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ESTIMATION OF ANTIBACTERIAL ACTIVITY OF MODIFIED PURINE ARABINOSIDES

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Summary

In this work, the influence of various concentrations of modified nucleosides 2-fluoro-arabinofuranosyl adenine, 2-amino-6-chloro-arabinofuranosyl purine and nucleotide 2-fluoro-arabinofuranosyl adenine monophosphate on conditionally pathogenic bacterial cultures in the exponential phase of their growth, in which there is an intensive growth of cells that actively consume oxygen and nutrients, was evaluated. *B. cereus* cells belong to gram–positive, obligate aerobes, and *P. Mirabilis* to gram-negative, facultative anaerobes. It was found that gram-positive culture *B. cereus* is more susceptible to the action of the studied compounds: when the concentration of the studied compounds reaches 10^{-3} M, a 30-40% slowdown in bacterial growth was found, while for *P. mirabilis*, 15-20% suppression of bacterial cell viability was noted at the same concentrations of compounds. It was also shown that the production of reactive oxygen species (ROS) in bacterial strains was enhanced in a dose-dependent manner when bacterial cells were cultured in the presence of all the compounds studied. The greatest increase in the level of ROS was found when bacterial cultures were cultured in the presence of fludarabine, which, however, does not completely correlate with the effect of inhibiting cell growth.

Keywords: antibacterial activity; modified nucleosides; ROS.

Introduction

A new era of medication treatment for infectious diseases has started with the widespread use of antibiotics in clinical practice. Nevertheless, less than a century has elapsed since a dramatic decline in the efficacy of anti-infective medications against pathogenic bacteria. Antibiotic resistance in microorganisms has emerged and spread quickly as a result of its widespread usage. Currently, more and more well-known and brand-new bacterial strains are developing resistance to the medications being utilized. According to some, humanity is about to enter a post-antibiotic period in which even common diseases or small wounds can be fatal [1, 2].

The widespread development of resistant microorganisms, which greatly affects the treatment of diseases brought on not only by bacteria but also by fungi, parasites, and viruses, is discussed in the World Health Organization's 2020 report [3]. Approximately 700,000 individuals per year lose their lives to diseases brought on by drug-resistant bacteria, and by 2050, that number may reach 10 million [4].

Antibiotic resistance in microorganisms can occur through a number of mechanisms, including changes in receptor structure, inactivation or degradation of the antibiotic by an enzyme (the oldest mechanism still effective against beta-lactam antibiotics), inhibition of absorption, or active removal of the antibiotic from the cell. There might be more, different mechanisms [1]. The location of the majority of resistance genes in plasmids allows for their heredity and horizontal

spread to other bacteria. Currently, it has been demonstrated that any known class of antibiotics, regardless of how they work, are susceptible to developing resistance [5, 6, 7].

Before the 1970s of the previous century, the majority of the antibiotics that are used today were discovered [8]. Due to the high time and money requirements for bringing a medicine to market as well as the lack of effective methods for finding active ingredients, there has been a low level of activity in the search for new antimicrobial compounds [9]. Additionally, the vast majority of antibiotics now in use have high levels of cytotoxicity, which restricts the circumstances in which they can be used. It is obvious that there is a pressing need to create new antibacterial medications with novel modes of action that can combat strains that are multidrug resistant.

Nucleic acid derivatives including nucleosides, nucleotides, and their modified analogues are among the understudied families of substances with potential antibacterial activity. Numerous biological processes, including the transmission of signals, the storage of genetic information, and the expression of genes, are influenced by these substances. All living things, including bacteria, depend on these mechanisms to survive. One of the most significant pharmacological classes used in clinical practice is modified nucleosides, which are mostly used as antiviral and anticancer agents [10]. However, information about their efficiency against microbes has been accumulating recently. Currently, nucleosides' antibacterial properties have been found in both their synthetic analogues and a number of natural substances [11, 12, 13]. Additionally, recognized nucleosides that have been or are now being utilized to treat various diseases have been revealed to possess antimicrobial characteristics [2, 14]. Therefore, the search for novel compounds among modified nucleosides and nucleotides that may have antibacterial activity, as well as the investigation of the molecular mechanisms behind these compounds' actions, are of basic and practical significance.

Materials and Methods

Synthesis of 2-amino-6-chloro-9-(β-D-arabinofuranosyl) purine (2-NH_2-6-Cl-araPur) (4)

Synthesis of 2-NH_2-6-Cl-araPur (**4**) was carried out according to the scheme shown in Figure 1.

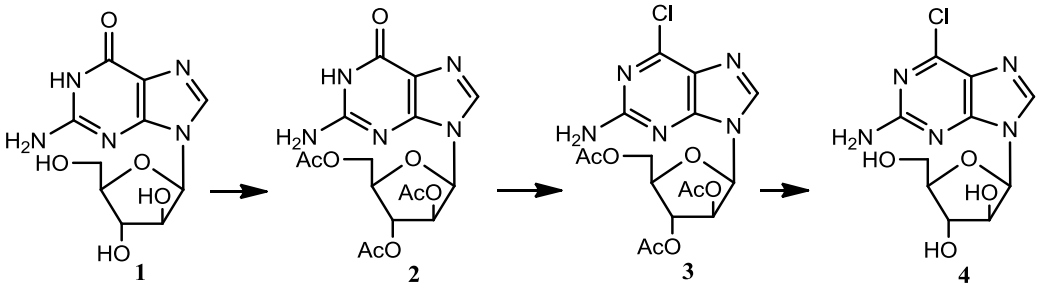

Figure 1. Scheme of synthesis of 2-amino-6-chloro-9-(β-D-arabinofuranosyl) purine

Treatment of triacetate **2** with phosphorus chloroxide in acetonitrile in the presence of benzyl triethylammonium chloride at 100-110 °C and subsequent treatment of the reaction mixture by (neutralization and treatment with a chloroform-water mixture) resulted in a 6-chloro derivative **3**, which was deacetylated without additional purification by the action of a solution of

potassium carbonate in methanol at 50-60 ° C. Subsequent treatment of the reaction mixture with Dauex 50x8 ion exchange resin (H^+ form) and activated carbon resulted in a deacetylated 6-chlorine derivative **4** in the form of an amorphous powder.

Synthesis of 2-fluoro-arabinofuranosyl adenine (fludarabine, 2-F-AraA) 9

Synthesis of 2-F-araA **9** was carried out according to the following scheme (Fig. 2).

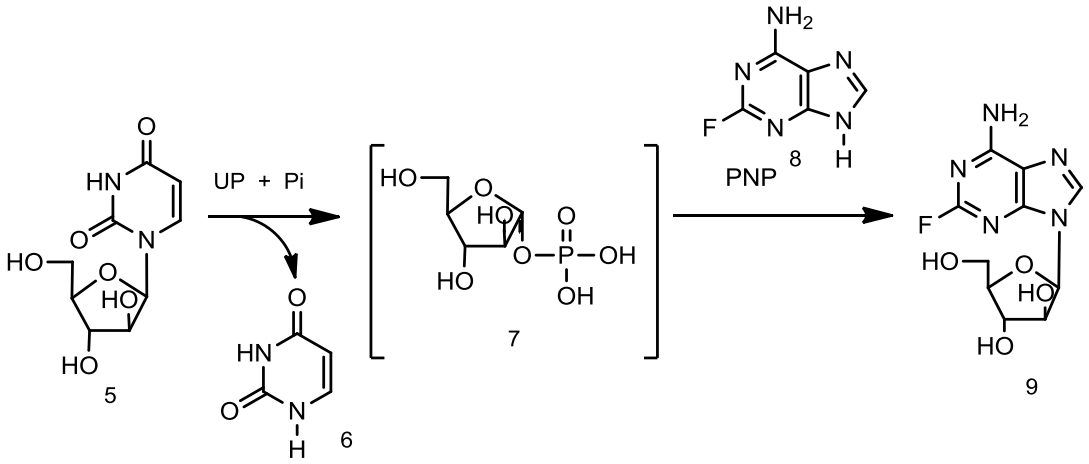

Figure 2. Scheme of synthesis of fludarabine **9**

The reaction mixture with arabinofuranosyluracil **1** and 2-fluoroadenine **4** in potassium phosphate buffer (pH 7.0) in the presence of uridine and purine nucleoside phosphorylases was incubated at 50 °C for 72 h with constant stirring. During the reaction, arabinofuranosyluracil **1** was converted into uracil **2** and ribose phosphate **3**, which reacted with 2-fluoroadenine **4** to form fludarabine **5**. At the end of the reaction, the distilled water was added to the mixture, heated to 90 °C and left to cool at 4 °C for crystallization of fludarabine. The resulting precipitate was filtered off and recrystallized from hot water. The crystalline precipitate of fludarabine **5** was filtered off, washed on a filter with water, ethyl alcohol, and dried to constant weight. The yield of fludarabine **5** was 43%.

Synthesis of 2-fluoro-arabinofuranosyl adenine monophosphate (fludarabine phosphate, 2-F-AraAMP) 12

Synthesis of 2-F-araAMP **12** was carried out according to the following scheme (Fig. 2).

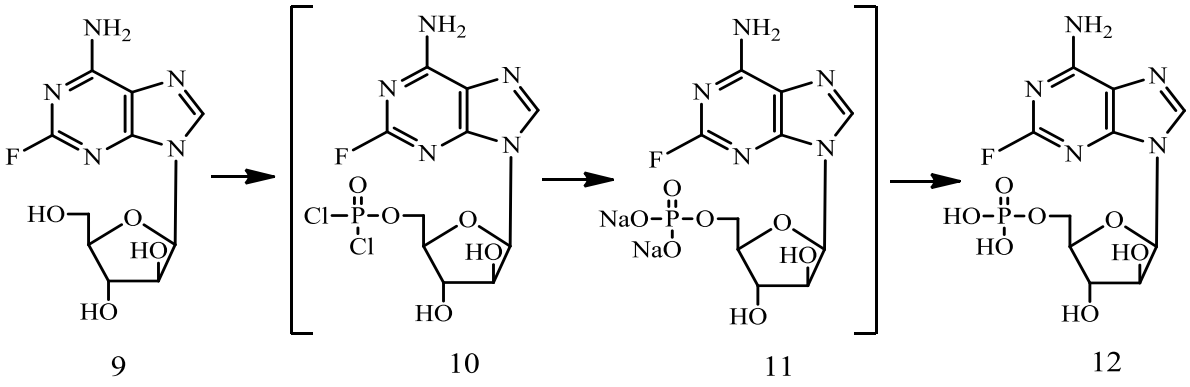

Figure. 3. Схема Scheme of synthesis of fludarabine phosphate **12**

Fludarabine **1** was added to a mixture of trimethyl phosphate and phosphorus oxychloride cooled to 0–4 °C, and the reaction mixture was stirred for 1–2 hours. Then the reaction mixture containing phosphorodichloridate **2** was poured onto crushed ice and 10 M sodium hydroxide solution was added to the resulting solution with stirring until neutral. The resulting solution containing the sodium salt of fludarabine phosphate **3** was concentrated in vacuum at a temperature of ≤30 °C to the half of the initial volume using a rotary evaporator and applied to a chromatographic column thermostatted at 22-25 ° C filled with Dowex 50Wx8 cation-exchange resin (200-400 mesh) in H^+-form. The column was eluted with water heated to 22–25 °C. The fractions containing fludarabine phosphate were collected and placed in a refrigerator for 18–20 hours. The precipitated fludarabine phosphate **4** precipitate was filtered off, washed on a filter with water, ethyl alcohol, and dried to constant weight. The product yield was 70%.

Bacteria Strains and Culture

The bacterial strains used in the study were *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.

Resazurin reduction assay

The resazurin metabolization experiments were performed in 96-well plates as described [16]. 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. The percentage of survival was established for wells containing nucleosides/nucleotides relative to control wells containing no compounds.

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. 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 CLARIOstar Plus (BMG Labtech, Germany) plate reader with an excitation wavelength of 485 nm. 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.

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:

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

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.

Results and discussion

Various concentrations of the modified nucleosides 2-fluoro-arabinofuranosyladenine, 2-amino-6-chloro-arabinofuranosylpurine, and nucleotide 2-fluoro-arabinofuranosyladenine monophosphate are assessed for their impact on the viability of opportunistic bacterial cultures of B. cereus (gram-positive) and P. mirabilis (gram-negative). When tested against bacterial cultures, all of the modified purine nucleosides and nucleotides exhibited dose-dependent inhibitory action (Fig. 4).

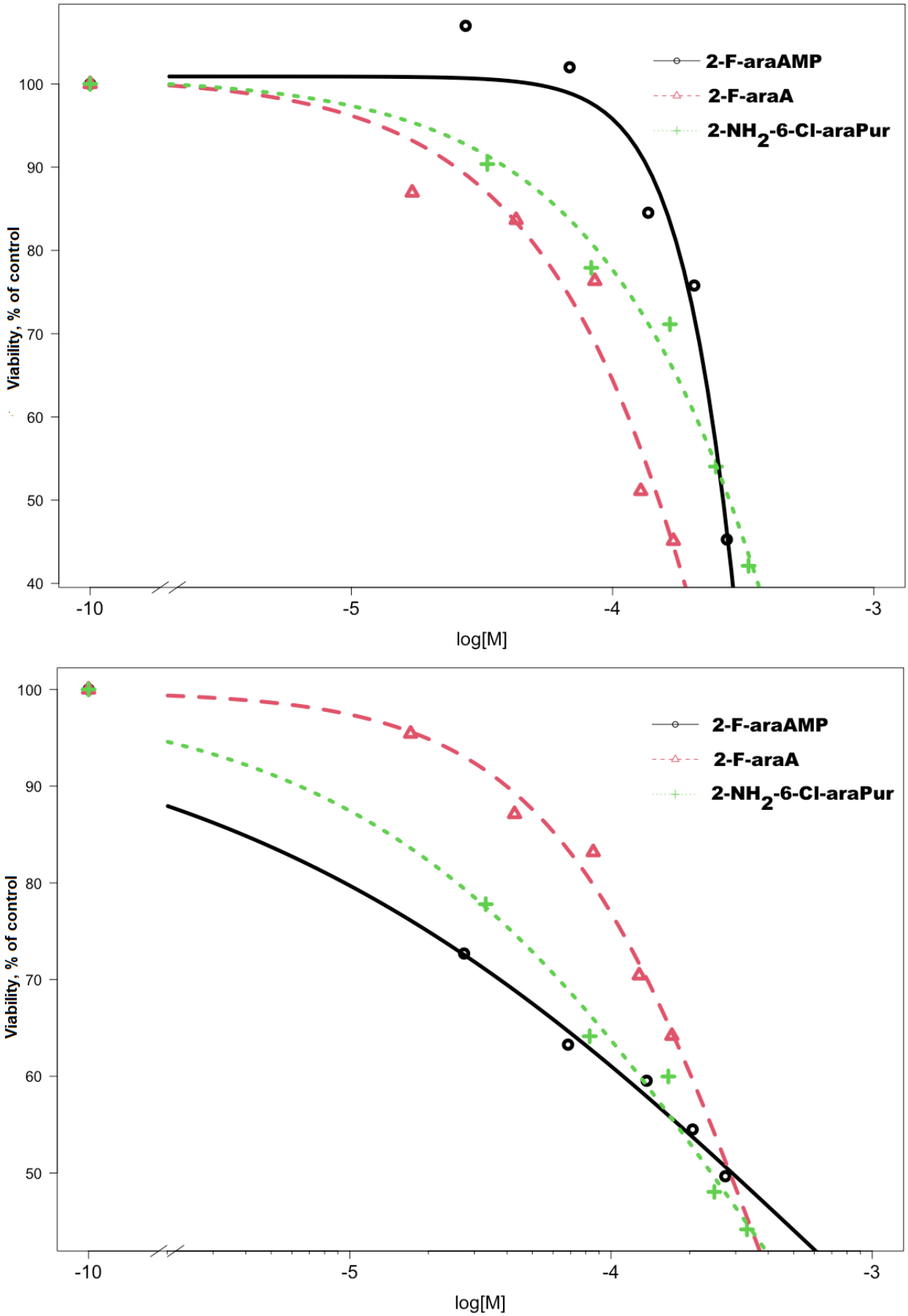

Figure 4. Effect of different concentrations of 2-F-araA, 2-F-araAMP и 2-NH_2-6-Cl-araPur on the viability of cells of bacterial cultures of *B. cereus* (top) and *P. mirabili*s (bottom)

According to the data shown in Figure 4, all of the investigated substances reduce the viability of bacterial cultures in the range of specified concentrations (10^{-5} - 10^{-4} M). According to the shape of the curves showing changes in cell viability in the concentration range (10^{-5}-10^{-4} M), the inhibitory effect on the growth of B. cereus cells becomes less effective in the order 2-F-AraA > 2-NH_2-6-Cl-araPur > 2-F-araAMP. The activity range for compounds, however, has the opposite character when taking into account the predicted values of ED50 for compounds when they act on B cells cereus: (2-F-araAMP - $5.5•10^{-4}$ M) > (2-NH_2-6-Cl-araPur - $2.5•10^{-3}$ M) > (2-F-AraA - $1.1•10^{-3}$ M).

The inhibitory action of the compounds diminishes in the order 2-F-araAMP > 2-NH_2-6-Cl-araPur > 2-F-AraA when the investigated compounds act at the same doses (10^{-5}-10^{-4} M) on *P. mirabilis* cell culture. If the computed ED_{50} values for the compounds under study are taken into account, the sequence of inhibitory activity will look like this: (2-F-AraA - $3.8•10^{-4}$ M) > (NH_2-6-Cl-araPur - $4.4•10^{-4}$ M) > (2-F-araAMP - $7.3•1010^{-4}$ M).

The data obtained indicate that *P. mirabilis* cells may be more sensitive to low concentrations of the chemicals under study. *B. cereus* culture cells' growth was suppressed by 30–40% while *P. mirabilis* culture cells' growth was reduced by 15-20% when compounds' inhibitory activity was examined at concentrations about 10^{-3} M, indicating a higher sensitivity of *B. cereus*.

It was also investigated how synthetic substances affected the amount of ROS in bacterial cultures. It is well known that the action of antibacterial medications can sometimes cause a rise in ROS levels, which leads to the death of bacterial cells in some instances. An increase in ROS can destroy iron-sulfur clusters, producing Fe^{2+} iron ions that then interact with hydrogen peroxide as a result (Fenton reaction). In this situation, the chain process of hydroxyl radical generation is triggered, which can directly harm intracellular proteins, lipids, and DNA.

B. cereus and *P. mirabilis* bacterial cultures were cultivated in the presence of the investigated compounds at the same concentrations, together with DCFH-DA as an unspecific probe to measure the level of ROS, to evaluate the viability of the reported mechanism of antibacterial activity. When all of the investigated chemicals were used to culture all of the bacterial cultures, it was demonstrated that the level of ROS increased significantly (Figure 5).

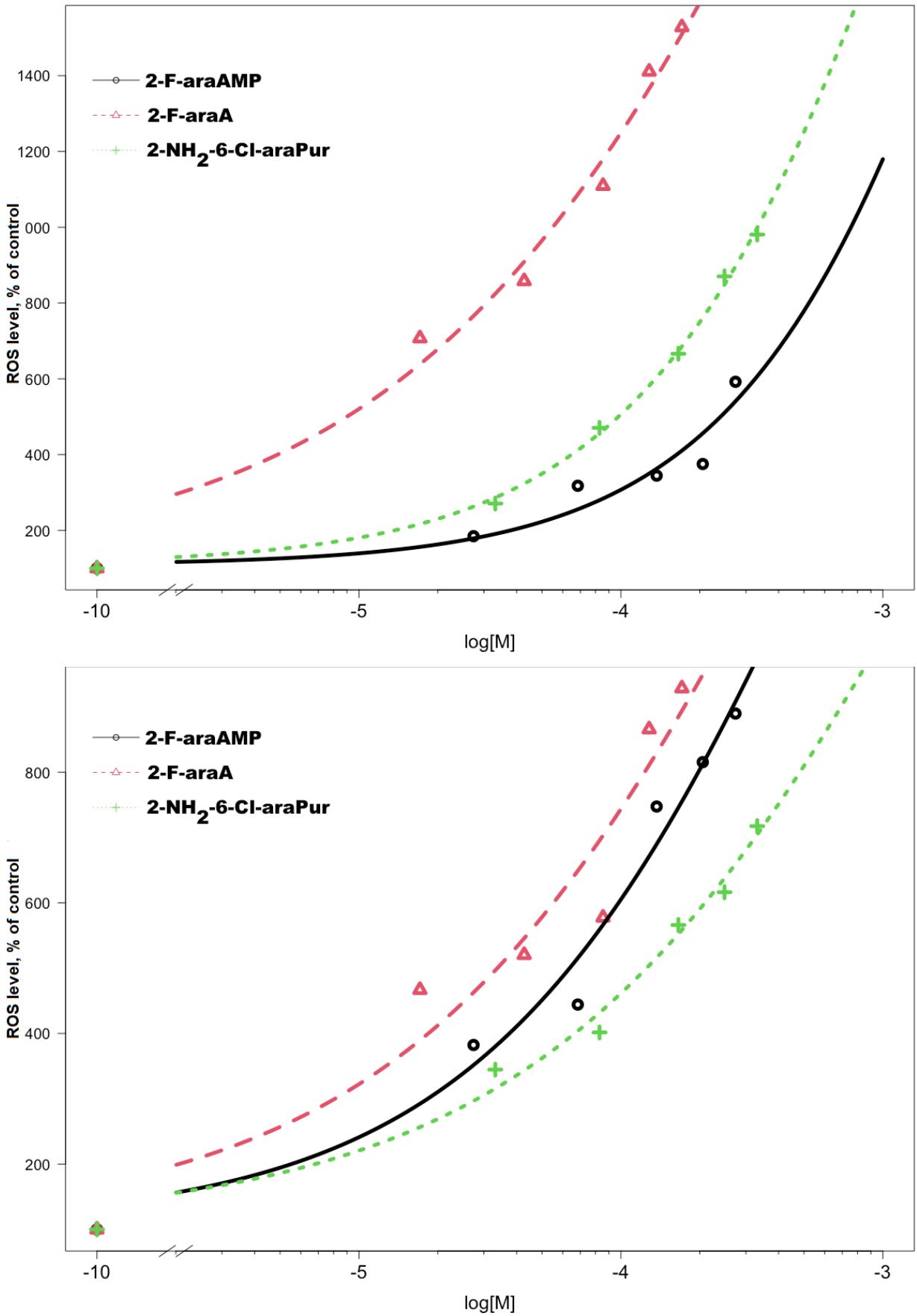

Figure 5. Effect of different concentrations of 2-F-araA, 2-F-araAMP и 2-NH_2-6-Cl-araPur on intracellular ROS levels in bacterial cultures of *B. cereus* (top) and *P. mirabilis* (bottom)

The information shown in Figure 5 allows for the drawing of several conclusions. First off, when it comes to raising the level of ROS in both bacterial cell cultures, fludarabine has been shown to be the most effective substance. Second, when acting on gram-positive cells of a *B. cereus* culture, fludarabine at all tested dosages more potently raised the quantity of ROS. Thirdly, after attaining concentrations of 0.1 μm for all of the chemicals under study in both bacterial cultures, an exponential rise in ROS levels was seen. These results are consistent with the ED_{50} values obtained from experimental data on the inhibition of cell growth. Fourth, fludarabine phosphate had the least effect on *B. cereus* in terms of causing an increase in the quantity of ROS of all the chemicals examined.

The increased generation of ROS has an indirect impact on the growth of the strains of the examined bacteria, it can be assumed based on the sum of the data obtained.

In gram-negative bacterial cultures, the outer lipopolysaccharide shell (LPS) protects against the toxicity of exogenous chemicals [16]. Bacteria can endure in an unfavorable environment thanks to this property. It has previously been demonstrated that reactive oxygen species and other harmful substances cannot pass through LPS physically or chemically [17]. As a result, bacterial strains that lack the ability to make LPS are more sensitive to external ROS than ones that can.

The lipopolysaccharide shell of gram-negative strains is a structure that the majority of gram-positive bacteria lack. This outer cell envelope, which serves as a physical barrier, can also operate as a chemical trap for ROS, which is brought on by the unsaturated fatty acids and proteins that make up this envelope and are known to be substances that actively react with ROS [18].

However, since they can be eliminated without causing cell death, gram-negative bacteria's LPS does not represent essential targets for the fatal action of ROS (formation of spheroplasts). Since the gram-positive and gram-negative bacteria's cell walls are fundamentally different from one another, it is reasonable to assume that once ROS pass the barrier, both gram-positive and gram-negative bacteria's targets and methods of cell lysis will be comparable or the same.

The findings demonstrated that modified purine nucleosides including 2-fluoro-arabinofuranosyladenine, 2-amino-6-chloro-arabinofuranosylpurin, and nucleotide 2-fluoro-arabinofuranosyladenine monophosphate had an impact on both gram-negative (*P. mirabilis*) and gram-positive (*B. cereus*) bacterial strains. Additionally, these findings show that the modified purine arabinosides under study and their biological activity share structural and functional links.

The research demonstrates that when bacterial cells were cultivated in the presence of all investigated substances, the production of ROS in bacterial strains was increased in a dose-dependent manner. The quantity of ROS increased significantly when fludarabine was present in the bacterial cultures, which is not entirely consistent with fludarabine's ability to limit bacterial cell development [19].

Conclusion

The study demonstrates that the modified purine nucleosides 2-fluoro-arabinofuranosyladenine, 2-amino-6-chloro-arabinofuranosylpurin, and 2-fluoro-arabinofuranosyladenine monophosphate are potent growth inhibitors of both gram-positive (*B. cereus*) and gram-negative (*P. mirabilis*) bacteria. Additionally, 2-F-araA, 2-F-araAMP и 2-NH_2-6-Cl-araPur can increase the generation of intracellular ROS. A more potent substance that can

both stimulate oxidative stress by increasing the production of ROS and impede the growth of bacterial culture cells is fludarabine phosphate.

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