RESEARCH PAPER

Modified Purine and Pyrimidine Nucleosides Exhibit ROS-Independent Bacteriostatic Activity and Quercetin-Mediated Attenuation of Hematotoxicity in Experimental Endotoxemia

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ABSTRACT

Background:

Antimicrobial resistance necessitates the exploration of non-conventional antibacterial targets. Modified nucleosides, established as antiviral and anticancer agents, represent an underexplored compound class for antibacterial applications, yet their mechanism of action in bacteria remains poorly understood.

Objectives:

To (1) characterise the bacteriostatic activity and structure–activity relationships of modified purine and pyrimidine nucleosides, (2) determine whether reactive oxygen species (ROS) generation is causally required for growth inhibition, and (3) evaluate whether quercetin co-administration can attenuate oxidative side effects without compromising antibacterial efficacy.

Methods:

Four modified nucleosides – fludarabine, 2-amino-6-chloro-arabinofuranosylpurine (2-NH2-6-Cl-araPur), cytarabine, and 1-(2′,3′,5′-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)uracil (TTU) – and their 5′-monophosphates were tested against Gram-positive (Bacillus cereus, Bacillus subtilis, Sarcina lutea) and Gram-negative (Escherichia coli, Proteus mirabilis) bacteria. IC_{50} values were determined by four-parameter log-logistic regression (six independent experiments per condition). Intracellular ROS were measured using the semi-quantitative DCFH-DA fluorimetric probe. A quercetin dissociation strategy was employed: equimolar quercetin was co-administered, and both ROS levels and IC_{50} values were compared using equivalence testing (two one-sided t-tests [TOST], bounds ±25%). Bacteriostatic activity was additionally confirmed under anaerobic conditions. Phenotypic enzymatic profiling (catalase, dehydrogenase, invertase, amylase, protease) was performed in B. subtilis. Systemic effects were assessed in vivo in a lipopolysaccharide (LPS)-induced endotoxemia model in Wistar rats (85 animals, 17 groups; hematology, hepatic markers, antioxidant defence). Multiple comparisons were controlled by Benjamini–Hochberg false discovery rate (FDR) correction.

Results:

All compounds inhibited bacterial growth dose-dependently (IC_{50} range: 4.6-645.0 μM). Two-way ANOVA confirmed that purine derivatives exhibited significantly greater activity than pyrimidine analogues ($F_{1,20} = 12.7$, $p = 0.002$); Gram-positive bacteria were more susceptible than Gram-negative species. Compounds induced a 2.0-10.0-fold increase in intracellular ROS; however, equimolar quercetin reduced ROS by 1.7-5.0-fold without altering IC_{50} values. TOST equivalence testing confirmed that IC_{50} values with and without quercetin were statistically equivalent for all 28 compound-strain combinations ($p < 0.05$ for equivalence). Furthermore, bacteriostatic activity was preserved under anaerobic conditions (IC_{50} values within 1.5-fold of aerobic values), providing additional evidence for a ROS-independent mechanism. Enzymatic profiling revealed a pattern consistent with metabolic stress: increased catalase activity (+25-35%), unchanged central metabolic enzymes (dehydrogenase, invertase), and decreased secretory enzyme activity (amylase –22-33%; protease –28%). In vivo, co-administration of quercetin with modified nucleosides during LPS-induced endotoxemia partially corrected hematotoxicity (hemoglobin closer to control by 9-27 percentage points), reduced cytolysis markers (ALT 1.2-1.4-fold decrease), and restored antioxidant parameters (TBARS decreased by 29-72%; SOD increased by 15-25%).

Conclusions:

Modified nucleosides exert bacteriostatic activity through a ROS-independent mechanism, as demonstrated by convergent evidence from quercetin dissociation experiments, anaerobic growth inhibition, and enzymatic profiling. The data are consistent with a model in which these compounds may interfere with bacterial nucleotide metabolism,

although direct demonstration of dNTP pool perturbation remains to be performed. Quercetin co-administration attenuates oxidative side effects without compromising antibacterial efficacy, supporting a rational combination strategy.
Keywords: modified nucleosides; bacteriostatic activity; reactive oxygen species; nucleotide metabolism; quercetin; endotoxemia; antimicrobial resistance; antimetabolites; equivalence testing.
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1. Introduction

The global spread of antimicrobial resistance (AMR) constitutes one of the most pressing biomedical challenges of the 21st century. Resistance emerges and disseminates rapidly because antibacterial therapy imposes strong selection on microbial populations, while horizontal gene transfer accelerates the acquisition of resistance determinants across species and ecological niches (Murray et al., 2022; O'Neill, 2016; World Health Organization [WHO], 2021). In addition to the direct clinical burden, AMR undermines modern medical practice by increasing the risk of complications in routine surgery, immunosuppressive therapy, intensive care, and the management of chronic diseases (Murray et al., 2022; WHO, 2021). Although antibacterial drugs transformed medicine in the 20th century, the pace of discovery of truly new antibacterial classes has slowed, while resistance to many legacy antibiotics continues to increase (Hutchings et al., 2019; Lewis, 2020). Consequently, there is an urgent need to expand the antibacterial target space and to develop compounds with biochemical modes of action distinct from those of widely used antibiotics.

A substantial proportion of clinically used antibacterial agents act on a limited set of cellular processes: cell wall biogenesis, protein synthesis, DNA topology (gyrase/topoisomerase), folate metabolism, or membrane integrity (Hutchings et al., 2019; Lewis, 2020). Conceptually, one route to diversify antibacterial mechanisms is to target highly conserved and essential metabolic hubs where even moderate perturbations can lead to loss of growth capacity and sensitisation to stress. Among such hubs, nucleotide metabolism is particularly attractive because nucleotides are indispensable not only for DNA replication and RNA transcription, but also for energy transfer (ATP/GTP), signalling (c-di-AMP, c-di-GMP), cofactor synthesis (e.g., NAD), and metabolic regulation. Importantly, the cellular nucleotide pool is tightly controlled; deviations in the concentration ratios of (d)NTPs, or the accumulation of non-canonical nucleotide species, can cause replication stress, transcriptional dysregulation, and genome instability (Jordheim et al., 2013; Nyhan, 2014; Parker, 2009). In bacteria, these effects are integrated through DNA damage and replication stress responses, including SOS-regulated pathways that couple DNA integrity, cell cycle progression, and survival (Imlay, 2013; Maslowska et al., 2019).

Nucleoside and nucleotide analogues are a mature class of bioactive compounds in antiviral and anticancer therapy, where their efficacy often depends on intracellular activation (sequential phosphorylation to the triphosphate form) followed by competition with endogenous nucleotides, inhibition of polymerases, and/or incorporation into nucleic acids (Jordheim et al., 2013; Parker, 2009). A key biochemical feature of many nucleoside analogues is that they function as antimetabolites, acting either as alternative substrates or as competitive inhibitors within nucleotide biosynthesis and salvage networks. While nucleoside analogues are classically associated with eukaryotic or viral targets, their antibacterial potential is increasingly recognised, particularly for analogues that can be processed by bacterial salvage enzymes and that interfere with nucleic acid synthesis or nucleotide homeostasis (Jordheim et al., 2013; Nyhan, 2014; Thomson & Lamont, 2019). However, compared with antiviral and anticancer contexts, the antibacterial mode-of-action space of nucleoside analogues remains underexplored and frequently lacks mechanistic anchoring at the biochemical level.

Bacteria maintain nucleotide homeostasis via interconnected de novo and salvage pathways. Salvage reactions recycle nucleobases and nucleosides from the environment or from intracellular turnover, using nucleoside phosphorylases, nucleoside kinases, and phosphotransfer reactions to generate nucleotides that feed DNA/RNA synthesis and metabolism (Nyhan, 2014). Because salvage enzymes and transport systems differ across taxa, susceptibility to nucleoside analogues may vary between Gram-negative and Gram-positive bacteria and between species, reflecting differences in membrane permeability, transporter repertoire, kinase specificity, and intracellular metabolic context (Nikaido, 2003). These considerations imply that a mechanistic interpretation of antibacterial activity in the nucleoside analogue class should explicitly address (i) activation plausibility through salvage enzymes, (ii) potential engagement of enzymes controlling nucleotide pools,

and (iii) the likelihood of replication/transcription stress as the proximal cause of growth inhibition.

A recurring theme in antibacterial research is the possible contribution of reactive oxygen species (ROS) to bacterial killing or growth arrest. ROS are generated as by-products of aerobic metabolism, and bacteria possess detoxification systems (superoxide dismutases, catalases, peroxidases) and regulatory circuits (e.g., OxyR/SoxRS) that limit oxidative damage (Imlay, 2013). A prominent hypothesis proposed that bactericidal antibiotics may induce a lethal oxidative burst, amplifying damage through iron-dependent Fenton chemistry and oxidative lesions in DNA, proteins, and lipids (Kohanski et al., 2007). However, this ROS-centric model has been challenged; multiple independent studies indicate that bactericidal activity can occur without a requirement for ROS, suggesting that oxidative stress may be a secondary phenotype associated with metabolic disturbance rather than the primary cause of growth inhibition or death (Keren et al., 2013; Liu & Imlay, 2013). In this context, ROS measurements are best interpreted as a stress readout unless causality is specifically tested – an approach we adopt in the present study.

We have previously reported preliminary data on the bacteriostatic activity of individual nucleoside analogues against selected bacterial species (Author et al., 2022a, 2022b). The present study substantially extends this work by (i) providing a systematic comparison of purine versus pyrimidine series with formal statistical analysis, (ii) introducing the quercetin dissociation experiment to directly test the ROS hypothesis, (iii) confirming ROS-independent activity under anaerobic conditions, (iv) adding phenotypic enzymatic profiling as an orthogonal readout of the metabolic response, and (v) evaluating the nucleoside–quercetin combination in an in vivo model of LPS-induced endotoxemia — none of which were addressed in our previous publications.

The specific questions addressed are: (i) Do modified nucleosides inhibit bacterial growth, and is this activity structurally dependent? (ii) Is ROS generation causally required for the bacteriostatic effect, or is it a secondary consequence? (iii) Can quercetin co-administration attenuate oxidative side effects without compromising antibacterial efficacy?

2. Materials and Methods

2.1. Compounds

The following modified nucleosides were used: fludarabine (2-fluoro-9-β-D-arabinofuranosyladenine), 2-amino-6-chloro-9-β-D-arabinofuranosylpurine (2-NH_2-6-Cl-araPur), cytarabine (1-β-D-arabinofuranosylcytosine, ara-C), and [1-(2′,3′,5′-tri-O-acetyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)]uracil (TTU). The corresponding 5′-monophosphates – fludarabine-5′-monophosphate (F-ara-AMP) and cytarabine-5′-monophosphate (ara-CMP) – and 3′,5′-cyclic cytidine monophosphate (cyclo-CMP) were also tested.

All nucleoside and nucleotide preparations were synthesised and purified (>98%) using published procedures. The synthesis of halogenated purine arabinofuranosides (fludarabine, 2-NH_2-6-Cl-araPur) followed general procedures for fluorinated and chlorinated nucleoside preparation developed by the group (Mikhailopulo et al., 1991). The arabinofuranosyl sugar configuration (present in fludarabine, 2-NH_2-6-Cl-araPur, and cytarabine) was introduced using enzymatic transglycosylation methodology with bacterial nucleoside phosphorylases (Zinchenko et al., 1990). Fludarabine-5′-monophosphate was synthesised enzymatically using recombinant bacterial kinases as described by Beresnev et al. (2017). The design of nucleoside triphosphate analogues as DNA polymerase inhibitors was informed by the group's prior work on 3′-fluoro-2′,3′-dideoxynucleoside 5′-triphosphates (Chedgeavadze et al., 1985). Structural identity and purity of all preparations were confirmed by ^{1}H-NMR, UV spectrophotometry, and thin-layer chromatography.

Quercetin (≥95% purity; Sigma-Aldrich, St. Louis, MO, USA) was used as the antioxidant reference compound. Stock solutions were prepared in dimethyl sulfoxide (DMSO) immediately before use; the final DMSO concentration in all assays did not exceed 0.5% (v/v), which was confirmed to have no effect on bacterial growth or ROS levels in solvent control experiments.

2.2. Bacterial Strains and Culture Conditions

The bacterial panel included Gram-positive (*Bacillus cereus, Bacillus subtilis*, and *Sarcina lutea* [syn. *Micrococcus luteus*]) and Gram-negative (*Escherichia coli, Proteus mirabilis*) organisms. Ehe bacteria used in this study were environmental and food-derived isolates. Primary isolation was performed using selective and differential media. Genus- and species-level identification was confirmed through standard microbiological protocols, including Gram staining, cellular morphology, and comprehensive biochemical profiling.

All strains were maintained on nutrient agar at 4 °C. Working cultures were prepared by inoculating a single colony into liquid nutrient broth (Himedia, India) and growing overnight at 37 °C with shaking (180 rpm).

For all experiments, overnight cultures were adjusted to an OD_{600} of 0.10 ± 0.02, corresponding to (1.0-1.5)*10^6 colony-forming units (CFU)/mL as verified by plating serial dilutions on nutrient agar in three independent calibration experiments per strain. This inoculum density is consistent with the recommendations of the Clinical and Laboratory Standards Institute for broth microdilution susceptibility testing (CLSI, 2018).

2.3. Bacteriostatic Activity Assay

Dose-response experiments were performed in flat-bottom 96-well polystyrene microplates (Sarstedt, Germany). Standardised bacterial suspensions (100 µL/well) were exposed to serial two-fold dilutions of test compounds (final concentration range: 10^{-5}-10^{-3} M; 100 µL/well) in triplicate. Positive controls (bacteria + DMSO vehicle) and negative controls (sterile broth + compound, to correct for compound-induced turbidity) were included on each plate. Plates were incubated at 37 °C for 18-24 h, and growth was quantified by measuring optical density at 600 nm (OD_{600}) using a Multiskan FC microplate reader (Thermo Scientific, Finland). Percent inhibition was calculated as:

$$\text{Inhibition (\%)} = [1 - (OD_{600}^{treated} - OD_{600}^{neg}) / (OD_{600}^{pos} - OD_{600}^{neg})] * 100$$

The half-maximal inhibitory concentration (IC_{50}) was calculated by fitting the concentration-response data to a four-parameter log-logistic model using nonlinear regression (GraphPad Prism 9.0, La Jolla, CA, USA). Each IC_{50} value represents the mean of six independent experiments, each performed in triplicate wells.

Inoculum effect control. To verify the absence of a clinically significant inoculum effect, IC_{50} values for fludarabine and cytarabine were determined at three inoculum densities (10^5, 10^6, and 10^7 CFU/mL) against *E. coli* and *S. lutea*.

Anaerobic growth inhibition. To test whether bacteriostatic activity requires aerobic metabolism, E. coli and B. subtilis were grown anaerobically using the GasPak EZ system (BD, USA) in the presence of fludarabine and cytarabine at their respective aerobic IC_{50} concentrations. Growth was monitored by OD_{600} after 24 h, and anaerobic IC_{50} values were determined as described above. Results are presented in Supplementary Table S2.

2.4. Quercetin Co-Administration and ROS Independence Assessment

For quercetin co-treatment experiments, equimolar concentrations of quercetin were added simultaneously with the nucleoside analogues at the onset of incubation. Both intracellular ROS levels and IC_{50} values were measured in parallel for each compound–strain combination in the presence and absence of quercetin.

The comparison of IC_{50} values ± quercetin was assessed in two complementary ways:

- difference testing: Paired t-test comparing IC_{50} values with and without quercetin for each compound–strain pair ($\alpha = 0.05$).
- equivalence testing: Two one-sided t-tests (TOST) with equivalence bounds of ±25% (Lakens, 2017). If the 90% confidence interval for the mean IC_{50} ratio fell entirely within the range 0.75-1.25, IC_{50} values were declared statistically equivalent.

Post hoc power analysis. For the IC_{50} comparison (n = 6 per group), the observed coefficient of variation of IC_{50} values (15-20%), and $\alpha = 0.05$, the study had >80% power to detect a ≥30% change in IC_{50} (G*Power 3.1.9.7; Faul et al., 2007). This confirms that the study was adequately powered to detect a biologically meaningful difference.

2.5. Intracellular ROS Measurement

Intracellular ROS levels were assessed fluorimetrically using the semi-quantitative probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich), according to established protocols (Kalyanaraman et al., 2012). DCFH-DA is a cell-permeant compound that is cleaved by intracellular esterases to yield 2′,7′-dichlorodihydrofluorescein (DCFH), which is subsequently oxidised by intracellular oxidant species (including H_2O_2, OH•, $ONOO^-$, and peroxidase-generated intermediates) to yield the fluorescent product DCF.

Bacterial cells were incubated with test compounds at their respective IC_{50} concentrations for 4 h at 37 °C, then loaded with DCFH-DA (10 µM, 30 min, 37 °C in the dark). Fluorescence was measured using a Fluoroskan Ascent FL microplate fluorimeter (Thermo Scientific; excitation 485 nm, emission 535 nm). Background fluorescence of compound-treated, DCFH-DA-free wells was subtracted from all readings to correct for potential autofluorescence of the test compounds. Results were expressed as relative fluorescence units (RFU) normalised to the untreated control (fold increase).

DCFH-DA is a semi-quantitative probe that detects multiple oxidant species; data are therefore reported as fold-change relative to untreated controls rather than absolute ROS concentrations (Kalyanaraman et al., 2012). We note that the central conclusion of this study – the dissociation between ROS levels and growth inhibition – is robust to any systematic bias in absolute ROS quantification, because it depends on the comparison of IC_{50} values under conditions of high versus reduced ROS, not on the absolute magnitude of the ROS signal.

2.6. Phenotypic Enzymatic Profiling

Enzymatic profiling was performed using B. subtilis ATCC 6633 as a representative organism, following exposure to test compounds at IC_{50} concentrations for 18 h. The following activities were measured as phenotypic readouts of distinct metabolic categories, not as direct assays of the proposed target enzymes:

- catalase activity (oxidative stress response): spectrophotometric assay based on the decomposition of hydrogen peroxide (H_2O_2) at 240 nm (Aebi, 1984); expressed as mmol H_2O_2 per gram of dry biomass per minute.
- dehydrogenase activity (respiratory chain function): reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to

formazan, measured spectrophotometrically at 485 nm (Trevors, 1984); expressed as µg formazan per gram of dry biomass per 24 h.

- invertase activity (carbohydrate metabolism): glucose release from sucrose hydrolysis, measured by the DNS method; expressed as mg glucose per gram of dry biomass per 24 h.
- amylolytic activity (secretory function): zone of starch hydrolysis on starch-agar plates after 48 h incubation, measured in mm.
- proteolytic activity (secretory function): zone of casein hydrolysis on milk-agar plates after 48 h incubation, measured in mm.

All enzymatic assays were performed in six independent replicates.

2.7. Animals and Experimental Design

All procedures involving animals were approved by the Ethics Committee …. (Protocol No. [XX], dated [XX.XX.20XX]) and were performed in accordance with Directive 2010/63/EU and national guidelines for laboratory animal use.

Male Wistar rats (n = 85; body weight 200-250 g; age 8-10 weeks) were obtained from the institutional vivarium, housed under standard conditions (12-h light/dark cycle, 22 ± 2 °C, 55 ± 10% humidity), and provided ad libitum access to standard chow and water. After a 7-day acclimatisation period, rats were randomly allocated into 17 groups of 5 animals each (Table 1).

Table 1. Experimental groups in the *in vivo* study.

Group	Treatment	n
1	Intact control (0.9% NaCl, i.p.)	5
2	LPS control (E. coli O111:B4, 5 mg/kg, i.p.)	5
3	LPS + fludarabine (400 mg/kg, i.p.)	5
4	LPS + F-ara-AMP (400 mg/kg, i.p.)	5
5	LPS + 2-NH_2-6-Cl-araPur (400 mg/kg, i.p.)	5
6	LPS + cytarabine (400 mg/kg, i.p.)	5
7	LPS + ara-CMP (400 mg/kg, i.p.)	5
8	LPS + cyclo-CMP (400 mg/kg, i.p.)	5
9	LPS + TTU (400 mg/kg, i.p.)	5
10	LPS + fludarabine + quercetin (5 mg/kg, i.p.)	5
11	LPS + F-ara-AMP + quercetin	5
12	LPS + 2-NH_2-6-Cl-araPur + quercetin	5
13	LPS + cytarabine + quercetin	5
14	LPS + ara-CMP + quercetin	5
15	LPS + cyclo-CMP + quercetin	5
16	LPS + TTU + quercetin	5
17	LPS + quercetin alone (5 mg/kg, i.p.)	5

Systemic inflammation was induced by a single i.p. injection of LPS from *E. coli* (Sigma-Aldrich; 5 mg/kg). After 7 days, test compounds were administered three times at 48-h intervals. Blood was collected 24 h after the last injection by cardiac puncture under isoflurane anaesthesia.

The nucleoside dose of 400 mg/kg was selected for mechanistic rather than translational purposes, to ensure measurable biochemical effects within the study timeframe. Using the allometric scaling factor recommended by the FDA (Reagan-Shaw et al., 2008), the human equivalent dose (HED) is approximately 65 mg/kg, which exceeds the clinical dose for fludarabine (~0.68 mg/kg in humans). This dose does not represent a proposed therapeutic dose and is consistent with rodent dosing in comparable mechanistic studies of nucleoside analogue toxicity (Galmarini et al., 2002).

2.8. Hematological and Biochemical Analyses

Hematological parameters were measured using an automated veterinary hematology analyser (Abacus Junior Vet, Diatron, Hungary) and included: hemoglobin concentration (g/dL), hematocrit (%), total leukocyte count ($\times 10^3/\mu L$), and lymphocyte count ($\times 10^3/\mu L$).

Serum biochemical markers were measured using an automated clinical chemistry analyser (BioChem SA, High Technology Inc., USA) and included: alanine aminotransferase (ALT, IU/L), aspartate aminotransferase (AST, IU/L), urea (mg/dL), total protein (g/dL), glucose (mg/dL), and chloride concentration (mmol/L).

2.9. Antioxidant Status Assessment

Liver tissue was homogenised in ice-cold phosphate-buffered saline (1:10 w/v) and centrifuged at 10,000g for 15 min at 4 °C. The following parameters were measured in the supernatant:

Thiobarbituric acid-reactive substances (TBARS): Marker of lipid peroxidation, measured spectrophotometrically at 532 nm (Ohkawa et al., 1979); expressed as µmol/mg protein.

Superoxide dismutase (SOD): Measured by the nitro blue tetrazolium (NBT) reduction method (Beauchamp & Fridovich, 1971); expressed as U/mg protein.

Catalase: Measured by the rate of H_2O_2 decomposition at 240 nm (Aebi, 1984); expressed as U/mg protein.

Glutathione peroxidase (GPx): Measured by the NADPH oxidation method (Paglia & Valentine, 1967); expressed as U/mg protein.

Protein content was determined by the Bradford method (Bradford, 1976).

2.10. Statistical Analysis

All statistical analyses and visualisations were performed in R (version 4.3.1; R Core Team, 2023) using the RStudio integrated development environment (version 2023.06; Posit Software, Boston, MA, USA). The following R packages were used:

- drc (version 3.0-1; Ritz et al., 2015) for fitting four-parameter log-logistic dose-response models and calculating IC_{50} values with 95% confidence intervals;

- TOSTER (version 0.8.2; Lakens, 2017) for two one-sided t-tests (TOST) with equivalence bounds of ±25%;
- car (version 3.1-2; Fox & Weisberg, 2019) for Levene's test of homogeneity of variances and Type II/III ANOVA;
- multcomp (version 1.4-25; Hothorn et al., 2008) for Tukey's honest significant difference (HSD) post hoc comparisons;
- stats (base R) for Shapiro–Wilk normality tests and Benjamini–Hochberg FDR correction (p.adjust, method = "BH");
- pwr (version 1.3-0; Champely, 2020) for post hoc power analysis;
- ggplot2 (version 3.4.3; Wickham, 2016), ggpubr (version 0.6.0; Kassambara, 2023), and pheatmap (version 1.0.12; Kolde, 2019) for figure generation.

Data are expressed as means ± standard deviations (SD). The normality of data distribution was verified by the Shapiro-Wilk test (shapiro.test()), and homogeneity of variances by Levene's test (leveneTest()).

Differences between groups were assessed by one-way ANOVA (aov()) followed by Tukey's HSD post hoc test (glht() with mcp()).

For the comparison of compound classes (purine vs. pyrimidine), a two-way ANOVA was performed on $\log_{10}$-transformed IC_{50} values with compound class and bacterial strain as factors (Anova(), Type II).

To control for multiple comparisons, the Benjamini–Hochberg FDR procedure was applied at $\alpha = 0.05$ (p.adjust(method = "BH")).

IC_{50} values were calculated by fitting concentration–response data to the four-parameter log-logistic model using drm() from the drc package. Equivalence of IC_{50} values ± quercetin was assessed by TOST using TOSTpaired() from the TOSTER package.

3. Results

3.1. Bacteriostatic Activity of Modified Nucleosides and Nucleotides

All tested compounds inhibited bacterial growth in a dose-dependent manner across the concentration range of 10^{-5}–10^{-3} M. The IC_{50} values determined from these curves are presented in Table 2.

Table 2. Bacteriostatic activity of modified nucleosides and nucleotides (IC_{50}, × 10^{-4} M).

Compound	*E. coli*	*P. mirabilis*	*B. cereus*	*S. lutea*
Purine series				
Fludarabine	0.20 ± 0.03	2.81 ± 0.35	2.70 ± 0.31	0.86 ± 0.10
F-ara-AMP	0.40 ± 0.05	2.96 ± 0.42	4.40 ± 0.52	1.08 ± 0.14
2-NH_2-6-Cl-araPur	2.20 ± 0.28	2.43 ± 0.30	4.00 ± 0.48	0.046 ± 0.007
Pyrimidine series				
Cytarabine	4.58 ± 0.55	6.45 ± 0.80	6.41 ± 0.75	1.79 ± 0.22
ara-CMP	2.69 ± 0.33	2.32 ± 0.45[a]	1.85 ± 0.24	0.59 ± 0.08
Cyclo-CMP	1.82 ± 0.22	4.04 ± 0.50	5.65 ± 0.68	1.79 ± 0.21
TTU	1.80 ± 0.23	2.94 ± 0.37	4.22 ± 0.50	1.20 ± 0.15

Note. Values are means ± SD of six independent experiments, each performed in triplicate. IC_{50} calculated by four-parameter log-logistic regression.

[a]The viability curve for ara-CMP against *P. mirabilis* deviated from the typical log-logistic relationship; this IC_{50} estimate is characterised by elevated uncertainty.

Two-way ANOVA on $\log_{10}$-transformed IC_{50} values revealed a significant main effect of compound class (purine vs. pyrimidine; $F_{1,20} = 12.7$, $p = 0.002$, $\eta^2_p = 0.39$) and a significant main effect of bacterial strain ($F_{3,20} = 8.4$, $p < 0.001$, $\eta^2_p = 0.56$). The interaction term was not significant ($F_{3,20} = 1.9$, $p = 0.16$), indicating that the greater potency of purine derivatives was consistent across all tested strains.

The IC_{50} range for purine compounds was (0.046-4.40)*10^{-4} M compared with (0.59-6.45)*10^{-4} M for pyrimidines. The most potent compound–strain combination was 2-NH_2-6-Cl-araPur against *S. lutea* ($IC_{50} = 4.6$ μM).

Tukey post hoc comparisons revealed that mean IC_{50} values for S. lutea were significantly lower than for all other strains ($p < 0.01$ for all pairwise comparisons, FDR-corrected). Across all compounds, Gram-positive bacteria (*S. lutea, B. cereus*) displayed lower IC_{50} values than Gram-negative species (*E. coli, P. mirabilis*), consistent with the well-established permeability barrier conferred by the outer membrane (Nikaido, 2003).

Comparison of nucleoside–nucleotide pairs revealed heterogeneous effects of phosphorylation. For the cytarabine/ara-CMP pair against *B. cereus*, the 5′-monophosphate was 3.5-fold more potent (IC_{50}: 6.41 vs. 1.85*10^{-4} M; $p < 0.001$), whereas differences between fludarabine and F-ara-AMP were not statistically significant for any strain. This heterogeneity is consistent with the complexity of intracellular nucleotide processing, where the net contribution of the phosphate group depends on cellular uptake, de-/rephosphorylation,

and competition with endogenous substrates (Jordheim et al., 2013).

IC_{50} values for fludarabine and cytarabine at inoculum densities of 10^5, 10^6, and 10^7 CFU/mL differed by less than 2-fold, confirming the absence of a significant inoculum effect for this compound class. This result is consistent with the antimetabolite mode of action, which does not involve enzymatic degradation of the compound by the target organism (Udekwu et al., 2009).

3.2. Intracellular ROS Induction and the Quercetin Dissociation Experiment

3.2.1. ROS Induction

All tested compounds induced dose-dependent increases in intracellular ROS across all bacterial strains, as measured by the DCFH-DA probe (Table 3).

Table 3. Intracellular ROS induction in bacterial cells exposed to modified nucleosides and nucleotides at IC_{50} concentrations (fold increase relative to untreated control).

Compound	*E. coli*	*P. mirabilis*	*B. cereus*	*S. lutea*
Purine series				
Fludarabine	3.5 ± 0.4	4.2 ± 0.5	3.8 ± 0.4	5.0 ± 0.6
F-ara-AMP	2.8 ± 0.3	5.0 ± 0.6	5.0 ± 0.5	2.5 ± 0.3
2-NH_2-6-Cl-araPur	4.0 ± 0.5	3.0 ± 0.4	3.2 ± 0.4	3.5 ± 0.4
Pyrimidine series				
Cytarabine	2.5 ± 0.3	4.5 ± 0.5	2.8 ± 0.3	10.0 ± 1.2
ara-CMP	3.0 ± 0.4	3.5 ± 0.4	4.5 ± 0.5	8.0 ± 0.9
Cyclo-CMP	3.2 ± 0.4	2.8 ± 0.3	2.5 ± 0.3	3.0 ± 0.4
TTU	3.0 ± 0.4	3.0 ± 0.4	2.0 ± 0.2	4.0 ± 0.5

Note. Data represent the ratio of DCFH-DA-derived fluorescence (RFU) in treated cells to untreated control, after subtraction of compound autofluorescence. Values are means ± SD of six independent experiments.

The highest ROS levels were recorded in *S. lutea* upon exposure to cytarabine (10.0 ± 1.2-fold) and ara-CMP (8.0 ± 0.9-fold). This strain also displayed the greatest susceptibility to growth inhibition (Table 2), initially suggesting a possible causal relationship between ROS generation and bacteriostatic activity. To test this hypothesis directly, we employed the quercetin dissociation strategy.

3.2.2. Quercetin Dissociation Experiment

The experimental logic was as follows: if ROS generation is a primary mechanism of growth inhibition, then effective reduction of intracellular ROS should attenuate the bacteriostatic effect (i.e., increase IC_{50} values). Conversely, if ROS are a secondary consequence of metabolic stress, their reduction should leave bacteriostatic activity unchanged.

Equimolar quercetin reduced intracellular ROS levels by 1.7-5.0-fold across all compound-strain combinations (Table 4). The greatest absolute reduction was observed in *B. cereus* cells exposed to F-ara-AMP (from 5.0 ± 0.5 to 1.0 ± 0.1-fold; 5.0-fold reduction).

Table 4. Effect of quercetin on intracellular ROS levels and IC_{50} values.

Strain	ROS without quercetin (fold range)	ROS with quercetin (fold range)	Fold ROS reduction	IC_{50} change
E. coli	2.5-4.0	1.4-2.0	1.75-2.0	None
P. mirabilis	3.0-5.0	1.5-2.5	2.0-3.0	None
B. cereus	2.0-5.0	1.0-3.1	1.6-5.0	None
S. lutea	2.5-10.0	1.9-3.3	1.3-3.0	None

Note. Ranges reflect variation across all seven compounds within each strain.

Despite the substantial reduction in ROS levels, IC_{50} values remained unchanged for all 28 compound-strain combinations. This was confirmed by three statistical approaches:

- paired t-tests: no significant differences between IC_{50} values ± quercetin for any combination ($p > 0.05$, all comparisons, FDR-corrected).
- TOST equivalence testing: IC_{50} values with and without quercetin were statistically equivalent for all 28 combinations ($p < 0.05$ for equivalence at ±25% bounds), confirming that the two conditions produced equivalent levels of growth inhibition.
- magnitude of difference: the mean IC_{50} difference between ± quercetin groups was <5% for all combinations, well within experimental variability.

The probability of observing zero significant IC_{50} changes across 28 independent tests, if ROS were the primary mechanism, is negligible. We conclude that ROS generation is a secondary consequence of the metabolic stress induced by modified nucleosides, not a causally required component of the bacteriostatic effect.

3.2.3. Anaerobic Confirmation

As an orthogonal test of the ROS-independence conclusion, bacteriostatic activity was assessed under anaerobic conditions for fludarabine and cytarabine against *E. coli* and *B. subtilis*. IC_{50} values under anaerobic conditions were within 1.5-fold of aerobic values for all four combinations, confirming that growth inhibition

does not require aerobic ROS generation. This finding is consistent with the results of Keren et al. (2013), who demonstrated that bactericidal antibiotic activity is preserved under anaerobiosis.

3.3. Phenotypic Enzymatic Profiling

The enzymatic profile of B. subtilis exposed to modified nucleosides at IC_{50} concentrations is presented in Table 5.

Table 5. Effect of modified nucleosides on enzyme activities in Bacillus subtilis (phenotypic readouts of metabolic state).

Enzyme (metabolic category)	Control	Purine nucleoside	Pyrimidine nucleoside	Change (%)
Catalase (oxidative stress), mmol H_2O_2/g/min	2.0 ± 0.1	2.5 ± 0.1*	2.7 ± 0.1*	+25 to +35
Dehydrogenase (respiration), µg formazan/g/24h	123 ± 1.2	121 ± 1.1	120 ± 1.5	n.s.
Invertase (carbohydrate metabolism), mg glucose/g/24h	13.1 ± 1.0	12.9 ± 0.8	12.8 ± 0.7	n.s.
Amylase (secretory function), zone mm	9 ± 0.2	7 ± 0.3*	6 ± 0.3*	-22 to -33
Protease (secretory function), zone mm	7 ± 0.2	5 ± 0.2*	5 ± 0.3*	-28

Note. * $p < 0.05$ vs. control (ANOVA + Tukey HSD, FDR-corrected); n.s. = not significant. Data are means ± SD of six independent replicates.

The enzymatic profile reveals a characteristic pattern:

- oxidative stress response activated: catalase activity significantly increased (+25-35%), consistent with the DCFH-DA data and indicative of intracellular H_2O_2 accumulation requiring adaptive detoxification.
- central energy metabolism preserved: dehydrogenase (marker of respiratory chain function) and invertase (marker of carbohydrate metabolism) were not significantly affected, ruling out generalised metabolic poisoning.
- secretory function suppressed: amylase (-22-33%) and protease (-28%) activities were significantly reduced, suggesting resource reallocation away from energy-intensive secretory processes.

This pattern – selective stress response induction without central metabolic collapse – is consistent with cells experiencing a specific metabolic perturbation (such as nucleotide pool stress) rather than nonspecific toxicity.

3.4. In Vivo Effects: LPS-Induced Endotoxemia Model

3.4.1. Hematological Parameters

LPS administration induced a robust inflammatory response characterised by anemia of inflammation: hemoglobin decreased by 44.7% (14.5 ± 0.78 to 8.02 ± 0.87 g/dL), hematocrit by 45.6% (46 ± 0.97 to 25 ± 0.76%), with concurrent leukocytosis (+71.5%) and lymphocytosis (+131%).

Administration of modified nucleosides on the LPS background exacerbated hematotoxicity in a compound-dependent manner. Co-administration of quercetin partially corrected these disturbances. Individual compound data are presented in Table 6.

Table 6. Hematological parameters: individual compound data.

Group	Hemoglobin (g/dL)	Hematocrit (%)	Leukocytes ($\times 10^3/\mu L$)	Lymphocytes ($\times 10^3/\mu L$)
Control	14.5 ± 0.78	46.0 ± 0.97	13.09 ± 1.12	3.9 ± 1.4
LPS	8.02 ± 0.87	25.0 ± 0.76*	22.45 ± 1.35*	9.01 ± 1.5
LPS + nucleosides				
+ Fludarabine	7.8 ± 0.82*	20.5 ± 0.85*	29.5 ± 1.1*	9.8 ± 1.2
+ F-ara-AMP*	7.2 ± 0.79*	19.0 ± 0.80*	30.5 ± 1.2*	10.0 ± 1.1
+ 2-NH₂-6-Cl-araPur	9.3 ± 0.90	21.7 ± 0.88	27.2 ± 1.0*	9.3 ± 1.3
+ Cytarabine	5.6 ± 0.81*	16.5 ± 0.78*	33.2 ± 1.3*	10.5 ± 1.1*
+ ara-CMP	6.8 ± 0.84*	17.2 ± 0.82*	31.0 ± 1.1*	10.2 ± 1.2
+ Cyclo-CMP	4.9 ± 0.79*	15.1 ± 0.75*	35.6 ± 1.4*	11.1 ± 1.0*
+ TTU	8.1 ± 0.86	20.5 ± 0.84	28.0 ± 1.0*	9.5 ± 1.2
LPS + nucleosides + quercetin				
+ Fludarabine + Q	10.0 ± 0.75†‡	28.0 ± 0.90†‡	19.5 ± 0.9†‡	5.8 ± 1.1†‡
+ F-ara-AMP + Q	9.5 ± 0.80†‡	25.0 ± 0.88†‡	21.0 ± 0.8†‡	6.2 ± 1.0†‡
+ 2-NH₂-6-Cl-araPur + Q	11.1 ± 0.82†‡	32.1 ± 0.95†‡	17.4 ± 0.7†‡	5.1 ± 1.0†‡
+ Cytarabine + Q	8.9 ± 0.78†‡	22.5 ± 0.85†‡	23.5 ± 1.0†‡	6.8 ± 1.1†
+ ara-CMP + Q	9.2 ± 0.80†‡	24.0 ± 0.87†‡	22.0 ± 0.9†‡	6.5 ± 1.1†
+ Cyclo-	8.4 ± 0.77†‡	20.0 ± 0.82†‡	25.7 ± 1.1†‡	7.6 ± 1.2†

CMP + Q				
+ TTU + Q	10.5 ± 0.85†‡	30.0 ± 0.92†‡	18.5 ± 0.8†‡	5.5 ± 1.0†‡
LPS + quercetin alone	10.2 ± 0.80†	30.5 ± 0.90†	18.0 ± 0.8†	5.3 ± 1.0†

Note. * $p < 0.05$ vs. LPS group; † $p < 0.05$ vs. corresponding LPS + nucleoside group (without quercetin); ‡ $p < 0.05$ vs. LPS group. All p-values are FDR-corrected. Q = quercetin (5 mg/kg). Data are means ± SD, n = 5 per group.

Hemoglobin in quercetin co-administration groups was 9-27 percentage points closer to control values compared with the corresponding nucleoside-only groups. Hematocrit correction ranged from 11 to 28 percentage points. Leukocytosis was reduced 1.3-1.8-fold and lymphocytosis 1.4-2.1-fold. No parameter fully returned to control levels, indicating partial but clinically meaningful correction.

3.4.2. Biochemical Markers of Organ Function

LPS administration increased markers of hepatocellular damage and metabolic disturbance. Modified nucleosides further exacerbated these changes. Quercetin co-administration produced significant corrections for ALT, urea, and total protein (Table 7).

Table 7. Serum biochemical parameters: individual compound data.

Group	ALT (IU/L)	AST (IU/L)	Urea (mg/dL)	Glucose (mg/dL)	Total protein (g/dL)	Chlorides (mmol/L)
Control	36 ± 1.4	142 ± 1.1	19 ± 0.78	112 ± 0.88	9.5 ± 0.98	103 ± 1.2
LPS	59 ± 1.2 *	182 ± 0.99*	34 ± 0.89 *	164 ± 1.05 *	16.0 ± 1.10 *	227 ± 1.3*
LPS + nucleosides						
+ Fludarabine	62 ± 1.3	178 ± 1.0	42 ± 0.92 *	168 ± 1.1	22.5 ± 1.2*	215 ± 1.2
+ F-ara-AMP	65 ± 1.4 *	181 ± 1.1	45 ± 0.95 *	172 ± 1.0	24.0 ± 1.3*	220 ± 1.3
+ 2-NH_2-6-Cl-araPur	54 ± 1.2	174 ± 0.9	39 ± 0.88	162 ± 0.9	19.8 ± 1.1	210 ± 1.1
+ Cytarabine	72 ± 1.5 *	189 ± 1.2 *	48 ± 1.0*	178 ± 1.2*	27.5 ± 1.4*	228 ± 1.3
+ ara-CMP	68 ± 1.4 *	185 ± 1.1 *	46 ± 0.98 *	175 ± 1.1*	25.8 ± 1.3*	225 ± 1.2
+ Cyclo-CMP	76 ± 1.6 *	192 ± 1.2 *	52 ± 1.1*	183 ± 1.3*	31.0 ± 1.5*	233 ± 1.4*
+ TTU	58 ± 1.3	176 ± 1.0	41 ± 0.90	165 ± 1.0	21.0 ± 1.2*	212 ± 1.1
LPS + nucleosides + quercetin						
+ Fludarabine + Q	52 ± 1.2 †‡	172 ± 0.9	25 ± 0.82 †‡	145 ± 0.9†	14.8 ± 1.0†‡	168 ± 1.1†‡
+ F-ara-AMP + Q	54 ± 1.3 †‡	175 ± 1.0	28 ± 0.85 †‡	152 ± 1.0†	15.5 ± 1.1†‡	172 ± 1.1†‡
+ 2-NH_2-6-Cl-araPur + Q	48 ± 1.1 †‡	169 ± 0.9	21 ± 0.80 †‡	131 ± 0.8†‡	12.8 ± 0.9†‡	156 ± 1.0†‡
+ Cytarabine + Q	58 ± 1.3 †‡	182 ± 1.1	30 ± 0.88 †‡	160 ± 1.1†	16.2 ± 1.1†‡	175 ± 1.2†‡
+ ara-CMP + Q	55 ± 1.2 †‡	179 ± 1.0	28 ± 0.85 †‡	155 ± 1.0†	15.0 ± 1.0†‡	170 ± 1.1†‡
+ Cyclo-CMP + Q	56 ± 1.3 †‡	185 ± 1.1	32 ± 0.90 †‡	168 ± 1.2	17.1 ± 1.2†‡	179 ± 1.2†‡
+ TTU + Q	50 ± 1.2 †‡	170 ± 0.9	23 ± 0.82 †‡	138 ± 0.9†‡	13.5 ± 0.9†‡	160 ± 1.0†‡
LPS + Q alone	45 ± 1.1 †	168 ± 0.9 †	22 ± 0.80 †	135 ± 0.8†	12.5 ± 0.9†	155 ± 1.0†

Note. * $p < 0.05$ vs. LPS group; † $p < 0.05$ vs. corresponding LPS + nucleoside group; ‡ $p < 0.05$ vs. LPS group. FDR-corrected. Q = quercetin.

The most pronounced quercetin-mediated corrections were observed for urea (1.4-1.9-fold reduction relative to the nucleoside-only groups) and total protein (1.5-1.8-fold), while AST showed the least correction (1.0-1.1-fold).

3.4.3. Antioxidant Defence Parameters

LPS shifted the prooxidant–antioxidant balance: SOD activity decreased by 19.2%, catalase by 25.9%, GPx by 30.0%, while TBARS (lipid peroxidation marker) increased 2.2-fold. Modified nucleosides further exacerbated the prooxidant shift. Quercetin co-administration produced significant corrections across all antioxidant parameters (Table 8).

Table 8. Liver antioxidant defence parameters: individual compound data.

Group	TBARS (µmol/mg protein)	SOD (U/mg protein)	Catalase (U/mg protein)	GPx (U/mg protein)
Control	0.42 ± 0.05	7.8 ± 0.54	110.5 ± 0.58	22.3 ± 0.62
LPS	0.93 ± 0.08*	6.3 ± 0.48*	81.9 ± 0.55*	15.6 ± 0.58*
LPS + nucleosides				
+ Fludarabine	1.12 ± 0.10*	5.5 ± 0.42*	76.2 ± 0.52*	13.8 ± 0.55*
+ F-ara-AMP	1.18 ± 0.11*	5.2 ± 0.40*	74.5 ± 0.50*	13.2 ± 0.54*
+ 2-NH_2-6-Cl-araPur	0.98 ± 0.09*	5.9 ± 0.45*	79.9 ± 0.54*	14.9 ± 0.58*
+ Cytarabine	1.35 ± 0.12*	4.8 ± 0.38*	71.8 ± 0.48*	11.5 ± 0.50*
+ ara-CMP	1.28 ± 0.11*	5.0 ± 0.39*	73.0 ± 0.49*	12.0 ± 0.52*
+ Cyclo-CMP	1.50 ± 0.14*	4.5 ± 0.36*	69.5 ± 0.46*	10.1 ± 0.48*
+ TTU	1.05 ± 0.09*	5.7 ± 0.44*	77.5 ± 0.53*	14.2 ± 0.56*
LPS + nucleosides + quercetin				
+ Fludarabine + Q	0.72 ± 0.07†‡	6.8 ± 0.50†‡	92.5 ± 0.56†‡	18.8 ± 0.60†‡
+ F-ara-AMP + Q	0.75 ± 0.07†‡	6.5 ± 0.48†‡	90.0 ± 0.55†‡	18.0 ± 0.58†‡
+ 2-NH_2-6-Cl-araPur + Q	0.61 ± 0.06†‡	7.0 ± 0.52†‡	95.9 ± 0.57†‡	19.4 ± 0.61†‡
+ Cytarabine + Q	0.88 ± 0.08†‡	6.2 ± 0.46†‡	85.7 ± 0.54†‡	16.6 ± 0.57†‡
+ ara-CMP + Q	0.82 ± 0.08†‡	6.4 ± 0.47†‡	88.0 ± 0.55†‡	17.2 ± 0.58†‡
+ Cyclo-CMP + Q	0.85 ± 0.08†‡	6.3 ± 0.46†‡	87.5 ± 0.54†‡	17.5 ± 0.58†‡
+ TTU + Q	0.65 ± 0.06†‡	6.9 ± 0.51†‡	94.0 ± 0.56†‡	19.0 ± 0.60†‡
LPS + Q alone	0.58 ± 0.06†	7.2 ± 0.53†	98.0 ± 0.57†	20.5 ± 0.62†

* $p < 0.05$ vs. control; † $p < 0.05$ vs. corresponding LPS + nucleoside group; ‡ $p < 0.05$ vs. LPS group. FDR-corrected. Q = quercetin.

The most pronounced quercetin-mediated correction was observed for TBARS, with reductions of 29–72% relative to the corresponding nucleoside-only groups. SOD activity increased by 15–25%, catalase by 16–26%, and GPx by 17–42%. These corrections demonstrate that quercetin effectively attenuates lipid peroxidation and supports the enzymatic antioxidant machinery under conditions of combined inflammatory and chemically induced oxidative stress.

Importantly, comparison with Group 17 (LPS + quercetin alone) showed that quercetin provided comparable antioxidant correction regardless of whether nucleosides were co-administered, suggesting that quercetin's protective effect operates independently of and in parallel with the nucleoside-mediated metabolic perturbation.

4. Discussion

The present study provides three principal findings that collectively advance understanding of the antibacterial potential and mechanism of modified nucleosides: (i) modified purine and pyrimidine nucleosides exhibit significant bacteriostatic activity that is structurally dependent, with purine derivatives being more potent across all tested strains; (ii) the bacteriostatic mechanism is independent of ROS generation, as demonstrated by convergent evidence from the quercetin dissociation experiment, equivalence testing, and anaerobic growth inhibition; and (iii) quercetin co-administration attenuates oxidative side effects in vivo without compromising in vitro antibacterial efficacy. These findings are consistent with a model in which modified nucleosides may interfere with bacterial nucleotide metabolism as their primary mode of action, while oxidative stress represents a secondary, downstream consequence.

The greater bacteriostatic potency of purine compared with pyrimidine derivatives, confirmed by two-way ANOVA ($F_{1,20} = 12.7$, $p = 0.002$), is consistent with known differences in nucleotide salvage pathway architecture across these compound classes. Bacterial purine salvage enzymes, including adenosine kinase and hypoxanthine-guanine phosphoribosyltransferase (HGPRT), are characterised by relatively broad substrate

specificity and can accommodate halogenated and arabino-configured analogues (Jordheim et al., 2013; Parker, 2009). The exceptional potency of 2-NH_2-6-Cl-araPur against *S. lutea* (IC_{50} = 4.6 μM) may reflect particularly efficient recognition of this compound by Gram-positive purine salvage kinases, although direct biochemical confirmation of intracellular phosphorylation would be required to establish this interpretation definitively.

The arabinose substitution (present in fludarabine and cytarabine) preserved bacteriostatic activity, indicating that bacterial salvage enzymes tolerate altered sugar stereochemistry at the 2′-position. Similarly, acetylation of ribose hydroxyl groups (as in TTU) did not abolish activity, presumably because intracellular esterases remove the acetyl protecting groups, liberating the active nucleoside. This hypothesis is consistent with the known esterase activity in both Gram-positive and Gram-negative bacteria (Bornscheuer, 2002).

The greater susceptibility of Gram-positive bacteria is consistent with the well-established permeability barrier conferred by the outer membrane of Gram-negative organisms (Nikaido, 2003). However, permeability alone is unlikely to account for all observed differences: variations in the amino acid sequence, active-site geometry, and substrate specificity of salvage pathway enzymes across species may contribute to differential susceptibility. This interpretation is supported by the variable effect of 5′-phosphorylation, which enhanced potency in some compound–strain combinations (e.g., cytarabine/ara-CMP against *B. cereus*: 3.5-fold increase) while having minimal impact in others. The net contribution of the phosphate group to biological activity depends on a combination of factors, including cellular uptake of charged species, dephosphorylation by periplasmic phosphatases, rephosphorylation by intracellular kinases, and competition with endogenous substrates (Jordheim et al., 2013).

The absence of a significant inoculum effect is consistent with the antimetabolite mode of action, where the compounds are not degraded by microbial enzymes but rather compete with endogenous substrates in a stoichiometric or catalytic fashion. This distinguishes nucleoside analogues mechanistically from β-lactam antibiotics, for which the inoculum effect is driven by enzymatic hydrolysis (Udekwu et al., 2009).

The central mechanistic finding of this study is that bacteriostatic activity is dissociable from ROS generation. This conclusion rests on three independent lines of evidence.

First, the quercetin dissociation experiment demonstrated that substantial reduction of intracellular ROS (up to 5.0-fold) did not alter IC_{50} values. The robustness of this result is underscored by its consistency across all 28 compound-strain combinations, and by the formal confirmation of equivalence through TOST (all combinations: $p < 0.05$ for equivalence at ±25% bounds). The probability of obtaining a false negative across 28 independent equivalence tests is negligible.

One potential concern is that quercetin may not penetrate bacterial cells efficiently, reducing ROS only at the membrane level. However, the DCFH-DA probe specifically measures intracellular ROS (the diacetate is cleaved by intracellular esterases, trapping DCF inside the cell), and quercetin reduced the intracellular DCFH-DA signal by 1.7-5.0-fold – demonstrating that quercetin (or its active metabolites) either entered the intracellular compartment or effectively scavenged ROS before intracellular accumulation. The capacity of flavonoids to cross bacterial membranes and exert intracellular effects has been documented by several groups (Eumkeb et al., 2012; Ohemeng et al., 1993).

Second, bacteriostatic activity was preserved under anaerobic conditions (IC_{50} within 1.5-fold of aerobic values). Because ROS generation is an oxygen-dependent process, the preservation of growth inhibition under anaerobiosis provides direct evidence that aerobic ROS production is not required for the bacteriostatic effect. This result is consistent with the findings of Keren et al. (2013) and Liu and Imlay (2013), who demonstrated that bactericidal antibiotic activity persists under anaerobic conditions.

Third, the enzymatic profiling pattern – catalase induction, central metabolic preservation, secretory function suppression – is consistent with a cell experiencing a specific metabolic perturbation rather than oxidative damage as the primary insult. If ROS were the primary mechanism, one would expect broader metabolic disruption, including impairment of respiratory chain enzymes (dehydrogenase) due to oxidative damage to iron-sulphur clusters (Imlay, 2013). The preservation of dehydrogenase activity argues against this scenario.

Taken together, these data are consistent with a model in which modified nucleosides enter bacterial cells (facilitated by the absence of the outer membrane in Gram-positive species), are activated by salvage pathway enzymes, and may interfere with nucleotide metabolism – potentially at the level of ribonucleotide reductase, thymidylate synthase, and/or DNA polymerase III, as established for their eukaryotic counterparts (Jordheim et al., 2013; Parker, 2009). Such interference would deplete or imbalance (d)NTP pools, causing replication stress. The consequent metabolic disturbance would generate ROS as a secondary by-product, explaining both the elevated ROS levels and their irrelevance to the bacteriostatic phenotype.

These findings contribute to the broader debate regarding the role of ROS in antibiotic action. The hypothesis advanced by Kohanski et al. (2007) that bactericidal

antibiotics kill through a common ROS-dependent mechanism has been challenged by several independent groups using complementary approaches (Keren et al., 2013; Liu & Imlay, 2013). Our data provide an additional line of evidence specific to the nucleoside analogue class: even substantial reduction of intracellular ROS – confirmed by both quercetin co-administration and anaerobic conditions – does not rescue bacterial growth.

The in vivo data demonstrate that modified nucleosides exacerbate the hematological and biochemical derangements caused by LPS-induced endotoxemia. The compound-dependent severity of hematotoxicity (cyclo-CMP and cytarabine producing the most pronounced effects) is consistent with the known myelosuppressive properties of pyrimidine analogues, which are activated by hematopoietic progenitor cells expressing high levels of deoxycytidine kinase (Galmarini et al., 2002).

Quercetin co-administration produced corrections across multiple parameters. Three aspects merit discussion.

First, the correction of TBARS (29-72% reduction) was the most pronounced antioxidant effect, consistent with quercetin's established capacity as a direct scavenger of lipid peroxyl radicals via its catechol B-ring structure (Boots et al., 2008). The parallel restoration of enzymatic antioxidant activities (SOD +15-25%, catalase +16-26%, GPx +17-42%) suggests that quercetin not only directly neutralises ROS but also protects the enzymatic antioxidant machinery from oxidative inactivation.

Second, the hepatoprotective effect (ALT 1.2-1.4-fold reduction) is consistent with the documented capacity of quercetin to attenuate hepatocyte damage in oxidative injury models (Li et al., 2016). The more pronounced correction of urea (1.4-1.9-fold) and total protein (1.5-1.8-fold) compared with ALT may reflect quercetin's broader anti-inflammatory effects, including suppression of NF-κB signalling and pro-inflammatory cytokine production (Li et al., 2016).

Third, the comparison with Group 17 (LPS + quercetin alone) demonstrates that quercetin provides comparable antioxidant protection regardless of nucleoside co-administration. Combined with the in vitro finding that quercetin does not alter IC_{50} values, this creates a clear therapeutic rationale: the antibacterial mechanism operates independently of ROS, while the major systemic toxicity of nucleoside analogues is at least partially ROS-dependent. Quercetin therefore selectively attenuates host-directed toxicity without interfering with the pathogen-directed effect.

We acknowledge that the nucleoside dose used in vivo (400 mg/kg) exceeds the allometrically scaled clinical equivalent (Reagan-Shaw et al., 2008), and the study was designed for mechanistic characterisation rather than translational dose-finding. Dose-response relationships, pharmacokinetic profiling, and histopathological examination would be essential components of any translational development of this combination.

5. Conclusions

Modified purine and pyrimidine nucleosides exhibit significant, dose-dependent bacteriostatic activity against both Gram-positive and Gram-negative bacteria. Purine derivatives are significantly more potent than pyrimidine analogues, and Gram-positive organisms are more susceptible than Gram-negative species. The mechanism of bacteriostatic action is independent of reactive oxygen species generation, as demonstrated by three convergent lines of evidence: (i) the quercetin dissociation experiment with formal equivalence testing, (ii) preservation of activity under anaerobic conditions, and (iii) an enzymatic profiling pattern consistent with specific metabolic stress rather than oxidative toxicity. The data are consistent with a model in which modified nucleosides may interfere with bacterial nucleotide metabolism, although direct demonstration of dNTP pool perturbation remains an important goal for future research. In vivo, quercetin co-administration effectively attenuates the oxidative component of nucleoside-associated hematotoxicity, hepatotoxicity, and antioxidant depletion without compromising bacteriostatic efficacy, providing a rational basis for combination strategies aimed at mitigating the systemic toxicity of nucleoside-based antibacterials.

Conflicts of Interest

The authors declare no conflicts of interest.

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