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# АНАЛИЗ КОНЦЕНТРАЦИОННО-ЗАВИСИМЫХ ЭФФЕКТОВ ТИОПУРИНОВ И ТИОНУКЛЕОЗИДОВ, ОКАЗЫВАЕМЫХ НА УРОВЕНЬ АКТИВНЫХ ФОРМ КИСЛОРОДА В КЛЕТКАХ K562

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***Аннотация.*** Тиопурины и их нуклеозидные производные широко используются при лечении онкозаболеваний, в частности лейкемии. Однако точные механизмы их действия, особенно в отношении производства активных форм кислорода (АФК), остаются невыясненными. В настоящей работе изучено влияние 6-меркаптопурина, 6-тиогуанина, 6-тиогуанозина и 2′-дезокси-6-тиогуанозина на уровни АФК в клетках хронического миелогенного лейкоза человека (клеточная линия K562). Определены уровни АФК при добавлении всех изученных соединений в диапазоне концентраций от $10^{-6}$ до $10^{-4}$ моль/л. Установлены различные закономерности индукции АФК среди соединений. Показано, что с ростом концентрации 6-меркаптопурина увеличивается уровень АФК, в то время как для 6-тиогуанина наблюдается обратная зависимость, при которой невысокие концентрации 6-тиогуанина приводят к более высокому уровню АФК. Нуклеозиды 6-тиогуанозин и 2′-дезокси-6-тиогуанозин оказывают менее значительное влияние на уровень АФК. Настоящее исследование позволяет лучше понять механизмы, лежащие в основе противоракового действия этих соединений, и разработать таргетные и более эффективные методы лечения на основе тиопурина.

***Ключевые слова:*** тиопурины; активные формы кислорода; АФК; клетки K562; противораковая активность; структурно-функциональная взаимосвязь; 6-тиогуанин; 6-тиогуанозин.

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# ANALYSIS OF CONCENTRATION-DEPENDENT EFFECTS OF THIOPURINES AND THIONUCLEOSIDES ON LEVELS OF REACTIVE OXYGEN SPECIES IN K562 CELLS

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***Abstract.*** Thiopurines and their nucleoside derivatives are widely used in cancer treatment, particularly for leukemia. However, their precise mechanisms of action, especially concerning reactive oxygen species (ROS) production, remain incompletely understood. This study investigated the effects of 6-mercaptopurine, 6-thioguanine, 6-thioguanosine, and 2′-deoxy-6-thioguanosine on ROS levels in K562 human chronic myelogenous leukemia cells. Using a 2′,7′-dichlorodihydrofluorescein diacetate essay, we measured ROS production across a concentration range of $10^{-6}$ to $10^{-4}$ mol/L. Our results revealed distinct patterns of ROS induction among the compounds. 6-Mercaptopurine showed a concentration-dependent increase in ROS levels, while 6-thioguanine exhibited a biphasic response with higher ROS production at lower concentrations. The nucleosides 6-thioguanosine and 2′-deoxy-6-thioguanosine demonstrated less pronounced effects on ROS levels. These findings provide valuable insights into the structure-activity relationships of thiopurines and thionucleosides, highlighting the complex interplay between their molecular structures and ROS-inducing properties. Our study contributes to a better understanding of the mechanisms underlying the anticancer effects of these compounds and may inform the development of more targeted and effective thiopurine-based therapies.

***Keywords:*** thiopurines; reactive oxygen species; ROS; K562 cells; anticancer activity; structure-activity relationships; 6-thioguanine; 6-thioguanosine.

## Introduction

Thiopurines, including 6-mercaptopurine and 6-thioguanine, have been widely used in the treatment of various disorders, particularly in the treatment of leukemia [1; 2]. In addition, nucleosides such as 6-thioguanosine and 2′-deoxy-6-thioguanosine have been studied for their antiproliferative effects and ability to induce apoptosis in cancer cells [3]. These compounds undergo extensive metabolism, which can be influenced by factors such as xanthine oxidase activity and thiopurine methyltransferase levels.

The metabolism of thiopurines is a complex process involving multiple enzymes and pathways. 6-Mercaptopurine is metabolises by xanthine oxidase to form 6-thiouric acid [4], a reaction that is also known to create reactive oxygen species (ROS) [5].

6-Thioguanine is metabolises to 6-thioguanosine monophosphate by hypoxanthine-guanine phosphoribosyltransferase [6], then phosphorylated to 6-thioguanosine triphosphate through a series of enzymatic reactions and is then incorporated into genomic DNA and methylated to form S6-methylthioguanine [7].

Nucleosides 6-thioguanosine and 2′-deoxy-6-thioguanosine have been studied for their ability to induce apoptosis in cancer cells. These compounds can be incorporated into genomic DNA, leading to the formation of mispairs that are processed by the DNA mismatch repair (MMR) pathway, resulting in cell cycle arrest, apoptosis, and autophagy [3].

Despite their widespread clinical use, significant gaps remain in understanding the pharmacological and toxicological pathways of thiopurines. Notably, a considerable proportion of patients with inflammatory bowel disease do not respond adequately to thiopurine therapy, underscoring the necessity for deeper insights into their mechanisms of action. One critical aspect of investigation is the role of ROS in thiopurine-induced apoptosis. Apoptosis, a prevalent form of programmed cell death (PCD), is characterises by morphological changes such as cell shrinkage and chromatin condensation, and biochemical events including caspase activation and DNA fragmentation. Apoptotic pathways can be caspase-dependent or caspase-independent, with the former involving extrinsic and intrinsic signaling pathways converging on caspase-3 activation. The intrinsic pathway encompasses pre-mitochondrial, mitochondrial, and post-mitochondrial phases, the latter involving mitochondrial membrane potential disruption and ROS production [8].

Some studies have indicated that both caspase-dependent and caspase-independent PCD pathways are activated following MMR processing of 6-thioguanine [9]. However, the detailed molecular mechanisms remain poorly understood.

In this study, we investigated the level of ROS in thiopurine-induced cell death in the K562 cell line following treatment with 6-mercaptopurine, 6-thioguanine, and the nucleosides 6-thioguanosine and 2′-deoxy-6-thioguanosine.

## Materials and methods

**Chemicals.** Studied compounds 6-mercaptopurine, 6-thioguanine (2-amino-6-mercaptopurine), 6-thioguanosine (2-amino-6-mercaptopurine riboside), 2′-deoxy-6-thioguanosine (6-thio-2′-deoxyguanosine) (fig. 1) were synthetised as described in [10][1].

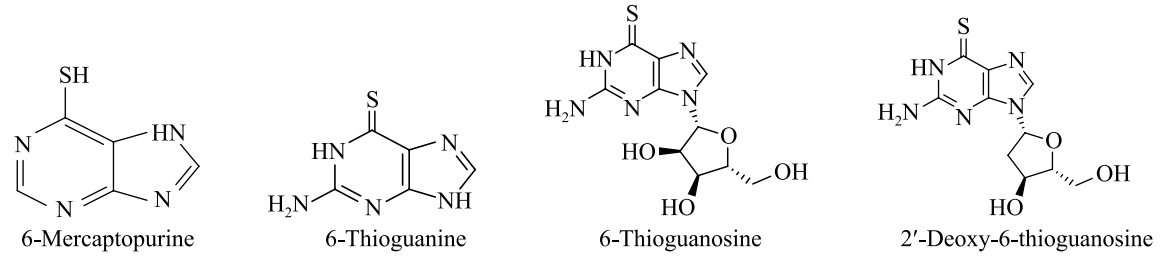


*Fig. 1.* Structures of studied nitrogen bases and nucleosides

**Detection of ROS (2′,7′-dichlorodihydrofluorescein diacetate essay).** Each experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10 % of the mean. For the resazurin reduction assay, a stock solution (1 % DMSO in the appropriate buffer with the tested compound) was prepared from which several dilutions were made with the appropriate buffer.

Human chronic myelogenous leukemia cell line (K562) was obtained from the Institute of Cytology of the Russian Academy of Sciences. The cell line was maintained in RPMI 1640 (*AppliChem*, Germany) with 20 % fetal calf serum (*HyClone*, USA). Cells were cultivated at 37 °C in a humidified atmosphere of 5 % $CO_2$.

ROS generation was analyses by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) [11]. For this assay cells were plated in triplicate in 96-well plates ($10^5$ cells per 1 well) and cultivated for 24 h overnight. At 70–80 % confluence cells were treated with studied compounds at concentrations $10^{-6}$–$10^{-4}$ mol/L and 10 μmol/L DCFH-DA and incubated for 2 h in 5 mmol/L PBS buffer. Cells used as control were incubated solely with 10 μmol/L DCFH-DA and the maximum used amount of the diluent DMSO. Fluorescence generation due to the hydrolysis of DCFH-DA to dichlorodihydrofluorescein (DCFH) by non-specific cellular esterases and the subsequent oxidation of DCFH by peroxides was measured at $\lambda_{ex}$ = 495 nm, $\lambda_{em}$ = 520 nm using a multi-detection microplate reader Synergy-4 (*BioTek Instruments Inc.*, USA).

**Statistical analysis.** The trials were repeated until three data sets (in triplicate) had been collected for each answer ($n$ = 3). All data are expressed as the median (interquartile range) and were analyses using the Kruskal – Wallis test for comparing more than two independent sets of samples.

When the Kruskal – Wallis test revealed significant differences between groups, the Wilcoxon rank sum post hoc test was performed to identify pairings of groups with statistically significant differences. A $p$-value of less than 0.05 indicates significance.

All statistical analyses were carried out using the *R-statistics software* (version 4.3.3).

## Results and discussion

ROS have important roles in intracellular signal transduction and redox homeostasis [12]. However, excessive ROS accumulation can exert toxic effects and cause oxidative stress, damaging main cellular components such as DNA, lipids and proteins, causing apoptosis [13–15]. To investigate whether thiopurine-induced apoptosis and autophagy was related to changes in the intracellular redox environment, we examined intracellular ROS production in thiopurine-treated K562 cancer cells.

The impact of 6-mercaptopurine, 6-thioguanine, 6-thioguanosine, and 2′-deoxy-6-thioguanosine on reactive oxygen species levels in K562 cells was assessed across a range of concentrations (0.000 000 1 to 0.000 1 mol/L). The results, expressed as a percentage of the control (untreated cells), are presented below and demonstrate a complex relationship between thiopurine exposure and ROS generation (fig. 2).

[1]Enzymatic synthesis of 6-thio-2′-deoxyguanosine and its phospholipid derivative / L. L. Birichevskaya [et al.] // Fiziko-khimicheskaya biologiya kak osnova sovremennoi meditsiny : abstr. of rep. of participants of the Int. sci. conf. dedicated to the 75th anniversary of the birth of prof. E. V. Barkovsky (Minsk, 21 May 2021) / ed. by V. V. Khrystalev, A. D. Taganovich, T. A. Khrystaleva. Minsk : Belarus. State Med. Univ., 2021. P. 39–41.

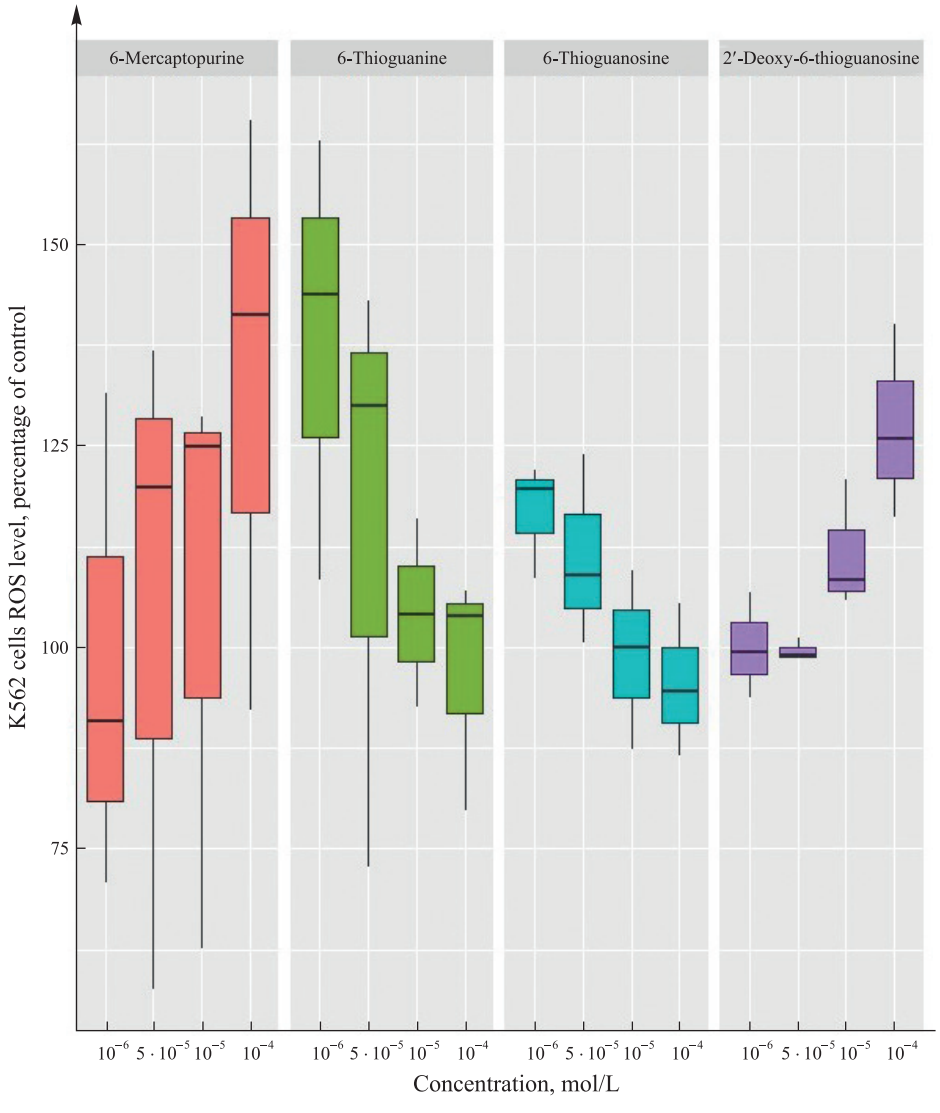


*Fig. 2.* Effect of different concentrations of 6-mercaptopurine, 6-thioguanine, 6-thioguanosine, and 2′-deoxy-6-thioguanosine on K562 cells ROS level
(every bar represents median, 25 and 75 % percentiles, minimal and maximal values)

Treatment with 6-mercaptopurine exhibited a clear correlation between increasing concentrations of 6-mercaptopurine and ROS levels. This trend is most pronounced at the highest concentration tested ($10^{-4}$ mol/L), where ROS levels are significantly elevated compared to the control. Notably, the response to lower concentrations is variable, suggesting a threshold effect for ROS induction. These findings suggest that 6-mercaptopurine is a potent inducer of ROS, with a notable impact on ROS production at higher concentrations.

Among the tested compounds, 6-thioguanine induces the highest median ROS level at the lowest studied concentration $10^{-6}$ mol/L. Interestingly, ROS levels decreased in concentration-dependent manner with increasing concentrations suggesting a biphasic response. This phenomenon indicates that lower concentrations of 6-thioguanine are more effective at stimulating ROS production, while higher concentrations may activate alternative cellular mechanisms that mitigate ROS generation.

6-Thioguanosine also demonstrated a general trend of decreased ROS production with increasing concentrations but exhibits a less dramatic effect on ROS levels compared to 6-thioguanine. This compound shows the least effect on ROS levels among the four tested. Although a slight increase in ROS is observed with decreasing concentrations, the changes are relatively minor and accompanied by high variability. These findings suggest that 6-thioguanosine may have a limited role in ROS-mediated cytotoxicity.

The effect of 2′-deoxy-6-thioguanosine on ROS levels was less consistent and generally less pronounced compared to the other thiopurines tested. At the highest concentration (0.000 1 mol/L), an increase in ROS levels was observed (125.9–140.0 %), but at lower concentrations, the impact on ROS production was minimal, fluctuating around the control levels.

The results suggest that thiopurine compounds exhibit varying effects on ROS production in K562 cells. Specifically, 6-mercaptopurine and 6-thioguanine are the most potent inducers of ROS, with distinct concentration-dependent responses. The nucleosides, 6-thioguanosine and 2′-deoxy-6-thioguanosine, have less pronounced effects on ROS levels.

Reactive oxygen species play a pivotal role in cancer cell death, acting as a double-edged sword in cellular processes [16]. While moderate levels of ROS are essential for various signaling pathways, excessive ROS accumulation can lead to oxidative stress, triggering apoptosis and other forms of programmed cell death [17]. Our findings on the effects of thiopurines and thionucleosides on ROS levels in K562 cells provide valuable insights into the potential mechanisms of their anticancer activity.

The observed variations in ROS production among the tested compounds suggest complex molecular correlations between thiopurine and thionucleoside administration and ROS levels. 6-Mercaptopurine, demonstrating a clear concentration-dependent increase in ROS, may exert its cytotoxic effects through direct ROS generation, potentially via its metabolism by xanthine oxidase [18]. This aligns with previous studies suggesting that 6-mercaptopurine-induced apoptosis is mediated by ROS-dependent mechanisms [19].

Conversely, the biphasic response observed with 6-thioguanine, characterises by higher ROS levels at lower concentrations, suggests a more nuanced mechanism. This phenomenon may be attributed to the activation of cellular antioxidant defenses at higher concentrations, potentially through the Nrf2-Keap1 pathway [20]. The decreased ROS production at higher concentrations could also indicate a shift towards alternative cell death mechanisms, such as DNA incorporation and subsequent mismatch repair processing [21].

The nucleosides, 6-thioguanosine and 2′-deoxy-6-thioguanosine, exhibited less pronounced effects on ROS levels compared to their base counterparts. This difference may be attributed to their distinct cellular uptake mechanisms and metabolic pathways [22]. The subtle variations in ROS production between these nucleosides suggest that structural differences, such as the presence or absence of the 2′-hydroxyl group, may influence their interaction with cellular redox systems or their incorporation into nucleic acids.

Our results contribute significantly to the understanding of structure-activity relationships in the anticancer activity of thiopurines and thionucleosides. The observed differences in ROS induction patterns among structurally related compounds highlight the importance of subtle molecular variations in determining their biological effects. For instance, the contrasting ROS profiles of 6-thioguanine and its nucleoside derivatives underscore the critical role of the sugar moiety in modulating cellular responses [23].

Moreover, the concentration-dependent effects observed, particularly the biphasic response of 6-thioguanine, emphasise the complexity of dose-response relationships in thiopurine pharmacology. This finding has important implications for optimising dosing strategies in clinical applications, suggesting that lower doses might be more effective in inducing ROS-mediated cytotoxicity in certain cases [1].

In conclusion, our study provides a comprehensive analysis of the ROS-inducing properties of thiopurines and thionucleosides in K562 cells, contributing valuable insights into their structure-activity relationships. These findings not only enhance our understanding of the molecular mechanisms underlying the anticancer effects of these compounds but also pave the way for the rational design of more effective and targeted thiopurine-based therapies. Future research should focus on elucidating the specific cellular pathways involved in the observed ROS modulation and investigating the potential synergistic effects of combining these compounds with other anticancer agents or ROS modulators.

## Conclusions

In conclusion, our comprehensive analysis of the ROS-inducing properties of thiopurines and thionucleosides in K562 cells has revealed important structure-activity relationships that contribute to our understanding of their anticancer mechanisms. The distinct patterns of ROS induction observed among structurally related compounds underscore the significance of subtle molecular variations in determining their biological effects. The concentration-dependent responses, particularly the biphasic behaviour of 6-thioguanine, highlight the complexity of dose-response relationships in thiopurine pharmacology and suggest potential strategies for optimising dosing regimens in clinical applications.

These findings not only enhance our understanding of the molecular mechanisms underlying the anticancer effects of thiopurines and thionucleosides but also provide a foundation for the rational design of more effective and targeted therapies. The differential ROS-inducing capabilities of these compounds, especially between the base forms and their nucleoside derivatives, offer new avenues for exploring their potential in combination therapies or as lead compounds for developing novel anticancer agents.

Future research should focus on elucidating the specific cellular pathways involved in the observed ROS modulation, investigating the potential synergistic effects of combining these compounds with other anticancer agents or ROS modulators, and exploring the implications of these findings in clinical settings and *in vivo* models.

Additionally, further studies on the relationship between ROS induction and other mechanisms of action, such as DNA incorporation and mismatch repair processing, could provide a more comprehensive understanding of thiopurine-mediated cytotoxicity.

Ultimately, this study contributes valuable insights to the field of cancer therapeutics and paves the way for the development of more precise and effective thiopurine-based treatments, potentially improving outcomes for patients with leukemia and other malignancies.

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