the SOS-or error-prone repair system.[4]

In order to compensate for the negative effects identified in the works of Cirz and Wigle et al.,[3,5] it was proposed to synthesize new compounds, which would be able to switch off the launch of SOS-response and thus increase the sensitivity of the bacterial population to antibiotics. Currently, several

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laboratories are conducting screening for SOS-response inhibitors that would prevent the rapid development of bacterial resistance to antibiotics. In the course of these studies, ATP-competitive nucleotide analogs,[6,7] polysulfated naphthyl compounds,[8] alpha-helical peptides,[9] and the variable valence metal cations [10] were investigated. Those compounds proved effective as inhibitors of RecA in vitro. However, none of these classes of compounds showed biological activity *in vivo*, being unable to penetrate the membrane barrier.[11]

In the study on the biological properties of the novel nitrobenzoxadiazole derivatives,[12] we described the synthesis of previously known 7-(1-methyl-3-pyrrolyl -)-4,6-dinitrobenzofuroxan (**1**), which possesses a high DNA-protective effect, and negates damage caused by the oxidating mutagen Dioxidine. Compound **1** includes both electron-excess (N-methylpyrrole) and electron-deficient (4,6-dinitrobenzofuroxan) fragments, resulting in significant intramolecular charge transfer and a high polarity of the structure **1** as a whole. In this study, we investigated the ability of this compound to inhibit the launch of SOS-response induced by ciprofloxacin of the fluorquinolone antibiotic group. This class of antibiotics is distinguished by the ability to start the SOS-repair (and, consequently, mutagenesis), without chemical modification of the bases, only by inhibiting the topoisomerase.[3,13,14] Our experimental approach implies using *E. coli*-based Lux-biosensors, responding to the activation of the SOS pathway with an increase in luminescence.[15] Through this approach, it became possible to identify the inhibitors, effectively, working not only in model cell-free systems, but also within bacterial cells. Shifts in spontaneous and induced mutation frequency of antibiotic resistance in *E. coli,* induced with substance-**1,** were determined by methods of classical genetics of microorganisms.

Materials and Methods

We thoroughly described the parameters of 7- (1-methyl-1H-pyrrol-3-yl)-4,6-dinitro-2,1,3-benzoxadiazolyl1-oxide (substance **1)** in our early papers.[12,16]

To analyze the expression of stress-inducible operons and mutagenesis we used recombinant strain of *Escherichia coli* MG1655 (pRecA-lux), with a plasmid containing luxCDABE operon from photobacteria *Photorhabdus luminescens*, put under control of PrecA promoter of *E. coli*. Biosensors with pRecA plasmids react to factors causing induction of the SOS-response in the cell.

A test compound was dissolved in DMSO (dimethylsulfoxide, analytical grade, Ugreaktiv, Russia) to a concentration of 10mg/ml. From this solution serial dilutions were then made in DMSO to 10^2mg/ml. Thus, the following concentrations were tested: 1; 0.1; 0.01 mg/ml. DMSO was used as a control for each dilution in respective concentrations.

Bioluminescent test

We used a slightly modified bioluminescent test protocol described in detail in our early papers.[12,15]

The culture of *E. coli* MG 1655 pRecA-lux was grown in LB broth. Bacteria were grown in liquid culture at 37 °C. The overnight culture was diluted with fresh medium to the density of 0.01 - 0.1 McFarland units (concentration $3 \cdot 10^7$- $3 \cdot 10^6$ cells/mL).

For growth on solid medium, LB-agar (LB + 20 g / liter of microbiological grade agar) was used. Both liquid and solid medium were supplemented with ampicillin (100 μg/ml).

The measurements were made using a DEN-1B («Biosan») densitometer. Then the suspension was grown for 2 hours to early logarithmic phase. Aliquots of the culture (90 μl) were transferred into the wells and they were added with 10 μl of the test preparation and 10 μl of the control inductor (except of control wells). The control wells were added with 10 μl of DMSO.

As an inducer to activate the recA promoter, we used ciprofloxacin solution (pharmaceutical grade, KRKA, Slovenia) in deionized water at a concentration of 4.10 mg /ml.

After treatment, the plate with the sample was placed into the luminometer and incubated at 30^0C. The intensity of the bioluminescence was measured every 10 - 15 minutes.

For luminescence measurements, microwell plate luminometer LM-01T (Immunotech Co.) was used.

SOS induction response factor (I^s) was calculated according to the formula:

$$I^s = {}^{L_e}/_{L_k} - 1$$

where: L_k - the intensity of the luminescence in the control sample (in arbitrary units);

L_e- luminescence intensity of the test sample (in arbitrary units).

We considered statistically significant excess of L_e over L_k as an indication of the SOS-inducing effect. The excess was measured with t-test, calculated in the Microsoft Excel program. Anti-SOS activity indicator (A,%) was calculated by the formula:

$$A = (1 - {}^{I_a}/_{I_p}) \times 100$$

where: I_a - factor SOS-response induction caused by the influence of the inhibitor under investigation, I_p- factor of ciprofloxacin SOS-response induction.

All experiments were performed in three independent replicates. Confidence intervals for protective activity value were calculated by the described method.[15, 16]

Determination of spontaneous and induced mutagenesis parameters

Cells of E.coli MG1655 (pRecA-lux) strain were inoculated into 4 ml of the LB medium and stirred evenly. The induced culture was divided into four equal aliquots. One aliquot served as a control. To the remaining three aliquots were added 100 μg of the substance 1 to the concentration of 1 mg/ml; 100 μg of ciprofloxacin was added to the concentration of 0.1 μg/ml; 100 μl of the mixture of ciprofloxacin and the substance 1 were added to reach the above concentrations.

Cultures were incubated at 37° C for 18-20 hours.

The resulting culture were diluted with fresh LB to the density of 1 McFarland unit ($3 \cdot 10^8$ CFU), and a number of serial 1:10 dilutions was prepared in saline. The optical density of the solution was measured using a DEN-1B densitometer («Biosan», Latvia)

About 100 ul were taken from the 1/10 dilution and plated onto the surface of plates with LB agar with 70 µg/ml of rifampicin to count the frequency of rifampicin-resistant mutants appearance. From the dilutions,10^{-6} and 10^{-5} 100 µl of the culture were plated to LB agar plates with no addition of rifampicin to count the baseline number of cells.[17] Luminescence measurements were performed in quadruplicates. Colony counting was carried out in 48 hours.

Survival rate was measured by the formula:

Survival rate, % = (n_e / n_c) 100%,

where: n_e - the number of colonies after incubation with the inducer, n_k - number of colonies after incubation without inducer.

The frequency of mutants resistant to rifampicin was calculated by the formula:

$F_m = n_a/a\ n_b$,

where: n - the number of colonies on the plate containing an antibiotic –containing medium; n_b - number of colonies on the medium without antibiotics; a - the dilution factor.

Statistical significance of the mutagenic effect was evaluated by statistically significant difference (t-test, $P < 0.05$) in the number of colonies between the experiment and control, considering dilution factor.

Results

Figures 1, 2 and 3 show the luminescence of biosensor cultures in the control, in the presence of ciprofloxacin and/or substance-**1** in concentrations of 0.01, 0.1 and 1mg/ml.

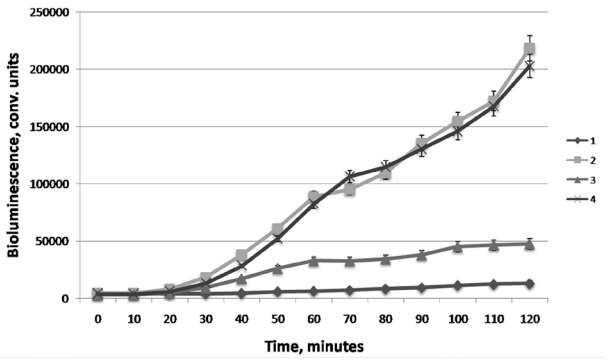

Fig. 1. *Luminescence of biosensor E. coli MG1655 (pRecA-lux) in the presence of Ciprofloxacinum with and without substance* **1** *(0.01 mg/ml).*

1 –control; 2 – 0.0001 mg/ml of Ciprofloxacinum; 3 – 0.01 mg/ml of substance **1;** *4 - Ciprofloxacinum with 0.01 mg/ml of substance* **1.**

As can be seen in the figures, the introduction of ciprofloxacin causes an increase in luminescence of cultures used in our study biosensor strain. That fact illustrates the activation of SOS-response. The maximum value of I_f obtained for the ciprofloxacin concentrations used in our experimental system was 16.06. Substance **1** in concentrations of 0.01, 0.1 and 1mg/ml also causes a weak SOS-induction. Maximum I_s values were 2.84, 1.95, 0.5, respectively. This effect could be explained by the interaction between substance **1** and RecA protein and/or other components of the SOS-reparation system. A lower concentration of substance **1** reduces the intensity of SOS-induction caused by ciprofloxacin. However, a statistically significant effect was observed in the first 60 minutes of incubation. Its absence in further processing seems to be due to bacteria metabolizing active ingredients.

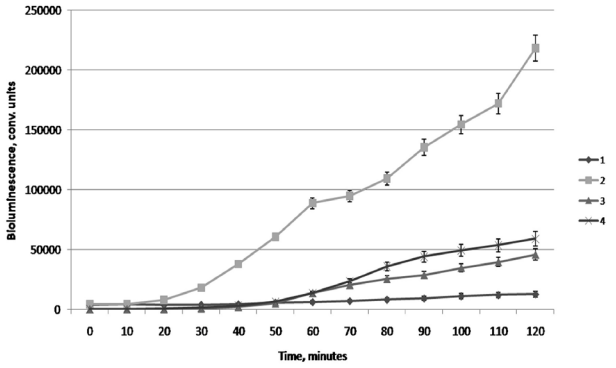

Fig. 2. *Luminescence of biosensor E. coli MG1655 (pRecA-lux) in the presence of Ciprofloxacinum with and without substance* **1** *(0.1 mg/ml).*

1 -control, 2 – 0.0001 mg/ml of Ciprofloxacinum; 3 – 0.1 mg/ml of substance **1;** *4 - Ciprofloxacinum with 0.1 mg/ml of substance* **1**

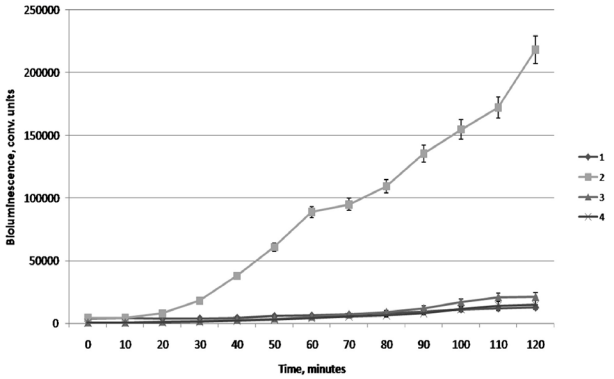

Fig. 3. *Luminescence of biosensor E. coli MG1655 (pRecA-lux) in the presence of Ciprofloxacinum with and without substance* **1** *(1 mg/ml).*

1 -control, 2 – 0.0001 mg/ml of Ciprofloxacinum; 3 – 1 mg/ml of substance **1;** *4 - Ciprofloxacinum with 1 mg/ml of substance* **1**

Concentrations of 0.1 and 1mg/ml gave more significant suppression of SOS-induction that was observed during the whole experiment. Thus, the anti-SOS effect of substance-**1** is generally dose-dependent, although the dependence is not linear (Fig.4).

Results of experiments on mutagenesis are given in Table 1. In our experimental system, substance 1 slightly (12%) but statistically significantly reduced the frequency of spontaneous mutagenesis. Ciprofloxacin more than doubled the frequency of mutants resistant to rifampicin. When ciprofloxacin and substance **1** were added together, a statistically significant mutagenic effect was also observed. However, in this case the increase in the frequency of mutations was about five times lower than in the case of a single action of ciprofloxacin.

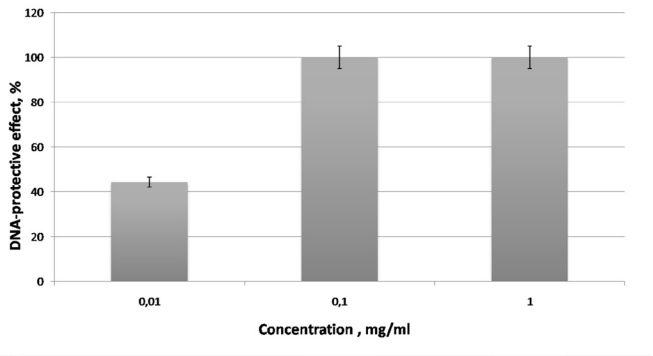

Fig. 4. *The maximum DNA-protective effect of substance* **1**

It should be noted that substance **1** slightly decreased the cell viability when compared to control. However, on the background of stress caused by ciprofloxacin, substance **1**, on the contrary, increased the survival rate of the bacteria. Therefore, the observed antimutagenic effect cannot be the result of an artifact associated with survival.

Discussion

Of considerable interest is the question of this phenomenon's mechanism. Besides DNA-protective activity, it was found earlier that substance-**1** has an ability to generate nitric oxide (II) *in vivo*.[12,16]

Another feature of this compound is its ability to exist in two isomeric forms, which are in **1** ⇌ **1'** equilibrium (Fig.5). This process of dynamic restructuring of the furoxan cycle is called 1,3-N-oxide tautomerism and may potentially facilitate its conformational adjustment to binding sites of biopolymers.

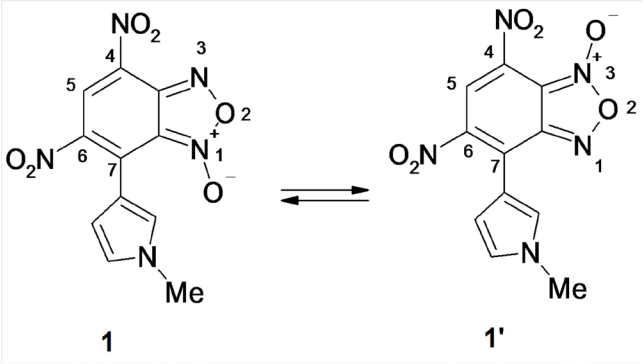

Fig. 5. *Two isomeric forms of substance* **1**

Figuring out how this activity relates to the ability to inhibit a RecA-mediated SOS-response will be the subject of our further research.

Extensive use of ciprofloxacin in medical and veterinary practice has led to the development of resistance up to 25% of species such as *Escherichia coli, Salmonella typhimurium, Streptococcus pneumoniae*, *Pasteurella multocida*, and *Staphylococcus aureus*.[18, 19] However, most of the standards of care for septic and infectious diseases include fluoroquinolones in general and ciprofloxacin in particular as the first-line drugs. That fact determines the utility of search for methods to reduce resistance.

Comparing our data on the biosensor test and the test for mutagenesis, it should be noted that the concentration of the substance **1** that causes the complete suppression of the Rec-induction did not block ciprofloxacin-induced mutagenesis completely. Apparently, the SOS-path is the main, but not the only, mechanism for the induction of mutations of antibiotic resistance by ciprofloxacin. However, based on our data, we can recommend using compound **1** as a starting point for the development of drugs that block mutagenesis associated with the emergence of antibiotic-resistant bacteria.

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Competing interests

The authors declare that they have no competing interests.

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