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In vitro Antimicrobial Activity Profile of Modified Pyrimidine Nucleosides Derivatives

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Background: The increase in prevalence of antimicrobial-resistant bacteria (ARB) is currently a serious threat, thus there is a need for new classes antimicrobial compounds to combat infections caused by these ARB. The growth inhibition ability of derivatives of the components of nucleic acids has been well-characterized but not for its antimicrobial characteristics.
Aims: To evaluate *in vitro* antimicrobial activity profile of modified pyrimidine nucleosides derivatives.
Methodology: Modified nucleosides arabinofuranosylcytosine (cytarabine, ara-C), [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)]uracil (TTU), and nucleotides cytarabine-5′-monophosphate (ara-CMP), and O^2,2′-cyclocytidine-5′-monophosphate (cyclocytidine monophosphate, cyclo-CMP) were synthetized and subsequentially checked for antibacterial activity. Bacterial cells characteristics were assessed by antiproliferative and the production of intracellular reactive oxygen species (ROS) assays.
Results: It was found that modified nucleosides ara-C, and TTU, and nucleotides ara-CMP, and cyclo-CMP were able to inhibit *Escherichia coli*, *Sarcina lutea*, *Bacillus cereus*, and *Proteus mirabilis* strains in a time and dose dependent manner via killing kinetics assay. Gram-negative (*E.*

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coli and *P. mirabilis*) bacteria stains were more sensitive to the exposure of TTU and cyclo-CMP and less sensitive to the exposure of ara-C and ara-CMP compared to gram-positive ones. The most effective cells growth inhibitor for gram-positive strains (*S. lutea, B. cereus*) was ara-CMP with ED_{50} = $5.2•10^{-5}$ M and $3.1•10^{-4}$ M, respectively. *Sarcina lutea* appeared to be the most sensitive bacteria strain to the exposure of all studied compounds. It was demonstrated that studied modified pyrimidine nucleosides derivatives enhanced the production of intracellular ROS over time (validated via DCFA-DA probe assay).

Conclusion: This study has revealed the mechanism of action of cytarabine, cyclocytidine monophosphate, and TTU as an antimicrobial agent for the first time, and has shown that these pyrimidine derivatives enhanced might be able to combat infections caused by *Escherichia coli*, *Sarcina lutea*, *Bacillus cereus*, and *Proteus mirabilis* in the future.

Keywords: antibacterial activity; modified nucleosides; ROS.

ABBREVIATIONS

AAAEs : aryl acid adenylating enzymes;
Ara : C, cytarabine – arabinofuranosylcytosine;
Ara : CMP – 5'-monophosphate of arabinofuranosylcytosine 5;
ARB : antimicrobial-resistant bacteria;
Cyclo : CMP, cyclocytidine monophosphate – 2'-cyclocytidine-5'-monophosphate;
DCFA : DA – 2'-7'-dichlorodihydrofluorescein diacetate;
FDA : Food and Drug Administration;
LPS : Lipopolysaccharide;
MHB : Mueller-Hinton Broth;
OxyRS : Oxidation Response Genes;
PBS : Phosphate Buffered Saline;
ROS : Reactive Oxygen Species;
SOS : "Son of Sevenless" Genes;
SoxRS : Superoxide Response Genes;
TTU : [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)] uracil.

1. INTRODUCTION

The introduction of antibiotics agents into clinical utilize proclaimed another age for medication. Nonetheless, not exactly a century later, the helpful adequacy of anti-infection agents is getting restricted attributable to the ascent of opposition in pathogenic microorganisms. The widespread use of antibiotics has led to the emergence and rapid spread of resistance in microorganisms. Nowadays, more and more known and new strains of bacteria are becoming immune to the drugs used. There is an opinion that humanity is entering the post-antibiotic era, when even common infections or small injuries can be life-threatening [1, 2]. The World Health Organization report 2020 says about the wide spread of resistant microorganisms, which significantly complicates the treatment of infections caused not only by bacteria, but also by fungi, parasites and viruses [3]. Every year, about 700 thousand people die from infections caused by drug-resistant bacteria, and this number may rise to 10 million by 2050 [4].

The resistance of a microorganism to an antibiotic can develop by several mechanisms: inactivation or degradation of the antibiotic by an enzyme (the most ancient mechanism effective against β-lactams), inhibition of absorption or active removal of the antibiotic from the cell (often used against tetracyclines, macrolides and fluoroquinolones) and changes in the structure of the receptor. Perhaps there are other, alternative, mechanisms [1]. Most resistance genes are localized in plasmids, which makes their heritability and horizontal transfer to other bacteria possible. At the moment, examples of resistance have been found for each class of antibiotics, regardless of their mechanism of action [5- 7].

Most of the currently used classes of antibiotics were discovered before the 70s of the last century [8]. Such a low activity in the search for new antimicrobial compounds can be explained by the high monetary and time costs for bringing the drug to the market, as well as a limited set of methods for identifying leading compounds [9]. In addition, the vast majority of existing antibiotics exhibit significant cytotoxicity, which limits the possibilities of their use. It is obvious that there is an urgent need to create new classes of antibiotics that will act on new targets and have activity against resistant strains of microorganisms.

One of the promising classes of antimicrobial compounds is derivatives of the components of nucleic acids: nucleosides, nucleotides, as well

as their analogues. These molecules are involved in a large number of biological processes, including, for example, the storage of genetic information, gene expression, energy metabolism and cell signaling. These processes are vital for all living organisms, including bacteria. Nucleoside analogues are one of the most important classes of drugs used in the clinic. The most common used nucleoside analogues are antiviral and antitumor agents [10]. However, recently there is more and more data on their effectiveness against microorganisms. At the moment, the inhibitory activity of nucleosides has been detected both among those isolated from natural sources and among synthetic analogues [11, 12, 13]. Antimicrobial properties have also been found in already known nucleosides that have been used or are being used to treat other diseases [2, 14]. There is not much data on clinical trials of nucleosides and/or nucleotides as antibacterial drugs. In this regard, their research can be an important step to start using them as full-fledged antibiotics.

2. MATERIALS AND METHODS

2.1 Experimental Procedures for the Synthesis of Compounds

2.1.1 Synthesis of arabinofuranosylcytosine

Synthesis of arabinofuranosylcytosine (ara-C) 4 was carried out according to the next scheme (Fig. 1).

An excess of chloro anhydride of acetylsalicylic acid 2 was added to the cytidine 1 suspension in acetonitrile and the reaction mixture was stirred at room temperature until the initial cytidine completely dissolved. The reaction mixture was added to the diethyl ether during stirring, and the precipitate was filtered out. Water was added to the precipitate, and the reaction mixture was kept at 80-90°C until the reaction was complete. The solution was cooled and the precipitated cyclocytidine 3 was filtered off. The precipitate was dissolved in water, an alkali solution was added to the resulting solution to pH of 10-12, and the mixture was kept until the initial cyclocytidine 3 was completely converted into arabinoside 4. The solution was neutralized by adding of hydrochloric acid, and the precipitated arabinofuranosylcytosine 4 was cooled and filtered off.

2.1.2 Synthesis of 5'-monophosphate of arabinofuranosylcytosine

Synthesis of 5'-monophosphate of arabinofuranosylcytosine 5 (ara-CMP) was carried out according to the next scheme (Fig. 2).

The reaction of cytidine 1 with phosphorus oxychloride in trimethyl phosphate led to the formation of cytidine-5'-phosphodichloridate 2, which was treated without isolation with chloro anhydride of
acetylsalicylic acid 3 in acetonitrile and then with water. The mixture was boiled until the intermediates were completely converted into cyclo-CMP 4, which was isolated in crystalline form using ion exchange chromatography using anion exchange resin Dowex 1×8 in acetate form.

The isolated cyclo-CMP 4 was purified by recrystallization from a water/alcohol mixture. The reaction of cyclo-CMP 4 with sodium hydroxide solution and subsequent treatment with hydrochloric acid solution to pH level 2-4 led to 5′-monophosphate of arabinofuranosylcytosine 5, which was purified by recrystallization from water.

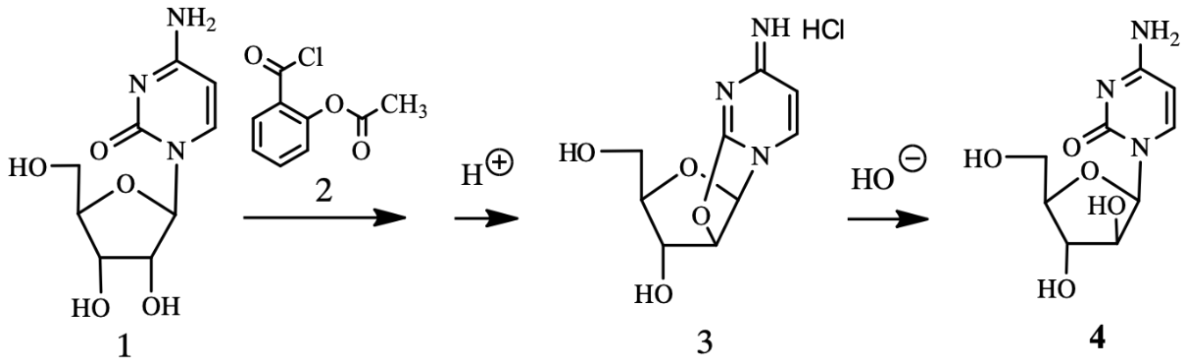

Fig. 1. Scheme of synthesis of arabinofuranosylcytosine

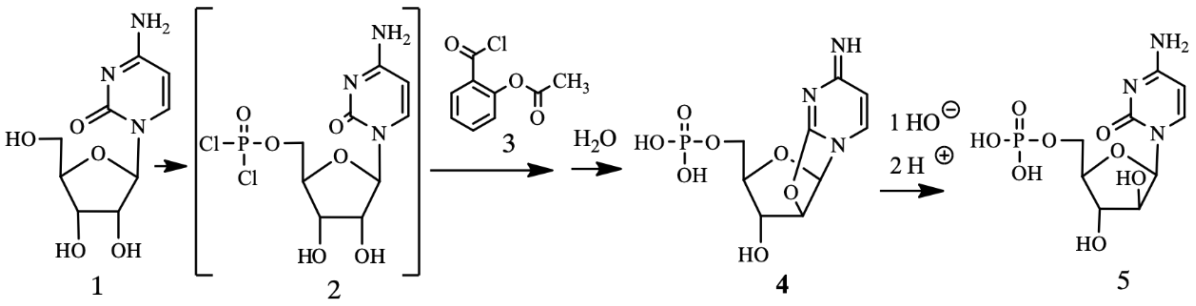

Fig. 2. Scheme of synthesis of 5'-monophosphate of arabinofuranosylcytosine

2.1.3 Synthesis of [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)]uracil

Synthesis of [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)] uracil (TTU) 3 carried out according to the scheme (Fig. 3) based on uridine 1.

The reaction of uridine 1 with acetic anhydride in acetonitrile in the presence of triethylamine and 4-N,N-dimethylaminopyridine leads to the formation of 1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)uracil 2 which was isolated as an amorphous powder. The interaction of triacetate 2 with phosphor(1H-1,2,4-triasol-1-yl)oxide previously obtained from 1,2,4-triazole and phosphorus oxychloride in acetonitrile in the presence of triethylamine leads to [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1H-1,2,4-triazol-1-yl)]uracil 3 which was isolated in crystalline form.

2.1.4 Synthesis of cyclocytidine-5′-monophosphate

A suspension of cytidine 1 in a mixture of phosphorus oxychloride ($POCl_3$) and trimethyl phosphate $(CH_3O)_3PO$ to the scheme (Fig. 4) was stirred at a temperature of 0-4°C until a homogeneous solution was obtained (~1.5-2 hours). Next a solution of chloro anhydride of acetylsalicylic acid 3 in acetonitrile was added to the resulting solution of phosphorodichloridate 2, and the reaction mixture was stirred at room temperature for 2 hours. Distilled water was slowly added to the reaction mixture with stirring, and the resulting hot solution was allowed to cool for 24-26 hours. The precipitated salicylic acid was filtered off, the filtrate was applied to a column with anion-exchange resin Dowex 1×2 (100-200 mesh) in acetate form. The column was eluted with distilled water, the fractions containing cyclocytidine-5'-monophosphate (cyclo-CMP) 4 were collected and evaporated to dryness on a rotary evaporator. The resulting precipitate was dissolved in a minimum volume of hot distilled water, and the solution was slowly added with stirring to a three-fold volume of ethyl alcohol. The formed precipitate of cyclocytidine-5'-monophosphate 4 was filtered off, washed with ethyl alcohol, and dried to constant weight. The product yield was 30%.

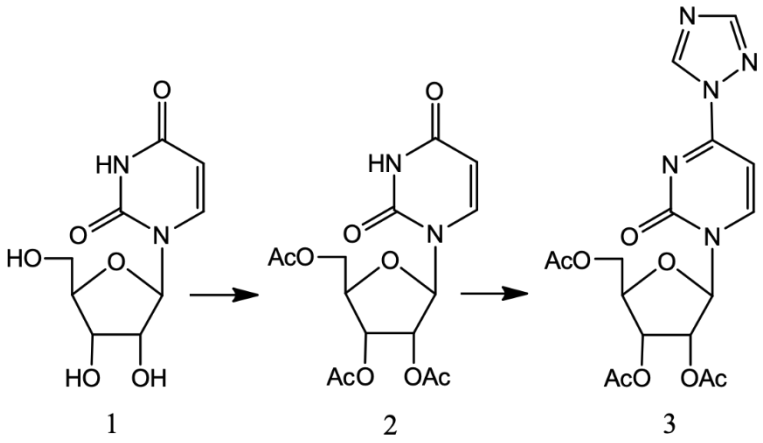

Fig. 3. Scheme of synthesis of [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)]uracil

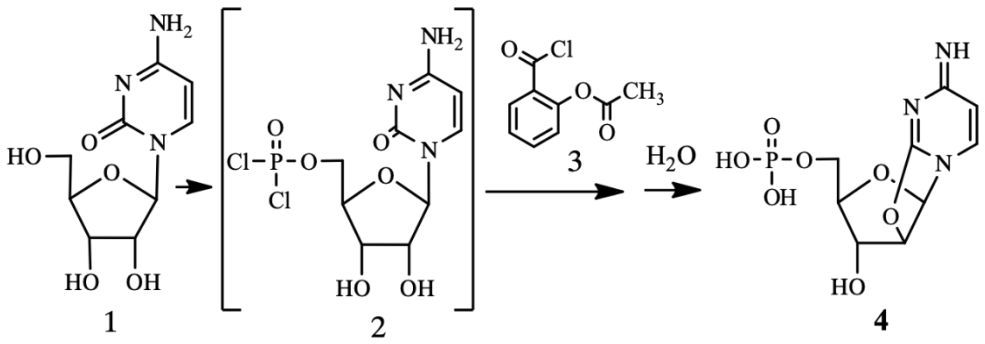

Fig. 4. Scheme of synthesis of cyclocytidine-5′-monophosphate

2.2 Bacteria Strains and Culture

The bacterial strains used in the study were *Escherichia coli*, *Sarcina lutea*, *Bacillus cereus*, and *Proteus mirabilis*. The bacterial colonies of different strains were transferred under aseptic conditions into a 10 mL MHB containing capped conical flask and incubated overnight at 37 °C. After 18-24 h of incubation, cells were centrifuged at 6000 rpm for 5 min, supernatant was discarded and cell pellet was resuspended in PBS followed by centrifugation. This removed debris and a clean bacterial suspension was obtained followed by suspending cells in MHB. The absorbance of the bacterial suspension prepared was recorded by UV–Visible spectrophotometer at 600 nm (OD_{600}). The cells were adjusted in the range of 0.15 to 0.2 OD_{600} which was considered to have cells at a concentration of 10^8 cells/mL. This suspension was further diluted to obtain a concentration of 10^7 cells/mL for testing nucleosides/nucleotides activity.

2.3 Resazurin Reduction Assay

The resazurin metabolization experiments were performed in 96-well plates as described [15]. Briefly, a volume of 10 µL of each suspension concentration was mixed with 200 µL of resazurin at a concentration of 20 µmol L^{-1} in phosphate buffered saline (PBS). The fluorescence (RFU) of microbial-generated resorufin was recorded at λ_{ex} = 520 nm/λ_{em} = 590 nm after in 60 min using a multi-detection microplate reader Synergy 4 (BioTek Instruments Inc., USA). Each concentration level was measured in hexaplicate and the mean ± SD was calculated. The percentage of survival was established for wells containing nucleosides/nucleotides relative to control wells containing no compounds.

2.4 Detection of Reactive Oxygen Species (ROS)

The production of ROS by bacterial strains after treatment with modified nucleosides/nucletides was evaluated using indicator 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, UK), which can detect a broad range of ROS including nitric oxide and hydrogen peroxide [12]. The adjusted bacterial culture (0.5 McFarland exponential phase bacteria culture) were treated with different concentrations of studied compounds in presence of DCFH-DA at a final concentration of 5 µM in 0.85% saline and incubated at 37 °C aerobically for 24 h. Untreated bacterial culture was served as a negative control. The fluorescence emission of DCFH-DA was measured at 525 nm using a Tecan microtiter plate reader with an excitation wavelength of 485 nm [13]. The background fluorescence of 0.85% saline and auto fluorescence of the bacterial cells incubated without the probe was measured to calculate the net fluorescence emitted from the assay itself. Experiment was conducted in triplicate.

2.5 Statistical Analysis

Bacterial survival data and associated nucleosides/nucleotides concentrations from resazurin and plating were then fit to *a* a log-logistic model with four parameters (*b*, *c*, *d*, *e*) LL.4 using R (GraphPad Software, Inc.), affording the dose-response curves:

$$\varphi(x) = c + \frac{d - c}{1 + e^{b\,(\log x - \log e)}}$$

The estimated parameters of the models have a definite physical meaning. In particular, for the log-logistic model, the parameters *c* and *d* determine the lower and upper horizontal

asymptotes of the sigmoid curve, *e* corresponds to the position of the inflection point, and *d* – to the angle of inclination in the transition region. Fitting of model parameters to the analyzed empirical data was carried out using the generalized method of minimizing the sum of squares of deviations of model forecasts from the observed values, taking into account specially selected weight coefficients.

Statistical analysis of the estimated parameters was carried out using Student's *t*-test, which tested the hypothesis of the equality of each coefficient to zero and calculated *p*-values that determine the achieved level of significance. The statistical significance of the model as a whole was verified by comparing it with a simple regression with a zero slope coefficient (the horizontal regression line corresponds to the absence of dose-effect dependence) by ANOVA.

3. RESULTS

3.1 EC_{50} and Killing Kinetics Studies of Modified Pyrimidine Nucleosides /Nucleotides

Killing kinetics was performed to evaluate the effect of different concentrations of modified nucleosides/nucleotides ara-C, ara-CMP, cyclo-CMP, and TTU on four bacterial strains for 24 h.

All studied modified pyrimidine nucleosides /nucleotides inhibit growth of exponential phase of all used bacterial strains in a dose and time dependent manner (Figs. 5-8).

S. Lutea culture treated with $4.2•10^{-4}$ M of ara-C achieved 90% reduction of bacteria cells growth after 24 h; while other bacterial cultures treated with the same concentration of ara-C achieved 55% reduction (*E. coli*) and 40% reduction (*B. cereus, P. mirabilis*) after 24 h, respectively (Fig. 5). ED_{50} of ara-C after *S. lutea* cultivation consisted the minimal value of $5.5•10^{-4}$ M, while for *E. coli* it was calculated the maximal ED_{50} value equaled to $3.4•10^{-3}$ M, the effectiveness with respect to *B. cereus* and *P. mirabilis* was similar and consisted $2.17•10^{-3}$ M and $2.29•10^{-3}$ M, respectively.

However, a different scenario was observed when *P. mirabilis* was treated with ara-CMP as there was only 25% reduction after incubated for 24 h at $3.0•10^{-4}$ M aerobically (Fig. 6). Actually, the effectiveness of ara-CMP with respect to all bacterial strains was higher. So, ED_{50} value after *S. lutea* cultivation was calculated as $5.2•10^{-5}$ M, while for *E. coli* it was detected the value $2.2•10^{-4}$ M, as for *B. cereus* and *P.mirabilis* $3.1•10^{-4}$ M and $4.5•10^{-3}$ M, respectively.

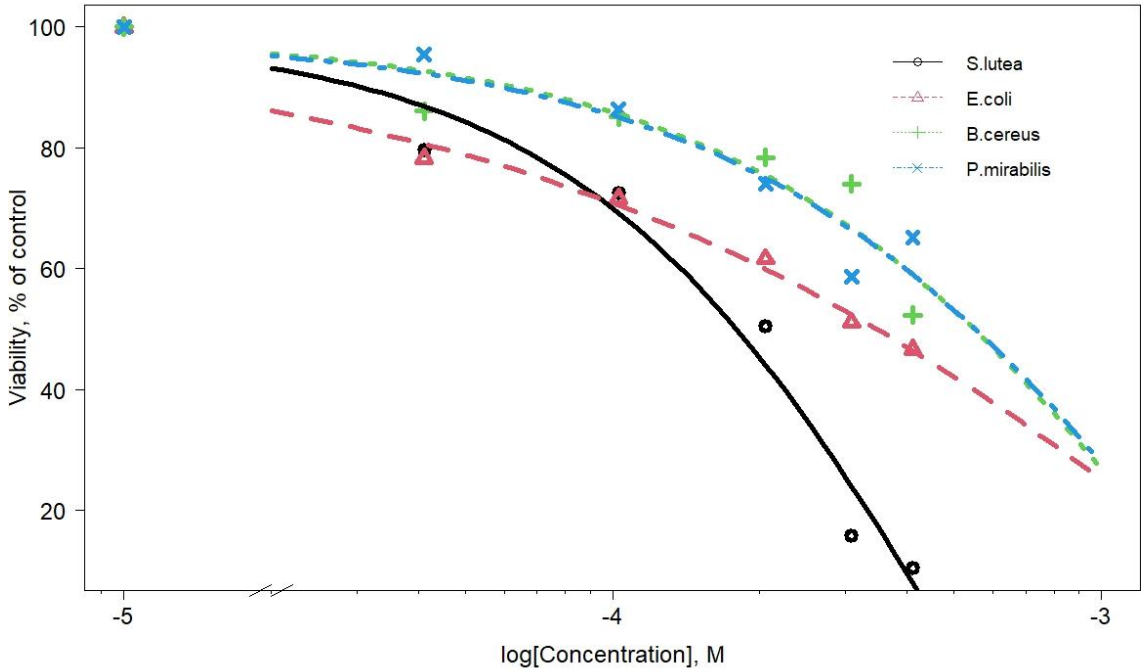

Fig. 5. Effect of different concentrations of arabinofuranosylcytosine against exponential phase *E. coli*, *S. lutea*, *B. cereus*, and *P. mirabilis* (incubated aerobically) at 37 °C for 24 h

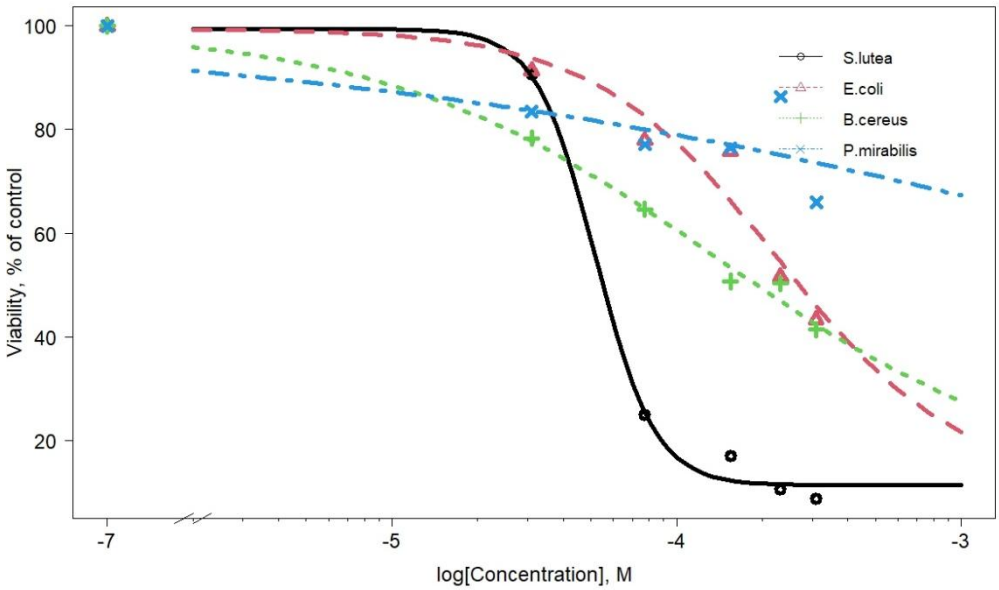

Fig. 6. Effect of different concentrations of 5'-monophosphate of arabinofuranosylcytosine against exponential phase *E. coli*, *S. lutea*, *B. cereus*, and *P. mirabilis* (incubated aerobically) at 37 °C for 24 h

S. lutea and E.coli cultures treated with $3.3•10^{-4}$ M of cyclo-CMP accomplished 90% and 80% reduction of bacteria cells growth after 24 h, respectively; while other bacterial cultures treated with the mentioned concentration of cyclo-CMP accomplished and it was 45% and 35% reduction r (*P. mirabilis, B. cereus*) after 24 h, individually (Fig. 7). ED_{50} of cyclo-CMP after *S. lutea* cultivation consisted the minimal value of $1.7•10^{-4}$ M, while for *E. coli* it was calculated the maximal ED_{50} value equaled to $1.5•10^{-4}$ M, the effectiveness with respect to *P. mirabilis* and *B. cereus* were close to each other and consisted $2.17•10^{-3}$ M and $2.29•10^{-3}$ M, separately.

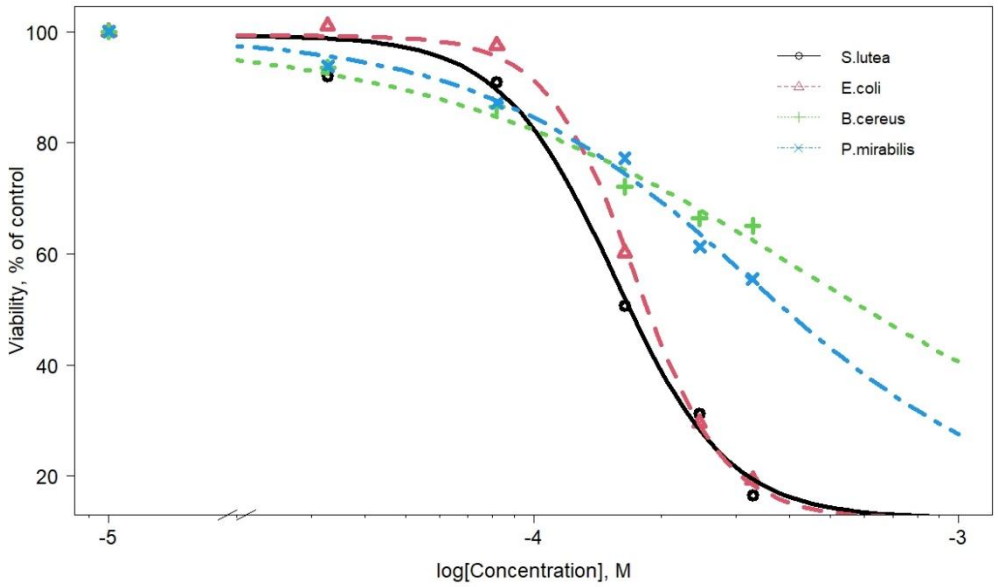

Fig. 7. Effect of different concentrations of cyclocytidine-5′-monophosphate against exponential phase *E. coli*, *S. lutea*, *B. cereus*, and *P. mirabilis* (incubated aerobically) at 37°C for 24 h

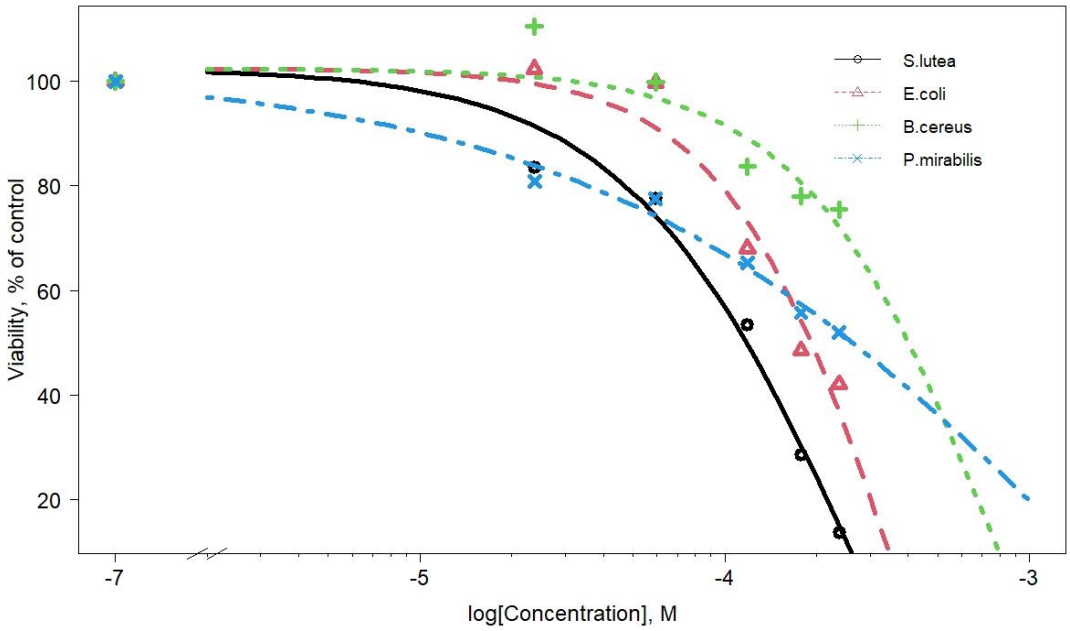

Fig. 8. Effect of different concentrations of [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)]uracil against exponential phase *E. coli*, *S. lutea*, *B. cereus*, and *P. mirabilis* (incubated aerobically) at 37 °C for 24 h

S. Lutea culture treated with $2.3•10^{-4}$ M of TTU achieved 90% reduction of bacteria cells growth after 24 h; while other bacterial cultures treated with the same concentration of ara-C achieved where : 60% reduction (*E. coli*), 50% reduction (*P. mirabilis),* and 25% (*B. cereus,*) after 24 h, (Fig. 8).

ED_{50} of TTU after *S. lutea* cultivation consisted the minimal value of $2.8•10^{-4}$ M, while for *E. coli* it was calculated the maximal ED_{50} value equaled to $3.6•10^{-3}$ M, the effectiveness with respect to *B. cereus* and *P. mirabilis* was similar and consisted $8.4•10^{-4}$ M and $1.7•10^{-3}$ M, respectively.

3.2 Effect of Modified Pyrimidine Nucleosides/Nucleotides on the Enhancement of ROS Production

It was hypothesized that in presence of modified pyrimidine nucleosides/nucleotides, the formation of ROS was enhanced in *E. coli*, *S. lutea*, *B. cereus*, and *P. mirabilis* which can damage the iron-sulphur clusters, thereby releasing ferrous ion. This iron can react with hydrogen peroxide in the Fenton reaction, causing a chain reaction, generating hydroxyl radicals which can directly damage intracellular DNA, lipids and proteins. Hence to validate the hypothesis, the intracellular ROS in all used bacteria strains was quantified prior and after modified pyrimidine nucleosides/nucleotides treatment in the subsequent experiments.

The production of ROS in healthy untreated bacterial cells is a natural side effect of aerobic respiration. These ROS can damage the RNA/DNA pool and also oxidizes lipid contents. Thus to protect themselves against the detrimental effect of ROS, bacteria are capable of producing enzymes (catalase and superoxide dismutase) to detoxify the ROS and having regulatory mechanisms (SoxRS, OxyRS and SOS regulons) to counteract the damage. To determine the effect of modified pyrimidine nucleosides/nucleotides on the enhancement of ROS production, *E. coli*, *S. lutea*, *B. cereus*, and *P. mirabilis* was treated with the same concentrations of studied compounds in presence of DCFH-DA, an unspecific probe for ROS. It was shown that the ROS production in bacteria strains was enhanced in a dose dependent manner when treated with all studied compounds.

The highest ROS level increase after ara-C was detected while cultivating with *S. lutea* what is highly correlated with the growth inhibition effect (Fig. 9A). There is a strong correspondence between ROS level and viability of *P. mirabilis* after cultivation with ara-CMP. Indeed, the lowest rates of both the ROS level and the growth

inhibition effect were detected in our experiments (Fig. 9B). Cultivating of *E. coli* and *S. lutea* with cyclo-C at the ED_{50} concentration leaded to the ROS burst (13- and 10-fold, respectively), what again correlates with the cell growth inhibition capacity of cyclic modified nucleoside (Fig. 9C). The ROS level in *B.cereus, E. coli* and *P.mirabilis* after TTU treatment showed the 6-fold increase but we haven`t detected any specify in its action on bacteria cells (Fig. 9D).
This suggests that the enhanced production of ROS has an indirect effect on the growth of bacteria strains.

4. DISCUSSION

Infectious diseases could be said to be making a come-back, due to chronic under-investment in anti-infective drug development, reduced acceptance of vaccines, and the increasing spread and severity of drug resistance [12,13]. Most antibacterial, antifungal, and antiparasitic drugs are decades old, and the continued lack of development threatens the treatability of many infectious diseases. Even when new therapies are proposed they are often developed from existing antimicrobial agents, e. g., new penicillins, new tetracyclines, diamidines, new minor groove binders etc. [14,15]. While such strategies can (temporarily) circumvent resistance, it was inherent to this approach that resistance to the class of compound was already widespread in the microbial populations targeted.

One class of drugs that are important from a clinical perspective is nucleoside analogues, a pharmacologically diverse class of drugs that arose from chemically modified natural ribose or 2′-deoxyribose nucleosides [14]. Nucleoside analogues are among the most important drugs in the clinical setting and are used widely as both anticancer and antiviral agents [15]. Nucleoside analogues mimic endogenous nucleosides, exploiting cellular metabolism and becoming incorporated into both DNA and RNA. Their structural similarity to nucleosides mand nucleotides involved in primary metabolism endows purine or pyrimidine nucleoside antibiotics with unique biochemical properties and capabilities; accordingly, these natural products can often be extremely influential to the internal workings of living organisms. Not surprisingly, significant effort has been directed to developing pyrimidine nucleosides natural products and derivatives as drugs. Indeed, a number of such compounds have seen clinical use for many years. For instance, carbocyclic nucleoside analogues, compounds in which a methylene group replaces the oxygen atom in the furanose sugar moiety, have a distinguished history as anti-infectious agents, including the Food and Drug Administration (FDA)-approved antiviral drugs abacavir, entecavir, and lobucavir, as well as the naturally occurring neplanocin and aristeromycin [16-18].

Several nucleoside antibiotics from various actinomycetes contain a high-carbon sugar nucleoside that is putatively derived via C-5′-modification of the canonical nucleoside. Two prominent examples are the 5′-C-carbamoyluridine- and 5′-C-glycyluridine-containing nucleosides, both families of which were discovered using screens aimed at finding inhibitors of bacterial translocase I involved in the assembly of the bacterial peptidoglycan cell wall [19]. The nucleoside antibiotic 5′-O-[N-(salicyl) sulfamoyl]adenosine (SAL-AMS) is the lead compound of a new class of antibiotics that targets iron acquisition through inhibition of aryl acid adenylating enzymes (AAAEs) in several pathogenic bacteria and is especially effective against *M. tuberculosis* [20].

It is known that the main mechanism of the damaging effect on eukaryotic cells under administration of antimetabolites is the excessive accumulation of reactive oxygen species as a result of activation of microsomal oxidation, respectively. The consequence of this is damage to the functioning of the antioxidant defense system (including its enzymatic and non-enzymatic links). In this regard, we assessed the level of reactive oxygen species formed in the bacteria cells under cultivation conditions with modified pyrimidine nucleosides/nucleotides.

In this study we analyzed the activity of some modified pyrimidine nucleotides/nucleosides against different bacteria strains, e. g. *E. coli (gram-negative, facultative anaerobe)*, *S. lutea (gram-positive, obligate aerobe)*, *B. cereus (gram-positive, facultatively anaerobe)*, and *P. mirabilis (gram-negative, facultative anaerobe).* The phase of exponential growth of bacterial culture was used in this work. Exponential phase culture consists of actively growing cells which consume readily available oxygen and nutrients for growth.

The gram-negative bacterial cell wall lipopolysaccharide coat (LPS) offers some protection from the toxic effects of exogenous agents [21]. This capacity enables these bacteria

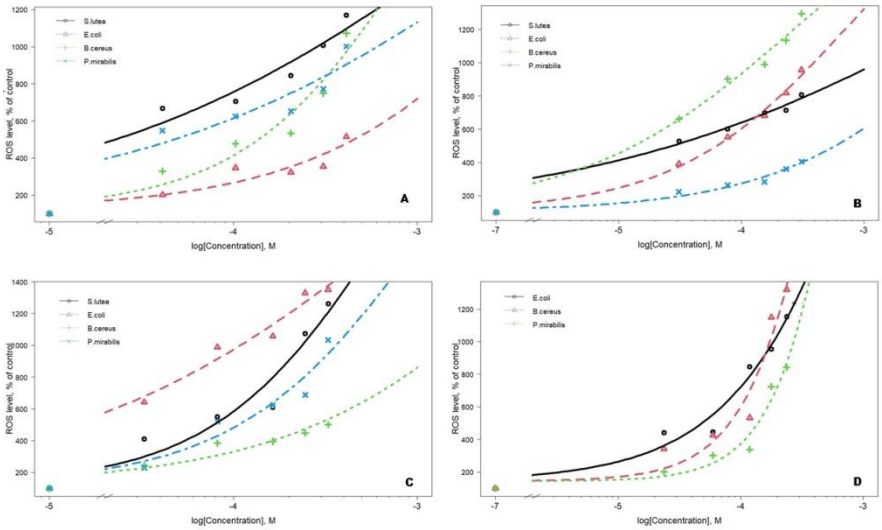

Fig. 9. Quantitation of intracellular ROS production by *E. coli*, *S. lutea*, *B. cereus*, and *P. mirabilis* after 24 h treatment with different concentrations of arabinofuranosylcytosine (A), 5'-monophosphate of arabinofuranosylcytosine (B), cyclocytidine-5′-monophosphate (C), and [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)]uracil (D) using the DCFA-DA probe

to survive in what otherwise must be considered hostile environments, such as mammalian intestines. The LPS has previously been shown to present a physical or chemical barrier through which ROS generated outside of cells must pass to interact with a vital target, such as membrane or cytoplasmic components [22]. As a result, some strains that fail to produce a large portion of the LPS have displayed greater sensitivity to exogenous ROS than do strains that retain this ability. Most gram-positive bacteria lack a protective structure analogous to the gram-negative LPS and the outer membrane in which it is anchored. In addition to possibly forming a structural barrier to penetration, this outer membrane may form a chemical trap for ROS; it is composed of unsaturated fatty acids and proteins, which are compounds known to react chemically with ROS [23]. The outer membrane and LPS of gram-negative bacteria do not, however, represent vital targets for the lethal action of ROS, since these can be removed without killing the cells (spheroplastformation). Because the cell wall structure of gram-positive and gram-negative bacteria represents the fundamental difference between these cells, once the barrier is crossed by ROS, the targets and mechanisms for cell killing for both gram-positive and gram-negative bacteria may be expected to be similar or identical.

Carotenoid pigments are known to physically quench ROS [24] and to protect bacteria against the lethal effects of photosensitization, whether by endogenous or exogenous photosensitizers [26]. Mathews-Roth and co-workers [25] have correlated the protective effects of carotenoids against photosensitization and singlet oxygen lethality in bacteria. Carotenoids also have been found to protect *Sarcina lutea* from killing by leukocytes, presumably by quenching singlet oxygen [27]. Administration of the carotenoid, –carotene has also been found to protect mice from lethal exposure to hematoporphyrin derivative and light and, in humans, to mitigate the photosensitivity associated with erythropoietic protoporphyria [28]. We have included for study a bacteria strain that produces high levels of carotenoid pigments in order to ascertain what protective effects the carotenoids may have against killing of these cells by exposure to pure exogenous ROS.

Our experiments showed that both gram-negative (*E. coli* and *P. mirabilis*) and gram-positive (*S. lutea* and *B. cereus*) bacteria stains were sensitive to the exposure of such modified pyrimidine nucleosides and/or nucleotides derivatives as ara-C, TTU, ara-CMP and cyclo-CMP. Besides that our results consider to set up some structure-function relationships in the range of modified pyrimidine nucleosides and/or nucleotides derivatives by the bacteria cell growth inhibition. Gram-negative (*E. coli* and *P. mirabilis*) bacteria stains were more sensitive to the exposure of TTU and cyclo-CMP and less

sensitive to the exposure of ara-C and ara-CMP compared to gram-positive ones. The most effective cells growth inhibitor for gram-positive strains (*S. lutea, B. cereus*) was ara-CMP. *Sarcina lutea* appeared to be the most sensitive bacteria strain to the exposure of all studied compounds.

Next it was shown that the ROS production in bacteria strains was enhanced in a dose dependent manner when treated with all studied compounds. The highest ROS level increase after TTU as well as after cyclo-CMP was detected while cultivating with the gram-negative strain of *E. coli* what is highly correlated with the cell growth inhibition effect. There was a strong correspondence between ROS level and viability of *B. cereus* strain after cultivation with ara-CMP.

5. CONCLUSION

Modified pyrimidine nucleosides and/or nucleotides derivatives like ara-C, TTU, ara-CMP and cyclo-CMP were found to be effective in inhibiting the growth of gram-negative (*E. coli* and *P. mirabilis*) and gram-positive (*S. lutea* and *B. cereus*) bacteria stains. Ara-C, TTU, ara-CMP and cyclo-CMP are able to enhance the production of intracellular ROS, moreover the more effective a pyrimidine derivative in the growth inhibition the more ROS species were caused to burst. This study has provided an insight that modified nucleosides and/or nucleotides might potentially be useful in treating infections caused by ARB.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It's not applicable.

ETHICAL APPROVAL

It's not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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