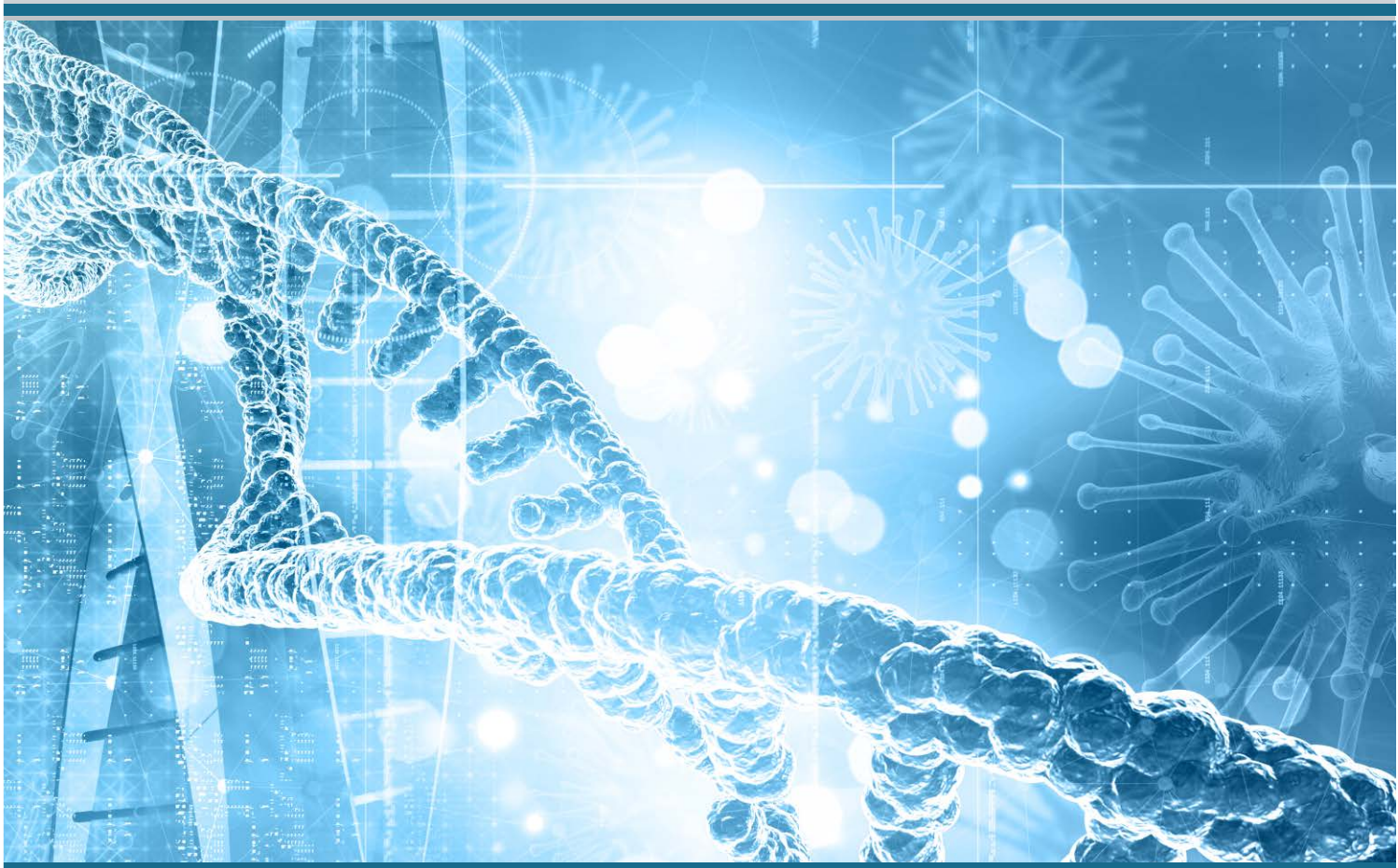
ISSN 2957-7349 (Print)
ISSN 2960-2327 (Online)

Республиканское научно-исследовательское
унитарное предприятие
«Институт биохимии биологически
активных соединений
Национальной академии наук Беларуси»

# БИОХИМИЯ И МОЛЕКУЛЯРНАЯ БИОЛОГИЯ

# BIOCHEMISTRY AND MOLECULAR BIOLOGY



ТОМ / VOL. 3 1(4)2024

# БИОХИМИЯ И МОЛЕКУЛЯРНАЯ БИОЛОГИЯ

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ISSN 2957-7349 (Print)
ISSN 2960-2327 (Online)

РЕЦЕНЗИРУЕМЫЙ НАУЧНО-ПРАКТИЧЕСКИЙ ЖУРНАЛ

*Основан в 2022 году*

**Учредитель**

Республиканское научно-исследовательское унитарное предприятие «Институт биохимии биологически активных соединений Национальной академии наук Беларуси»

**Журнал входит в Перечень научных изданий Республики Беларусь для опубликования результатов диссертационных исследований**

*Издано при финансовой поддержке Белорусского республиканского фонда фундаментальных исследований*

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тел.: +375 152 55-87-78,
e-mail: journal@ibiochemistry.by

Официальный сайт журнала
https://ibiochemistry.by

**Подписные индексы:**
для индивидуальных подписчиков
для ведомственных подписчиков

Отпечатано в типографии УП «ИВЦ Минфина»
Подписано в печать 07.04.2024.
Формат 60×84/8. Бумага офсетная.
Гарнитура TimesNewRoman. Печать цифровая.
Усл. печ. л. 30,69. Уч.-изд. л. 28,71.
Тираж 75 экз. Заказ 170.
ЛП № 02330/89 от 3 марта 2014 г.
Ул. Кальварийская, 17, 220004, г. Минск.

*В номере:*



Том 3

1 (4)/2024

# Biochemistry and Molecular Biology

ISSN 2957-7349 (Print)
ISSN 2960-2327 (Online)

PEER-REVIEWED
SCIENTIFIC AND PRACTICAL
JOURNAL

*Founded in 2022*

**Founder**

Republican Scientific Research Unitary Enterprise "Institute of Biochemistry of Biologically Active Compounds of the National Academy of Sciences of Belarus"

**The Journal is included in The List of Journals for Publication of the Results of Dissertation Research in the Republic of Belarus**

*Published with the financial support from the Belarusian Republican Foundation for Fundamental Research*

**Address of the Editorial Office:**
230023 Grodno, 7 Antoni Tyzenhauz Square,
Belarus Institute of Biochemistry of Biologically Active Compounds of the National Academy of Sciences of Belarus,
phone: +375 152 55-87-78,
e-mail: journal@ibiochemistry.by
Website – https://ibiochemistry.by

**Subscription indexes**
For individuals
For legal entities

Printed at UE "IVC Minfina"
It is sent of the press 07.04.2024
Format 60x84/8. Offset paper.
TimesNewRoman. Offset printing.
Prinded pages 30,69.
Publisher's signatures 28,71.
Circulation 75 copies. Order 170.
LP № 02330/89 of 3 March 2014
17, Kalvaryiskaya str., Minsk 220004

*In this issue:*



Vol. 3

1 (4)/2024

# РЕДАКЦИОННАЯ КОЛЛЕГИЯ / EDITORIAL BOARD

УДК 615.281.9: 577.113.4

# THE POTENTIAL OF MODIFIED NUCLEOSIDES AND NUCLEOTIDES AS NOVEL ANTIBACTERIAL AGENTS: AN IN VITRO STUDY OF BACTERIAL GROWTH INHIBITION AND OXIDATIVE STRESS INDUCTION

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*Belarusian State University, ISEI BSU, Minsk, Republic of Belarus*

**Background.** The overuse of antibiotics in healthcare has caused the rise of antibiotic resistance. New antibacterial drugs are urgently needed to fight resistant bacteria. Nucleic acid derivatives show potential as antimicrobial agents.

**Objective.** The objective of this research is to evaluate the antibacterial characteristics of modified nucleosides and nucleotides in an *in vitro* setting.

**Materials and methods.** Research studied modified nucleosides/nucleotides from purine/pyrimidine bases. *P. mirabilis* and *B. cereus* cultures were used. Growth rate assessed via resazurin metabolism in 96-well plates. DCFH-DA probe measured ROS levels in treated cultures.

**Results.** The results demonstrated that all the examined compounds exhibited dose-dependent inhibitory effects on the growth of studied bacterial cultures. The incorporation of the antioxidant quercetin into the reagent mixture did not alter the efficacy of the compounds in suppressing bacterial cell growth. However, there was a universal elevation in intracellular ROS levels across all bacterial cultures exposed to the studied compounds, indicating that the antibacterial activity of these compounds may be related to their capacity to induce oxidative stress in bacterial cells.

**Conclusions.** The study demonstrated that modified nucleosides and nucleotides have promising antibacterial properties and may be useful as novel antibacterial agents.

**Keywords:** modified nucleosides, nucleotides, antibacterial activity, reactive oxygen species (ROS), oxidative stress, multidrug-resistant bacterial strains.

**For citation:** Sysa AG, Kvasyuk EI, Shihad A, Gritskevitch ER. The potential of modified nucleosides and nucleotides as novel antibacterial agents: an in vitro study of bacterial growth inhibition and oxidative stress induction. *Biochemistry and Molecular Biology.* 2024, vol. 3, no 1(4). pp. 211–219.

## Introduction

The extensive utilization of antibiotics in clinical settings marks the onset of a new era in the pharmacological management of infectious ailments. Nonetheless, within a relatively short timeframe, the efficacy of anti-infective agents against pathogenic microorganisms has notably diminished. The misuse of antibiotics has precipitated the emergence and swift dissemination of resistance among microorganisms [1, 2]. Presently, an escalating array of both known and novel bacterial strains are acquiring resistance to conventional therapeutic agents. As posited by a prevailing hypothesis, society is transitioning towards a post-antibiotic epoch where commonplace illnesses and minor injuries may pose fatal risks [3].

The majority of antibiotics in use today were identified prior to the 1970s, reflecting a limited exploration for novel antimicrobial agents attributed to the substantial financial and temporal commitments essential for drug commercialization, along with restricted approaches for pinpointing bioactive compounds [4]. Furthermore, a considerable proportion of current antibiotics exhibit notable cytotoxic effects, constraining their applicability. Evidently, there is a pressing demand for the discovery of fresh antibacterial medications featuring novel modes of action to combat multidrug-resistant bacterial strains [5].

One of the understudied categories of substances with promising antimicrobial properties comprises derivatives of nucleic acid constituents: nucleosides, nucleotides, and their altered analogs. Nucleotides and nucleosides serve as the fundamental units of DNA and RNA, participating in protein synthesis, acting as cofactors in various biochemical pathways, and regulating the function of enzymes involved in nucleotide metabolism.

Modifying either the nucleic base or the sugar component of the nucleoside has the potential to impact enzyme recognition inhibition and the overall efficacy of the nucleoside in combating various pathogens. The utilization of nucleic acid derivatives and analogues has become increasingly prevalent in the realms of anticancer, antiviral, and, to a lesser

extent, antifungal therapies, as evidenced by studies conducted by A. E. J. Yssel et al. [6] and E. De Clercq et al. [7]. Notably, the realm of antibacterial activity has recently been unveiled in natural nucleosides, as highlighted in the research by Z. Cui [8] and M. Abbas et al. [9], along with their artificially synthesized counterparts studied by G. Seydlova et al. [10] and M. R. Bockman et al. [11]. This emerging field is currently experiencing rapid growth and significant advancements, as indicated by the works of M. Serpi et al. [12] and S. D. Negrya et al. [13].

The exploration of novel compounds exhibiting potential antibacterial properties within the realm of modified nucleosides and nucleotides, alongside the investigation into the molecular mechanisms underlying their efficacy, holds significant fundamental and practical relevance.

The objective of this research was to evaluate the antibacterial characteristics of various altered nucleosides and nucleotides in an *in vitro* setting. Additionally, the study aimed to examine their interactions with antioxidants to ascertain the potential for their application as drug substrates.

## Material and Methods

The research focused on the modified nucleosides and nucleotides derived from purine and pyrimidine bases. All compounds were synthetized as described in [14, 15]. Purine nucleoside analogs included halogenated compounds at the nitrogenous base, such as 2-fluoro-arabinofuranosyladenine (fludarabine) and 2-amino-6-chloro-arabinofuranosylpurine (2-$NH_2$-6-Cl-ara-Pur). Pyrimidine nucleosides encompassed sugar-modified arabinofuranosylcytosine (cytarabine, ara-C), featuring arabinose in lieu of ribose, and [1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)]uracil (TTU), distinguished by modifications in both the carbohydrate fragment (three acetate groups) and the nitrogenous base (triazole at the 4th position) (Figure 1).

Gram-negative (*P. mirabilis*, *E. coli*) and gram-positive (*B. cereus*) bacterial cultures were incubated at 32°C for 24 hours. Cells were centrifuged, resuspended in pH 7.4 buffer, and $OD_{600}$ was measured. The initial concentration of $10^8$ cells/ml was diluted to $10^7$ cells/ml for testing compound activity.

Bacterial cultures' viability was assessed using resazurin metabolism in 96-well plates following Travnikova et al.'s method [16]. A 10 μL cell suspension with modified nucleosides/nucleotides was incubated for 24 h, then mixed with 200 μL of 20 μmol/L resazurin in pH 7.4 phosphate buffer. After 60 min, fluorescence intensity of resorufin (λex = 520 нм, λem = 590 нм) was measured. Each concentration was tested in six replicates, and inhibitory effect was calculated as the ratio of fluorescence intensity in test wells to control wells without compounds.

A fluorescent probe DCFH-DA was used to measure intracellular ROS levels in bacterial cultures treated with test compounds. The cultures were incubated with DCFH-DA at 5 μm concentration in saline for 24 hours at 37 °C. ROS levels were determined fluorometrically at excitation 485 nm and emission 525 nm. Negative control included cultures without test compounds. The experiment was conducted in triplicate [17].

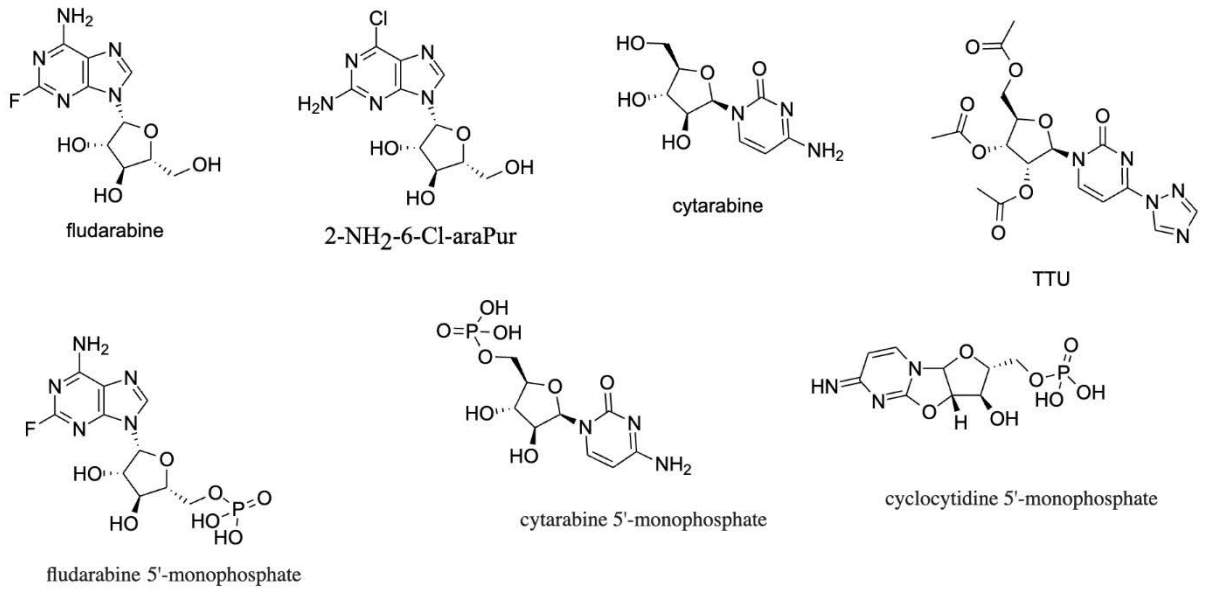


Figure 1 – The structure of the modified nucleosides and nucleotides used in the work

Рисунок 1 – Структурные формулы исследованных модифицированных нуклеозидов и нуклеотидов

## Results and Discussion

All examined modified nucleosides and nucleotides demonstrated dose-dependent inhibitory effects on bacterial cultures during the exponential growth phase.

The impact of varying concentrations of modified purine nucleosides and a nucleotide in the absence of an antioxidant (A, B) compared to when equimolar amounts of an antioxidant are included (C, D) on the proliferation and antioxidative condition of *B. cereus* bacterial cells is studied.

Based on the data illustrated in Figure 2A, within the specified concentration range ($10^{-5}$–$10^{-3}$ M), all examined compounds led to a decline in *B. cereus* growth. The patterns of changes in cell growth suggest that the potency of the inhibitory impact on *B. cereus* growth, as indicated by the $IC_{50}$ values, diminished in the fludarabine series ($1.48 \cdot 10^{-4}$ M) > fludarabine monophosphate ($2.64 \cdot 10^{-4}$ M) ≈ 2-$NH_2$-6-Cl-ara-Pur ($2.77 \cdot 10^{-4}$ M).

Next, the impact of varying concentrations of modified purine nucleosides and a nucleotide in the absence of an antioxidant (A, B) compared to when equimolar amounts of an antioxidant are included (C, D) on the proliferation and antioxidative condition of *P. mirabilis* bacterial cells is studied.

When the *P. mirabilis* cell culture is subjected to the tested substances at the same concentrations, the effectiveness of the compounds in inhibiting diminished in the order of 2-$NH_2$-6-Cl-ara-Pur ($2.43 \cdot 10^{-4}$ M) > fludarabine ($2.81 \cdot 10^{-4}$ M) ≈ fludarabine monophosphate ($2.96 \cdot 10^{-4}$ M) as illustrated in figure 3A.

Upon examination of the suppressive effects of substances at a concentration of approximately $10^{-5}$ M, a decrease in growth of *B. cereus* cell culture by 5–10% and P. mirabilis cell culture by 10–20 % was observed. This suggests a heightened susceptibility of P. mirabilis cells to minimal levels of the substances tested.

It is well-established that in certain instances, the demise of bacterial cells ensues from an elevation in reactive oxygen species levels induced by antibacterial agents. The heightened presence of reactive oxygen species can result in impairment to iron-sulfur clusters, leading to the liberation of $Fe^{2+}$ ions, which subsequently engage with hydrogen peroxide in the Fenton's

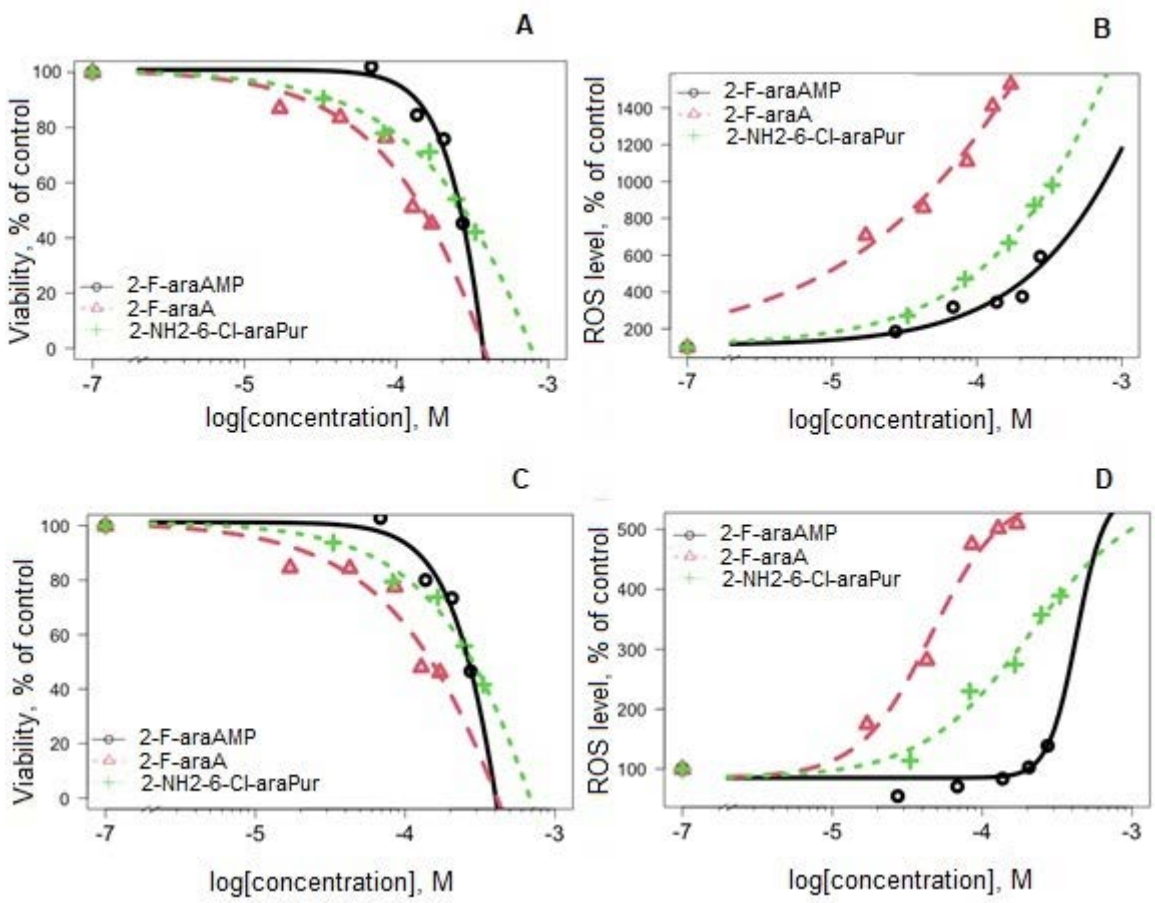


Figure 2 – Effects of different concentrations of modified purine nucleosides and a nucleotide on bacterial cells *B. cereus* growth and antioxidant status with and without equimolar quercetin

Рисунок 2 – Влияние различных концентраций модифицированных пуриновых нуклеозидов и нуклеотида на рост и антиоксидантный статус бактериальных клеток *B. cereus* с эквимолярными антиоксидантами и без них

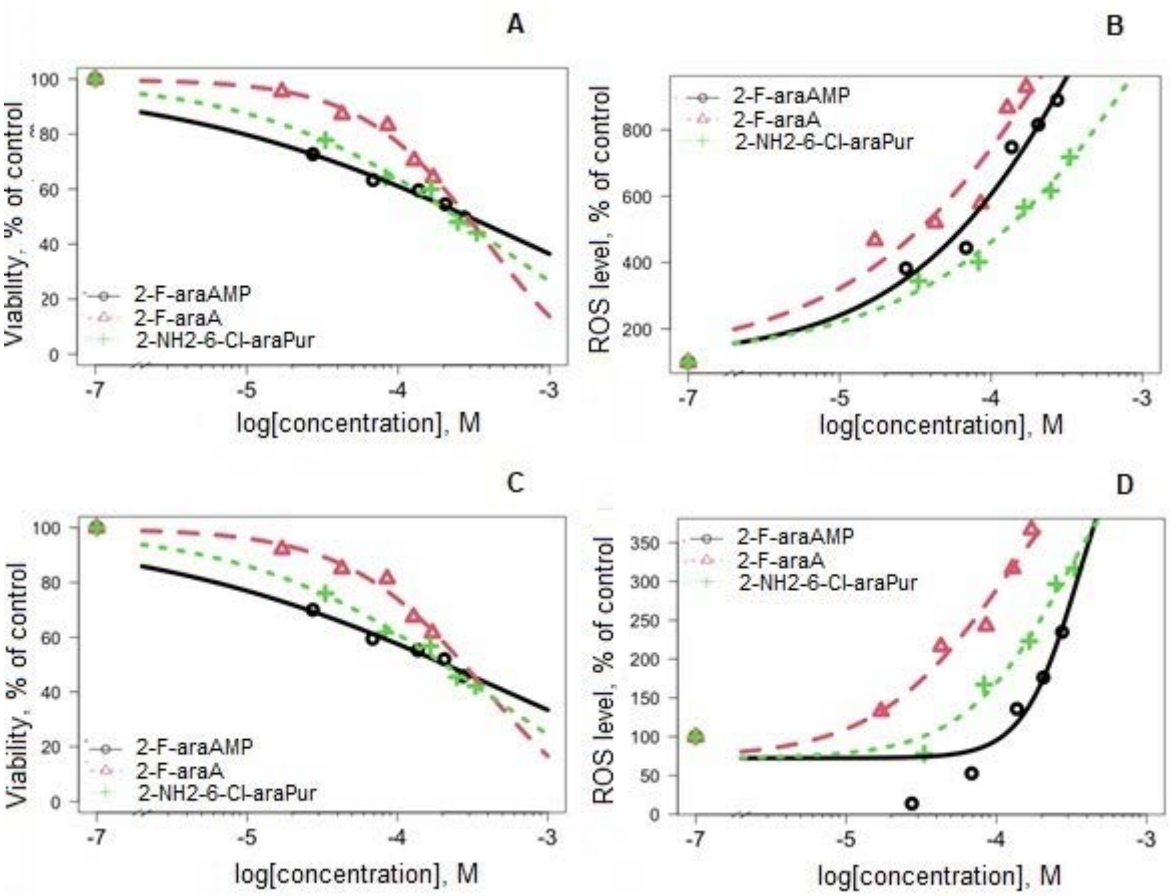


Figure 3 – Effects of different concentrations of modified purine nucleosides and a nucleotide on bacterial cells *P. mirabilis* growth and antioxidant status with and without equimolar quercetin

Рисунок 3 – Влияние различных концентраций модифицированных пуриновых нуклеозидов и нуклеотида на рост и антиоксидантный статус бактериальных клеток *P. mirabilis* с эквимолярными антиоксидантами и без них

reaction. Consequently, a cascade process is initiated, generating hydroxyl radicals that have the potential to directly harm intracellular DNA, lipids, and proteins. To investigate the plausibility of the aforementioned antibacterial action mechanism, bacterial cultures were grown in the presence of the specified compounds at equivalent concentrations, with DCFH-DA utilized as a non-specific indicator to quantify reactive oxygen species levels. The findings demonstrated a substantial increase in reactive oxygen species levels across all bacterial cultures when cultivated with the compounds under study (See Figures 2B, 3B).

A number of conclusions can be drawn from the data presented in Figures 1B and 2B. First, fludarabine proved to be the most effective compound in increasing the level of reactive oxygen species when it acted on both bacterial cell cultures. Secondly, fludarabine demonstrated a more pronounced elevation in reactive oxygen species levels across all concentrations when targeting Gram-positive *B. cereus* cells. Moreover, a notable exponential surge in reactive oxygen species levels was observed for both bacterial cultures upon reaching concentrations of 0.1 μM for all compounds investigated, aligning with the $IC_{50}$ values derived from growth inhibition data. Notably, fludarabine phosphate exhibited the lowest efficacy in inducing reactive oxygen species levels in *B. cereus* cultures, while 2-amino-6-chloro-arabinofuranosylpurine was the most effective in *P. mirabilis* cultures among the compounds examined.

The incorporation of the antioxidant quercetin into the reagent mixture did not alter the efficacy of the compounds in suppressing bacterial cell growth (Figures 2C and 3C). Concurrently, there was a universal elevation in reactive oxygen species levels across all bacterial cultures exposed to the studied compounds; however, growth dynamics were comparatively subdued in the presence of the antioxidant (Figures 2D and 3D). Notably, the most potent combination, fludarabine with quercetin, led to a modest five-fold rise in intracellular reactive oxygen species levels (a three-fold reduction compared to the antioxidant-free experiment), while the least effective combination, fludarabine monophosphate with quercetin, exhibited minimal impact on reactive oxygen species levels.

Next, the impact of varying concentrations of modified pyrimidine nucleosides and nucleotides in the absence of an antioxidant (A, B) compared to when equimolar amounts of an antioxidant are included (C, D) on the proliferation and antioxidative condition of *B. cereus* bacterial cells was studied.

The trends observed in cell growth alterations suggest a diminishing efficacy in the inhibitory impact of pyrimidine derivatives on *B. cereus* cell growth in the following order: ara-CMP (IC50=1.85 · $10^{-4}$ M) > TTU ($IC_{50}$ = 4.22 · $10^{-4}$ M) > cyclo-CMP ($IC_{50}$ = 5.65 · $10^{-4}$ M) > ara-C ($IC_{50}$ = 6.41 · $10^{-4}$ M). Concurrently, the capacity of these compounds to induce reactive oxygen species production was escalated accordingly (Figures 4A and 4B).

The following investigation examines the influence of different levels of modified pyrimidine nucleosides and nucleotides on the growth and antioxidant status of P. mirabilis bacteria in two scenarios: without an antioxidant (A, B) and with equimolar quantities of an antioxidant (C, D).

Under the influence of pyrimidine series compounds at equivalent concentrations on *P. mirabilis* cells, the inhibitory efficacy of the compounds showed a decreasing trend within the series TTU ($IC_{50}$ = 2.94 · $10^{-4}$ M) > cyclo-CMP ($IC_{50}$ = 4.04 · $10^{-4}$ M) > ara-C ($IC_{50}$ = 6.45 · $10^{-4}$ M) > ara-CMP ($IC_{50}$ = 2.32 · $10^{-2}$ M). Furthermore, the pattern of changes in cell viability in the presence of ara-CMP deviated from the dose-response models established for the other compounds (Figure 5A).

The minimal rise in reactive oxygen species occurred in the presence of ara-CMP (4-fold), contrasting with ara-C and ara-CMP which led to a 10-fold and cyclo-CMP an 8-fold elevation in reactive oxygen species levels, as depicted in Figure 5B.

Quercetin did not impact the antibacterial activity of modified nucleosides and pyrimidine nucleotides (Figures 4C and 5C); however, it attenuated their capacity for upregulation (Figures 4D–5D). The most potent compound in inhibiting the growth of *B. cereus* cells, ara-CMP, when combined with quercetin, led to a seven-fold increase in intracellular reactive oxygen species levels (a 1.7-fold decrease compared to the experiment conducted without the antioxidant). Notably, cyclo-CMP exhibited relatively lower levels

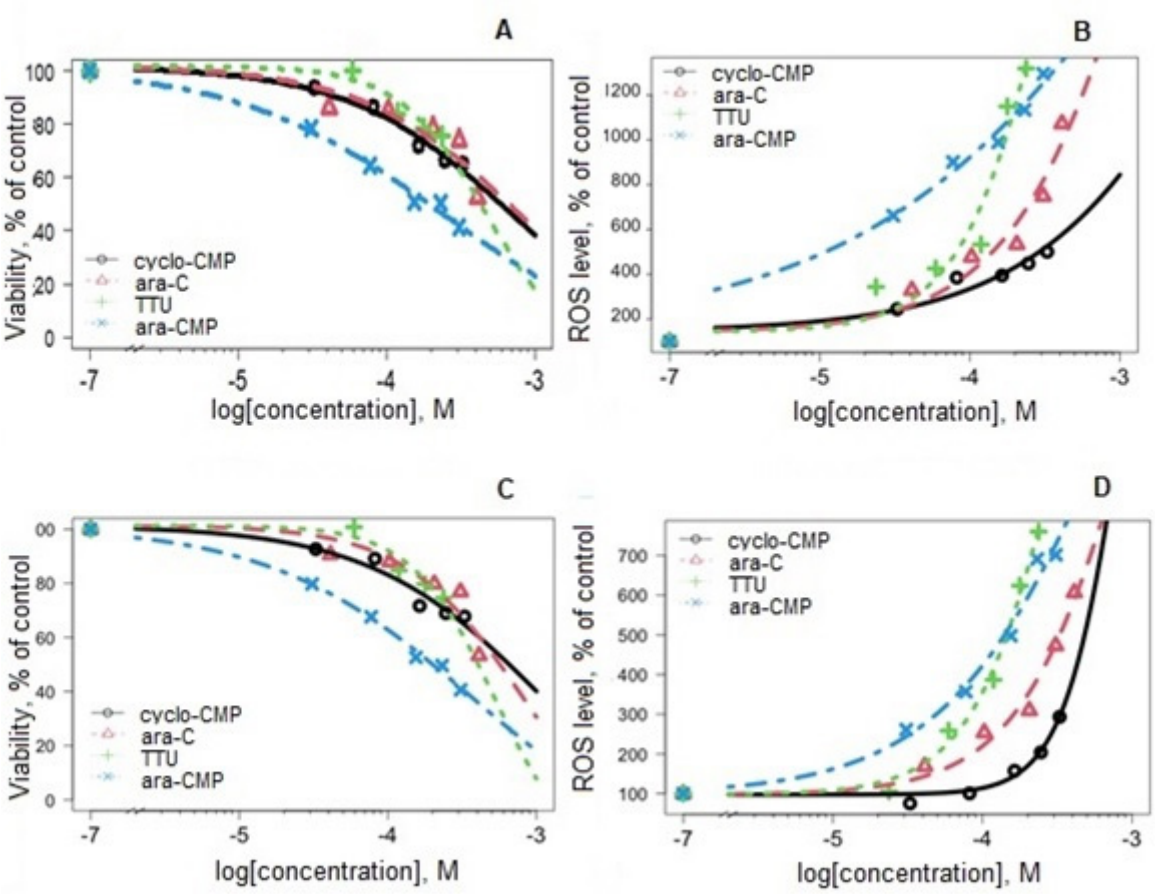


Figure 4 – Effects of different concentrations of modified pyrimidine nucleosides and nucleotides on bacterial cells *B. cereus* growth and antioxidant status with and without equimolar quercetin

Рисунок 4 – Влияние различных концентраций модифицированных пиримидиновых нуклеозидов и нуклеотидов на рост и антиоксидантный статус бактериальных клеток *B. cereus* с эквимолярными антиоксидантами и без них

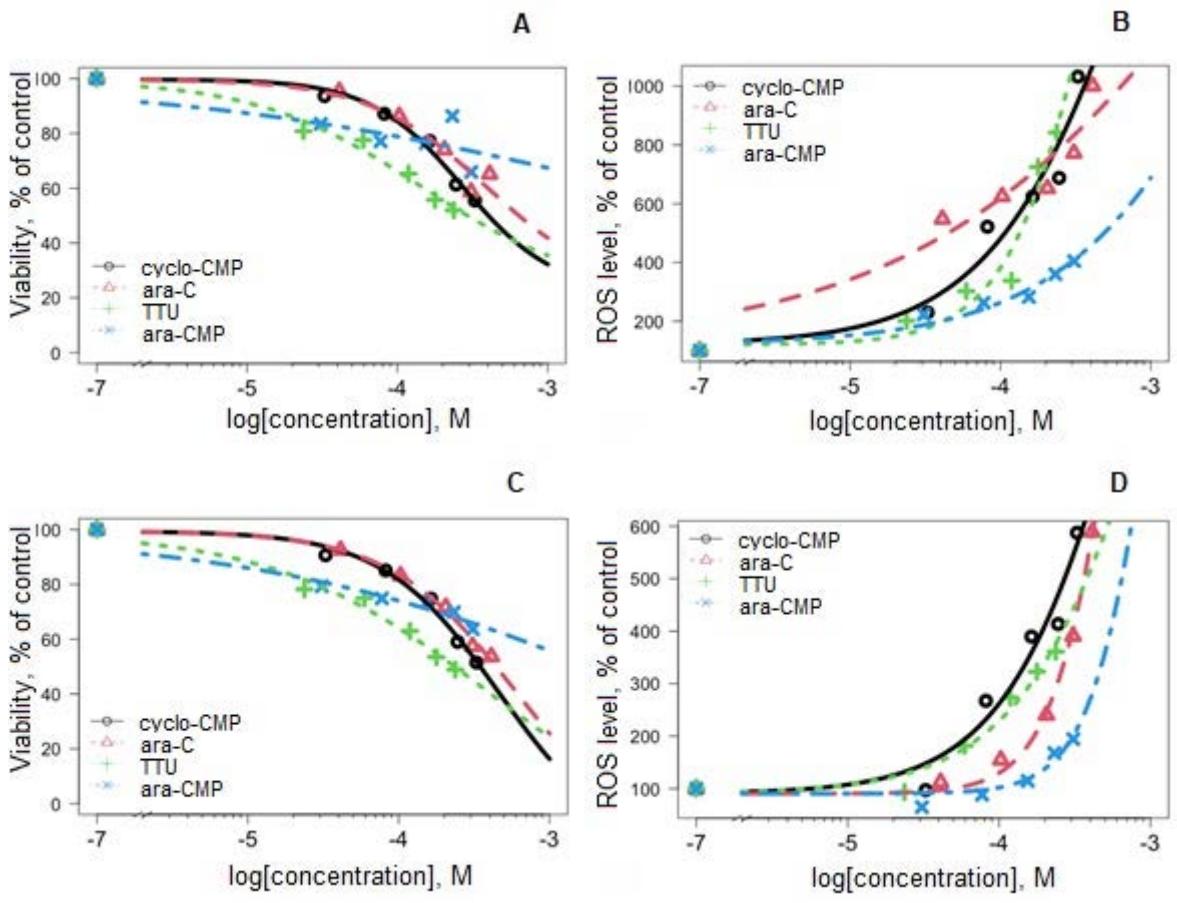


Figure 5 – Effects of different concentrations of modified pyrimidine nucleosides and nucleotides on bacterial cells *P. mirabilis* growth and antioxidant status with and without equimolar quercetin

Рисунок 5 – Влияние различных концентраций модифицированных пиримидиновых нуклеозидов и нуклеотидов на рост и антиоксидантный статус бактериальных клеток *P. mirabilis* с эквимолярными антиоксидантами и без них

of reactive oxygen species: a four-fold increase under cultivation conditions without quercetin and a three-fold increase in the presence of the antioxidant (Figures 4B and 4D).

In the Gram-negative *P. mirabilis* culture, the compound ara-CMP exhibited the least impact on the relative levels of reactive oxygen species within the cells. A four-fold increase was observed without the addition of an antioxidant, and a two-fold increase was noted in its presence, aligning with the antibacterial activity data. Across the studied compounds at concentrations of $10^{-4}$ M and above, similar efficacy was observed concerning oxidative stress levels, with a rise in reactive oxygen species levels by 7–10 times. Notably, a higher sensitivity to the antioxidant quercetin was observed during the cultivation of P. mirabilis with ara-C at lower concentrations (below $10^{-4}$ M), resulting in only a 1.5-fold increase in reactive oxygen species levels, which is five times lower compared to a system without quercetin.

The outcomes derived from our investigation indicate that each of the substances demonstrates encouraging antibacterial efficacy, a quality that can be linked to their capacity to trigger the generation of reactive oxygen species within bacterial cellular structures.

The mechanisms of action of these compounds, although requiring further investigation, are thought to involve interference with DNA synthesis, disruption of electron transport chains, and induction of DNA damage responses, ultimately leading to the generation of ROS and damage to intracellular components. Indeed, the generation of reactive oxygen species is a significant factor in the cytotoxic effects of these compounds. ROS, which include species such as superoxide radicals, hydrogen peroxide and hydroxyl radicals, are highly reactive molecules that can cause damage to various cellular components, including proteins, lipids, and DNA. This damage can ultimately lead to cell death.

The observation that quercetin reduced levels of reactive oxygen species in bacterial cells exposed to the altered nucleosides and nucleotides is a noteworthy discovery that may be linked to its antioxidative characteristics. Quercetin's capacity to eliminate harmful free radicals, impede ROS generation, and

stimulate the activity of antioxidant enzymes probably plays a role in diminishing ROS concentrations within bacterial cells. Furthermore, this finding could have significant implications in the field of bacterial biology, particularly in understanding the mechanisms of oxidative stress in bacterial cells. The ability of quercetin to modulate oxidative stress in bacterial cells could also open up new avenues for the development of novel antimicrobial agents.

The specificity of action of these compounds towards bacterial cells is thought to be due to differences in cell wall structure, variations in metabolic pathways, and faster proliferation rates compared to human cells. However, further investigation is necessary to confirm and fully elucidate the selectivity of these compounds towards bacterial cells.

## Conclusions

The extensive utilization of antibiotics and the rapid emergence of antibiotic resistance have highlighted the urgent need for the discovery of new antibacterial agents. In this context, modified nucleosides and nucleotides, which serve as the fundamental units of DNA and RNA, have emerged as promising candidates due to their potential to impact enzyme recognition and overall efficacy against various pathogens. In our study, we evaluated the antibacterial properties of various altered purine and pyrimidine nucleosides and nucleotides in an in vitro setting and examined their interactions with antioxidants.

Our findings revealed that all examined modified nucleosides and nucleotides demonstrated dose-dependent inhibitory effects on the growth of both Gram-negative (*P. mirabilis*) and Gram-positive (*B. cereus*) bacterial cultures. Furthermore, the presence of antioxidants did not significantly alter their antibacterial activity but led to a universal elevation in intracellular reactive oxygen species levels across all bacterial cultures. These results suggest a potential antibacterial mechanism involving the generation of reactive oxygen species, leading to damage to intracellular DNA, lipids, and proteins.

Among the tested compounds, fludarabine proved to be the most effective in increasing reactive oxygen species levels and demonstrated a more significant impact on Gram-positive *B. cereus* cells. The other examined compounds, such as 2-$NH_2$-6-Cl-ara-Pur and fludarabine monophosphate, also induced notable increases in reactive oxygen species levels, albeit to a lesser extent than fludarabine.

In the context of modified pyrimidine nucleosides and nucleotides, the trends observed in cell growth alterations suggested a diminishing efficacy in the inhibitory impact of pyrimidine derivatives on bacterial cell growth, with ara-CMP being the most effective. Additionally, the capacity of these compounds to induce reactive oxygen species production escalated accordingly.

In conclusion, this study provides valuable insights into the antibacterial properties of modified nucleosides and nucleotides and their potential interaction with antioxidants. Our findings contribute to the growing body of evidence supporting the exploration of these compounds as potential antibacterial agents, particularly in the context of the global antibiotic resistance crisis. Future research should focus on the elucidation of the molecular mechanisms underlying the antibacterial properties of these compounds and their potential for clinical application.

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# ПОТЕНЦИАЛ МОДИФИЦИРОВАННЫХ НУКЛЕОЗИДОВ И НУКЛЕОТИДОВ КАК НОВЫХ АНТИБАКТЕРИАЛЬНЫХ АГЕНТОВ: *IN VITRO* ИССЛЕДОВАНИЕ ИНГИБИРОВАНИЯ РОСТА БАКТЕРИЙ И ИНДУКЦИИ ОКИСЛИТЕЛЬНОГО СТРЕССА

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**Введение.** Чрезмерное использование антибиотиков в здравоохранении привело к росту устойчивости к антибиотикам. Для борьбы с устойчивыми бактериями срочно необходимы новые антибактериальные препараты. Производные нуклеиновых кислот демонстрируют потенциал в качестве противомикробных средств.

**Цель исследования.** Оценка антибактериальной эффективности модифицированных нуклеозидов и нуклеотидов в условиях *in vitro*.

**Материалы и методы.** В работе изучались модифицированные нуклеозиды/нуклеотиды на основе пуриновых/пиримидиновых оснований. Использовали бактериальные культуры *P. mirabilis* и *B. cereus*. Скорость роста оценивали по метаболизму резазурина, уровни АФК в бактериальных клетках оценивались с использованием зонда DCFH-DA.

**Результаты.** Показано, что все исследованные соединения оказывали дозозависимое ингибирующее действие на рост исследованных бактериальных культур. Включение антиоксиданта кверцетина в смесь реагентов не изменяло эффективность соединений в подавлении роста бактериальных клеток. Однако во всех бактериальных культурах, подвергшихся воздействию изучаемых соединений, наблюдалось повсеместное повышение внутриклеточных уровней АФК, что указывает на то, что антибактериальная активность этих соединений может быть связана с их способностью вызывать окислительный стресс в бактериальных клетках.

**Заключение.** Исследование показало, что модифицированные нуклеозиды и нуклеотиды обладают потенциальными антибактериальными свойствами и могут быть полезны в качестве новых антибактериальных средств.

**Ключевые слова:** модифицированные нуклеозиды, нуклеотиды, антибактериальная активность, активные формы кислорода (АФК), окислительный стресс, штаммы бактерий с множественной лекарственной устойчивостью.

**Для цитирования.** Потенциал модифицированных нуклеозидов и нуклеотидов как новых антибактериальных агентов: *in vitro* исследование ингибирования роста бактерий и индукции окислительного стресса / А. Г. Сыса [и др.] // Биохимия и молекулярная биология. – 2024. – Т. 3, № 1(4). – С. 211–219.

*Поступила 11.04.2024*

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