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EFFECTS OF PYRROLIDINEDIONE-THIAZOLIDINONE HYBRID MOLECULES ON THE GENOTOXICITY *IN VITRO*

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Background. *Pyrrolidinedione-thiazolidinone hybrid molecules represent a promising class of anticancer candidates; however, a comprehensive evaluation of their genotoxic and mutagenic safety profile is essential before further preclinical development.* ***Objectives.*** *This work aimed to investigate the genotoxic potential of pyrrolidinedione-thiazolidinone hybrid molecules Les-6287 and Les-6294 that possessed antineoplastic activity.* ***Methods.*** *The mutagenic potential was evaluated using the Ames bacterial reverse mutation test with Salmonella typhimurium strains TA98 and TA100 in the presence and absence of metabolic activation (S9 fraction). The effects at the chromosomal level were assessed using the Allium cepa anaphase-telophase chromosome aberration assay. Primary DNA strand break induction was quantified using the alkaline comet assay.* ***Results.*** *Les-6287 and Les-6294 at 10 and 100 µM concentrations do not produce a mutagenic activity exceeding 1.6 in either S. typhimurium strain TA98 or TA100, with or without S9-mediated metabolic activation. In the A. cepa anaphase-telophase assay treatment with Les-6287 and Les-6294 did not result in a statistically significant elevation in chromosomal aberration frequency relative to the negative control (2.8%), with observed values ranging from 3.3% to 4.3% across all tested concentrations. No significant alterations in the mitotic index were recorded. The alkaline comet assay revealed no significant increase in primary DNA damage, with percent tail DNA of 1.3-1.9% in treated peripheral blood mononuclear cells.* ***Conclusion.*** *Pyrrolidinedione-thiazolidinone hybrid molecules Les-6287 and Les-6294 at concentrations up to 100 µM do not pose a genotoxic or mutagenic risk under the tested experimental conditions. Further targeted safety assessments are needed prior to progressing Les-6287 and Les-6294 to preclinical evaluation as candidate antitumor agents.*

K e y w o r d s: pyrrolidinedione-thiazolidinone hybrid molecules; genotoxicity; Ames test; anaphase-telophase test; alkaline comet assay.

Cancer remains a leading cause of global mortality, with nearly 20 million new cases and 9.7 million deaths reported annually, driven significantly by breast, lung, and colorectal carcinomas [1]. While traditional chemotherapy remains a cornerstone of treatment, its clinical utility is frequently limited by tumor heterogeneity, the development of multi-drug resistance (MDR), and severe systemic toxicities, as well as the genotoxic potential [2-4]. To address these challenges, medicinal chemistry has shifted toward molecular hybridization. This strategy links multiple pharmacophoric units into a single scaffold to inhibit complementary oncogenic pathways while optimizing pharmacokinetic profiles. Among the versatile "scaffolds" used in this approach are five-membered nitrogen- and sulfur-containing heterocycles, specifically thiazoles, thiazolidinones, and pyrazoles. These rings provide the structural rigidity and electronic diversity necessary for high-affinity non-covalent interactions with crucial targets such as receptor tyrosine kinases (EGFR, VEGFR-2), cyclin-dependent kinases (CDKs) [5-6], and topoisomerases [7]. However, the development of these highly cytotoxic agents presents a dual challenge: researchers must rigorously distinguish between therapeutically in-

tended apoptosis in malignant cells and undesirable genotoxicity in normal somatic tissues. DNA damage caused by direct-acting or metabolically activated mutagens can lead to permanent somatic mutations and secondary malignancies, making early-stage toxicological profiling a regulatory and scientific imperative.

The thiazolidinone-based scaffolds are present in several pharmacologically important agents, including ponesimod (FDA-approved for the treatment of multiple sclerosis, sphingosine-1-phosphate (S1P) receptor 1 modulator), rosiglitazone and pioglitazone (FDA-approved anti-diabetic, PPAR-γ agonists), and etozoline (a loop diuretic marketed in Europe) [8]. One of the promising avenues in the rational design of 4-thiazolidinone-based antitumor agents involves the incorporation of a cinnamalum (2-chloro-3-(4-nitrophenyl)-2-propenylidene) pharmacophoric fragment. Finiuk et al. demonstrated that hybrid pyrrolidinedione-thiazolidinone hybrid molecules 1-(4-hydroxyphenyl)-3-[5-[2-chloro-3-(4-nitrophenyl)prop-2-enylidene]-4-oxo-2-thioxothiazolidine-3-yl]pyrrolidine-2,5-dione (Les-6287), 1-(4-chlorophenyl)-3-[5-[2-chloro-3-(4-nitrophenyl) prop-2-enylidene]-4-oxo-2-thioxothiazolidine-3-yl] pyrrolidine-2,5-diones (Les-6294) exerted growth-inhibitory activity against leukemia, breast, cervical, colon, lung carcinoma, and glioblastoma cell lines, with IC_{50} values in the range of approximately 1-10 μM at 72 h of exposure; moreover, these compounds were found to trigger mitochondria-dependent apoptosis in acute leukemia T-cells [9], tongue squamous carcinoma [10], and breast carcinoma cells [11] and to induce DNA damage in Jurkat T-cells [9]. However, some derivatives, such as the investigational agent 5F 203, demonstrate tumor-selective genotoxicity by acting as ligands for the Aryl Hydrocarbon Receptor (AhR). This triggers a biochemical cascade that induces cytochrome P450 1A1 (CYP1A1), which then bioactivates the compound into electrophilic intermediates that form DNA adducts selectively in tumor cells, sparing normal breast epithelial tissue [12]. Conversely, minor structural shifts in 2-aryl-substituted benzothiazoles can lead to direct, non-selective mutagenicity. Derivatives incorporating o-nitro, m-bromo, or 4-pyridyl groups (such as BT-11 to BT-15) have shown statistically significant increases in revertant colonies in the Ames test (Salmonella typhimurium strains TA98 and TA100) and induced chromosomal damage, such as sister chromatid exchanges, in healthy human lymphocytes [13]. In addition, chemotherapeutic drugs gemcitabine and topotecan have been shown to exert cytotoxic and genotoxic effects in murine bone marrow [2]. Mitoxantrone and melphalan at 70 μM concentration elevated the number of micronucleated lymphocytes in the micronucleus test [14].

Genotoxicity assessment represents a critical and state-of-the-art safety evaluation component in pharmaceutical development, as genotoxicity assays provide valuable insights into both the genotoxic potential and putative carcinogenicity of chemotherapeutic agents [15]. In the context of drug development, positive findings obtained through well-established genotoxicity testing platforms, including the Ames test, the mouse lymphoma assay (MLA), and the *in vitro* micronucleus (MN) assay or chromosomal aberration (CA) assay, carry substantial regulatory and safety significance [15].

Therefore, we aimed to investigate the genotoxicity profile of pyrrolidinedione-thiazolidinone hybrid molecules 1-(4-hydroxyphenyl)-3-[5-[2-chloro-3-(4-nitrophenyl)prop-2-enylidene]-4-oxo-2-thioxothiazolidine-3-yl]pyrrolidine-2,5-dione (Les-6287), 1-(4-chlorophenyl)-3-[5-[2-chloro-3-(4-nitrophenyl)prop-2-enylidene]-4-oxo-2-thioxothiazolidine-3-yl]pyrrolidine-2,5-diones (Les-6294) that possessed the anticancer activity.

Materials and Methods

Studied pyrrolidinedione-thiazolidinone hybrid molecules. The synthesis and characterization of 1-(4-hydroxyphenyl)-3-[5-[2-chloro-3-(4-nitrophenyl)prop-2-enylidene]-4-oxo-2-thioxothiazolidine-3-yl]pyrrolidine-2,5-dione (Les-6287), 1-(4-chlorophenyl)-3-[5-[2-chloro-3-(4-nitrophenyl)prop-2-enylidene]-4-oxo-2-thioxothiazolidine-3-yl]pyrrolidine-2,5-diones (Les-6294) (Fig. 1) was reported by Finiuk et al. [9]. The 100 mM stock solutions of the studied compounds were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA).

Ames mutagenicity assay. The potential mutagenic activity of the studied derivatives was evaluated using the Ames test [16, 17]. Two histidine-auxotrophic plasmid-bearing strains of *S. typhimurium* L., TA100 and TA98, were employed, both of which are capable of reverting to prototrophy upon exposure to mutagenic agents. Strain TA98 (his D3052, rfa, uvrB, +R, pKm101) was used to detect frameshift mutations. In contrast, strain TA100 (his G46, rfa, uvrB bio, pKm101), which harbours a point

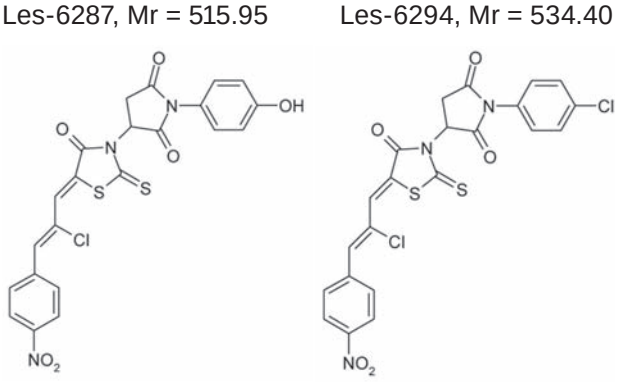

Fig. 1. Structural formulas of studied pyrrolidinedi-one-thiazolidinone hybrid molecules Les-6287 and Les-6294

mutation in the histidine operon (missense mutation his G46), was used for the detection of base-pair substitution mutations. Both *S. typhimurium* strains were obtained from the Microorganism Collection of the Department of Genetics and Biotechnology, Ivan Franko National University of Lviv (Lviv, Ukraine).

Mutagenicity was assessed using a semi-quantitative incorporation method, in which test substances were introduced directly into a semi-solid agar overlay. Overnight cultures of *S. typhimurium* (1-2 × 10^9 cells per ml) were incubated for 30 min at 37°C with studied derivatives at final concentrations of 10 and 100 µM per dish, and doxorubicin (Sigma-Aldrich, St. Louis, MO, USA) at final concentrations of 10 µM per dish, in the presence or absence of a rat liver microsomal activation fraction (S9 mix). All experimental procedures involving animals were conducted in accordance with internationally accepted bioethical standards and the principles of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). The animal experiments with rats of the Vistar line for obtaining the microsomal fraction for the Ames test were approved by the Bioethics Committee of the Institute of Cell Biology of the National Academy of Sciences of Ukraine (Protocol No 2025-1, dated 05 February 2025). Following incubation, the bacterial suspensions were combined with molten top agar (37-42°C) composed of 0.5% agar (Sfera Sim, Ukraine), 0.5% NaCl (Sfera Sim, Ukraine), 50 µM biotin (Sigma-Aldrich, St. Louis, MO, USA), and 50 µM L-histidine (Sigma-Aldrich, St. Louis, MO, USA), pH 7.4, and the mixture was subsequently poured onto Petri dishes containing minimal agar medium (1.5% agar in Vogel-Bonner E medium supplemented with 2% L-glucose (Sfera Sim, Ukraine)). The experiments were repeated in triplicate.

Positive controls were included for each strain as follows: benzidine (100 µg/dish; Sigma-Aldrich, St. Louis, MO, USA) served as the positive control for strain TA98 in the presence of S9; N-methyl-N′-nitro-N-nitrosoguanidine (MNNG; 1 mg/dish; Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control for strain TA100 in the presence of S9; and sodium azide (1.5 µg/dish; Sigma-Aldrich, St. Louis, MO, USA) served as the positive control for strain TA100 in the absence of S9; and 4-Nitroquinoline N-oxide (4-NQO, 0.5 µg/dish; Sigma-Aldrich, St. Louis, MO, USA). Following 48 h of incubation at 37°C, L-histidine revertant colonies were enumerated on each plate. The mutagenic potential (mutagenic activity, MA) of the test substances was determined by comparing the number of revertant colonies observed under experimental conditions with that of the negative control, which represents the spontaneous reversion frequency of the respective bacterial strains [16, 17].

Allium ana-telophase chromosome aberration assay. Genotoxicity assessment using *Allium cepa* was conducted in accordance with a previously described protocol [18]. The assay is based on the detection of chromosomal aberrations arising in meristematic root tip cells of *Allium cepa* L. following germination in the presence of the test compounds at two concentrations (10 µM, corresponding to the IC_{50} and µM, corresponding to 10× IC_{50} determined for tumor cells), alongside positive control compounds comprising sodium azide (10 µM) and doxorubicin (10 µM). Seeds were germinated at 22°C for 72 h. Harvested root tips were fixed in a 3:1 (v/v) mixture of ethanol and glacial acetic acid (Sfera Sim, Ukraine) for 24 h and subsequently stored in 70% ethanol until further processing. Before staining, roots were rinsed with distilled water and macerated in 1 M HCl (Sfera Sim, Ukraine) for 10 min. Chromosomes were stained with 1% aceto-orcein solution (Sigma-Aldrich, USA) for 15 min. Cells were examined at various stages of the cell cycle at 10×10 magnification under a light microscope. A minimum of 1,000 cells per experimental point was scored. The experiments were repeated in triplicate.

To evaluate the cyto- and genotoxic potential of the test compounds, the following parameters were determined: mitotic index (MI, %) = (P+M+A+T)/(I+P+M+A+T)×100%; and chromosomal aberration frequency (%) = N/(A+T)×100%, where P, M,

A, T, and I denote the number of cells in prophase, metaphase, anaphase, telophase, and interphase, respectively, and N represents the total number of chromosomal aberrations recorded in anaphase and telophase cells.

Isolation of human peripheral blood mononuclear cells. All experimental procedures involving human peripheral blood mononuclear cells (PBMCs) were conducted in accordance with protocols approved by the Bioethics Committee of the Institute of Cell Biology, National Academy of Sciences of Ukraine (Protocol No. 2025-1, February 5, 2025). Written informed consent was obtained from all donors before participation. PBMCs were isolated from venous blood samples of healthy adult volunteers that were collected into plastic K_3EDTA-containing vacutainer tubes. PBMCs were isolated by density gradient centrifugation using Gradisol G (Polfa, Poland) and washed twice in 0.9% sodium chloride solution as described previously [9, 19]. Isolated PBMCs were maintained in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and a 1× penicillin/streptomycin antibiotic cocktail (all reagents sourced from Sigma-Aldrich, USA) at 37°C in a humidified atmosphere comprising 95% air and 5% CO_2.

Alkaline DNA comet assay. Cells were seeded in 24-well plates at a density of 1×10^6 cells/ml and exposed to Les-6287, Les-6294 (both at 10 μM and 100 μM), and doxorubicin (10 μM) for 24 h. The alkaline comet assay was carried out in accordance with a previously established protocol [9, 20]. Following electrophoresis, slides were stained with ethidium bromide (10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) and visualised under a Zeiss fluorescence microscope. Quantitative analysis of DNA damage was performed using CaspLab software (version 1.2.3b2; CASPLab, Wrocław, Poland), with percent tail DNA and the Olive Tail Moment (OTM) determined as established parameters of genotoxic damage [20].

Statistical analysis. All data were analyzed using GraphPad Prism 9 (GraphPad Software, USA) and are expressed as mean (M) ± standard deviation (SD). Inter-group comparisons were performed using one-way analysis of variance (ANOVA). A *P*-value of < 0.05 was considered statistically significant.

Results and Discussion

The effect of Les-6287 and Les-6294 on S. typhimurium revertants formation in the Ames mutagenicity test. The mutagenic potential of novel pyrrolidinedione-thiazolidinone hybrid molecules Les-6287 and Les-6294 at concentrations of 10 and 100 μM was assessed using the Ames test with *S. typhimurium* strains TA100 and TA98 under and without S9 metabolic activation. The S9 fraction used in the present study primarily reflects hepatic Phase I biotransformation, largely mediated by cytochrome P450 (CYP450) enzymes, which catalyze oxidative, reductive, and hydrolytic reactions that can convert pro-mutagenic compounds into their reactive, DNA-damaging metabolites [21]. A compound was considered capable of inducing point mutations if it met the following criteria: (a) a reproducible, concentration-dependent increase in the number of revertant colonies per plate relative to the concurrent control, reaching at least a two-fold increase at one or more concentrations in at least one bacterial strain, with or without metabolic activation (S9); and (b) a dose–response relationship across the tested concentration range [22].

Exposure to the reference mutagens benzidine, nitrosoguanidine, 4-nitroquinoline N-oxide, and sodium azide produced a statistically significant increase in the number of *S. typhimurium* revertants, confirming the sensitivity and validity of the test systems employed. Under these positive control conditions, MA values ranged from 3.7 to 4.9 (Fig. 2, *A-D*). In contrast, treatment with compound Les-6287 at both tested concentrations of 10 and 100 μM yielded MA values of 1.3-1.5 in strain TA98 (Fig. 2, *A*) and 1.2-1.5 in strain TA100 in the absence of metabolic activation (Fig. 2, *B*). In the presence of the S9 microsomal fraction, MA values of 1.1–1.3 and 1.2–1.5 were recorded for strains TA98 and TA100, respectively (Fig. 2, *C-D*). These values fall within the non-mutagenic range, indicating the absence of mutagenic activity for the derivatives Les-6287 and Les-6294 at 100 μM under all tested conditions. The chemotherapeutic doxorubicin yielded MA values of 1.9-2.8 across both strains. The MA of 1.8 was found under the action of doxorubicin on strain TA98 in the absence of S9 (Fig. 2, *A*). The higher MA value increased to 2.8 in strain TA98 in the presence of S9 (Fig. 2, *C*). Based on the obtained results, doxorubicin could be classified as a potentially mutagenic agent.

Taken together, the pyrrolidinedione-thiazolidinone hybrid molecules Les-6287 and Les-6294 tested in this study demonstrated no mutagenic activity in *S. typhimurium* strains TA98 and TA100,

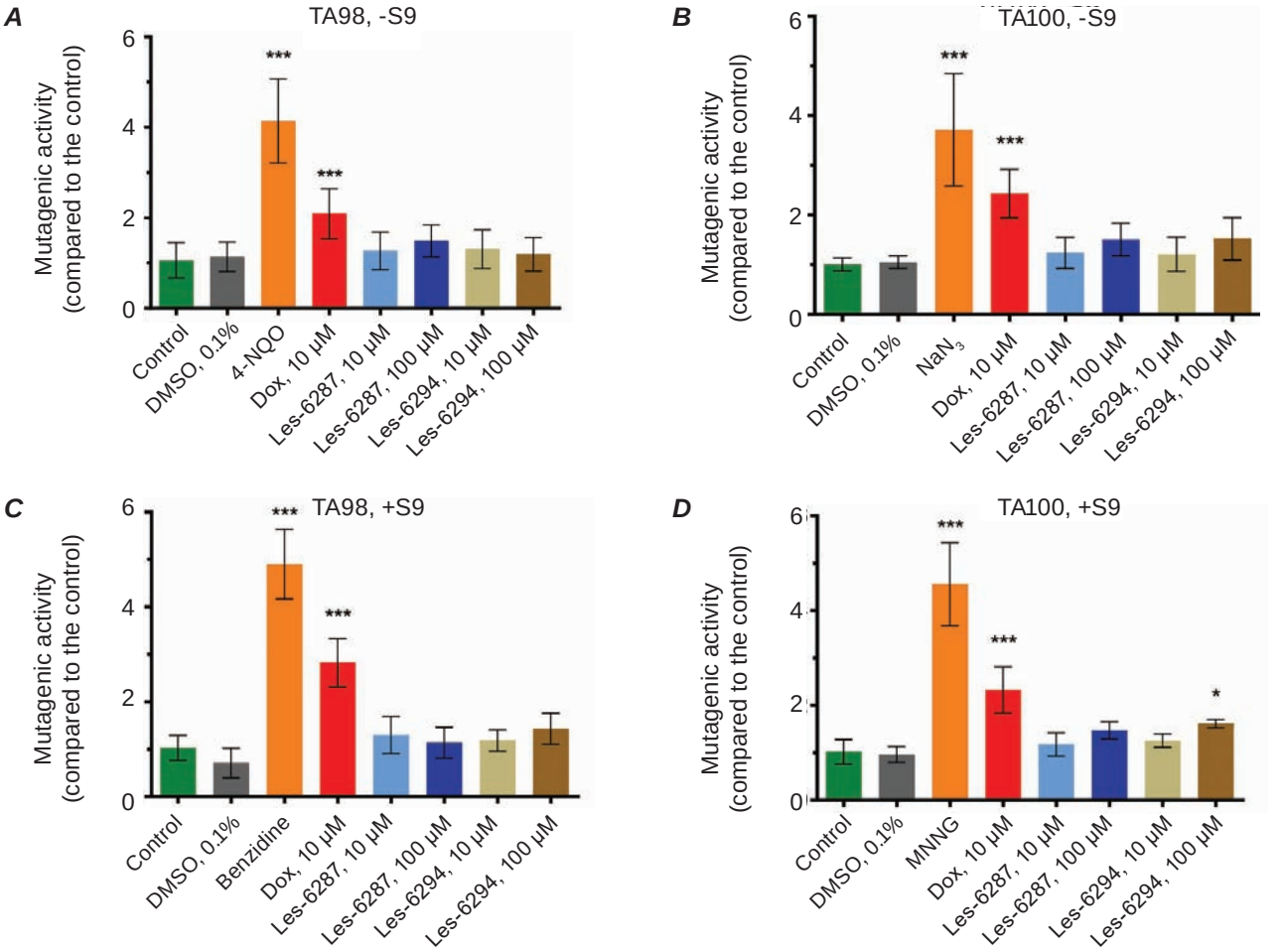

*Fig. 2. Results of determining the mutagenic activity of studied compounds on S. typhimurium strains TA98 and TA100 without (**A**, **B**) and (**C**, **D**) with metabolic activation by the microsomal fraction S9. Benzidine, nitrosoguanidine (MNNG), 4-nitroquinoline N-oxide (4-NQO), and sodium azide (NaN3), known mutagens, were used as positive control substances. Data are presented as M ± SD, n = 3 of three independent experiments. *P < 0.05; ***P < 0.001 compared to the control (non-treated cells)*

either with or without metabolic activation by the S9 fraction.

The effect of Les-6287 and Les-6294 on chromosomal aberrations in anaphase-telophase assay. The ana-telophase assay in *A. cepa* was employed to assess the genotoxic properties of Les-6287 and Les-6294 (Fig. 3). In the negative control group – comprising meristematic cells of *A. cepa* germinated in distilled water – the baseline frequency of chromosomal aberrations was 2.8%, which is attributable to spontaneous mutations inherent to *A. cepa* meristematic tissue. Among the tested compounds, neither Les-6287 nor Les-6294 induced pronounced elevation in chromosomal aberration frequency. Treatment with Les-6287 elicited a tendency to increase in aberrations, rising from 3.3% at 10 μM to 4.1% at 100 μM. Les-6294 demonstrated a similar tendency to increase in aberrations from 3.6% to 4.3%. Doxorubicin significantly elevated the number of chromosomal aberrations to 8.2%, and sodium azide to 7.1% (Fig. 3, *A*). The predominant types of aberrations observed following exposure to the studied compounds were micronuclei and anaphase bridges.

Exposure to increasing concentrations of the studied compounds, ranging from 10 to 100 μM, resulted in a statistically nonsignificant reduction in the mitotic index (MI) under higher concentrations of Les-6287 and Les-6294 (Fig. 3, *B*). Les-6287 at 100 μM reduced the MI to 13.6%, and Les-6294 to 13.8%, compared with a control value of 16.0%. No-

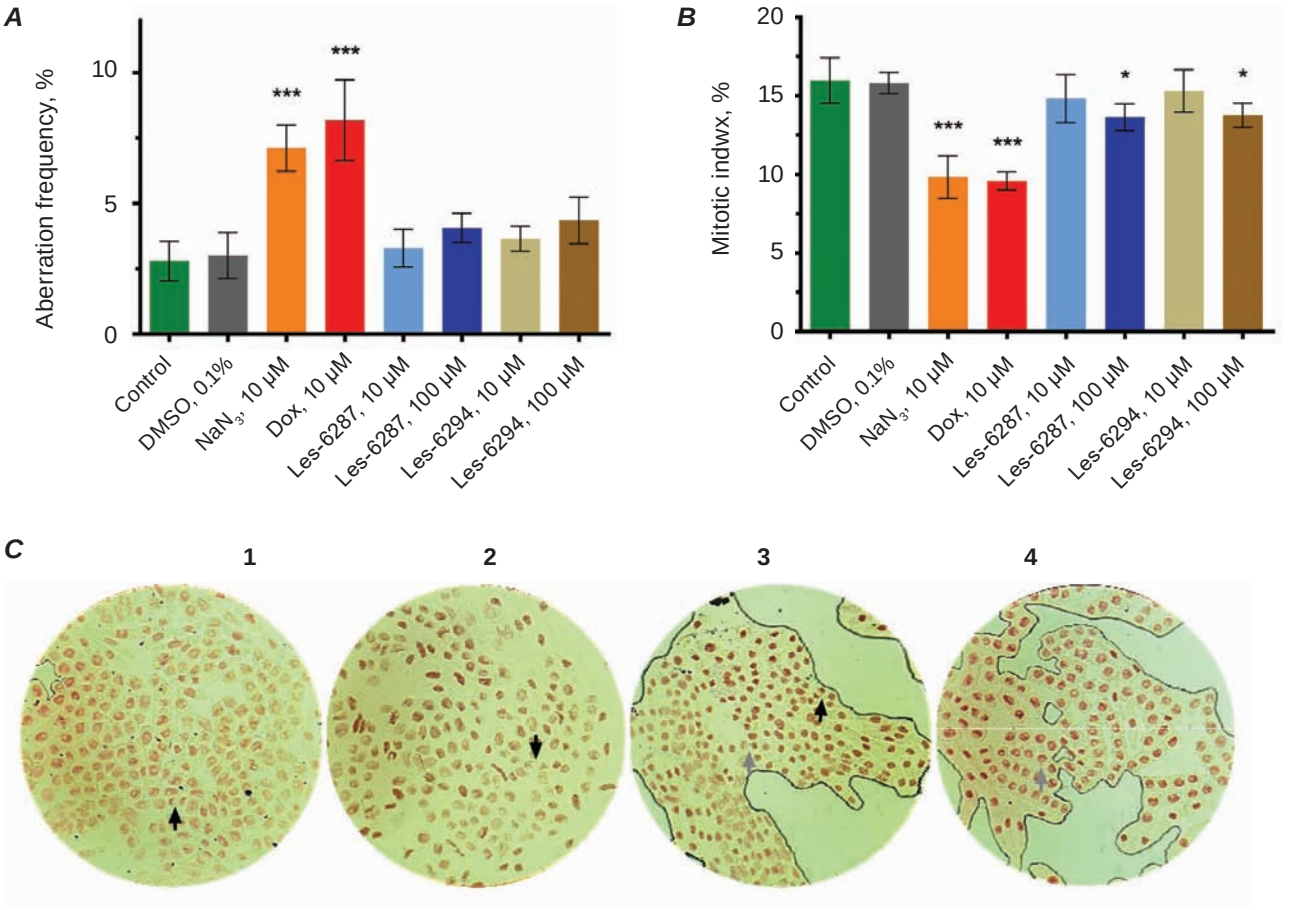

*Fig. 3. The effect of studied compounds on induction of chromosomal aberrations (**A**, **C**) and mitotic index (**B**) in Allium cepa meristema root cells: 1 – bridge (identified by black arrow) caused by Les-6287 at 10 μM, 2 – bridge (identified by black arrow) caused by Les-6294 at 10 μM; 3 – micronuclei (identified by grey arrow) and bridge (identified by black arrow) caused by sodium azide (NaN_3) at 10 μM; 4 – micronuclei (identified by grey arrow) caused by doxorubicin (Dox) at 10 μM; photos were made at 10x10 microscope magnification. Data presented as M ± SD, n = 3 of three independent experiments. *P < 0.05; ***P < 0.001 compared to the control (non-treated cells)*

tably, the reference compound doxorubicin progressively reduced the MI to 9.6%, and sodium azide to 9.8% (Fig. 3, *B*).

The chromosomal aberration frequencies for Les-6287 and Les-6294 at 10 and 100 μM did not reach statistical significance compared with the control at any tested concentration.

The DNA-damaging activity of Les-6287 and Les-6294. The structural integrity of DNA is fundamental to the maintenance of normal cellular function and proliferation. During the S phase of the cell cycle, DNA damage triggers the inhibition of replication origin firing, thereby attenuating replication fork progression and potentially giving rise to DNA double-strand breaks (DSBs) – among the most cytotoxic forms of genomic lesions [23]. When the extent of DNA damage surpasses the capacity of cellular repair mechanisms, programmed cell death is initiated [15]. In this context, a comprehensive evaluation of the dual nature of DNA-damaging agents is critical for achieving an optimal balance between chemotherapeutic efficacy and systemic toxicity, with particular regard to genotoxic risk. PBMCs were selected as the primary in vitro model for assessing the DNA-damaging activity of the studied derivatives because. PBMCs are non-transformed, diploid human cells with intact DNA repair machinery and a stable karyotype, they are more suitable than genomically unstable malignant cell lines for reliably detecting genotoxic potential and represent the standard OECD-validated system for micronucleus and comet assays [24]. It should be

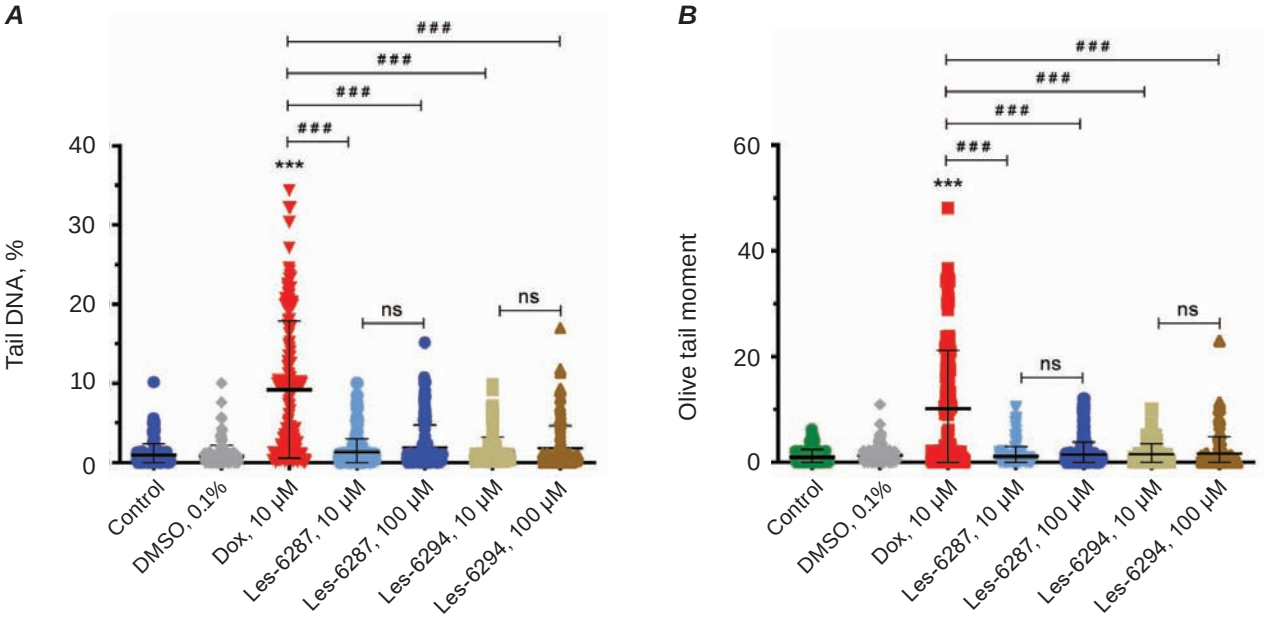

*Fig. 4. The level of the DNA damage, expressed as the percentage of DNA in the tail of comets (**A**) and Olive Tail Moment (**B**), in isolated PBMCs treated for 24 h with the studied compounds or with DMSO (vehicle control, equivalent to the concentration used at 100 µM compound treatment). Data are presented as M ± SD, n = 3 of three independent experiments. ***P < 0.001 compared to untreated (control) cells, ###P < 0.001 compared to cells treated with Dox, ns – non-significant changes*

noted that extrapolation to cancer cells is limited by their altered DNA damage repair, differing proliferation rates, drug efflux, and metabolic profiles relative to PBMCs. Thus, the application of PBMCs might provide a relevant indicator of toxic/genotoxic risk of chemicals to normal, non-target tissue, which is essential given the intended clinical translation of these hybrid compounds.

Compounds Les-6287 and Les-6294 cause a non-significant concentration-dependent elevation in DNA damage in isolated PBMCs, as assessed by the alkaline comet assay. At a concentration of 10 µM, treatment with compound Les-6287 resulted in a mean tail DNA of 1.3% and an Olive Tail Moment (OTM) of 1.1, whereas exposure at 100 µM resulted in a tail DNA of 1.9% and an OTM of 1.4 (Fig. 4, 5). A similar effect was identified under the Les-6294 treatment. This compound at a concentration of 10 µM resulted in a mean tail DNA of 1.4% and an Olive Tail Moment (OTM) of 1.5, whereas exposure at 100 µM produced the tail DNA of 1.8% and an OTM of 1.7. For comparative purposes, the reference compound doxorubicin was evaluated under identical conditions. At 10 µM, doxorubicin yielded a mean tail DNA of 9.2% and an OTM of 10.2 (Fig. 4, 5). These data suggest a superior genotoxic potency of doxorubicin relative to Les-6287 and Les-6294. In untreated control PBMCs, the level of DNA damage was 0.9% tail DNA with an OTM of 0.9, values that were closely mirrored in DMSO-treated cells (tail DNA: 0.8%; OTM: 1.3), confirming that the vehicle had no discernible effect on genomic integrity.

Collectively, these data demonstrate that Les-6287 and Les-6294 induce non-significant dose-dependent increases in DNA damage in isolated PBMCs compared with both untreated and vehicle-treated control levels and are markedly lower than the genotoxic response induced by doxorubicin.

The genotoxic and mutagenic potential of the investigated pyrrolidinedione-thiazolidinone hybrid molecules was evaluated using the Ames bacterial reverse mutation test, the *Allium cepa* anaphase-telophase chromosome aberration assay, and the alkaline comet assay. Collectively, the results obtained across all three test systems demonstrated that the tested compounds did not exhibit mutagenic activity or induce significant DNA damage under the experimental conditions employed.

In the Ames mutagenicity assay, none of the pyrrolidinedione-thiazolidinone hybrid molecules

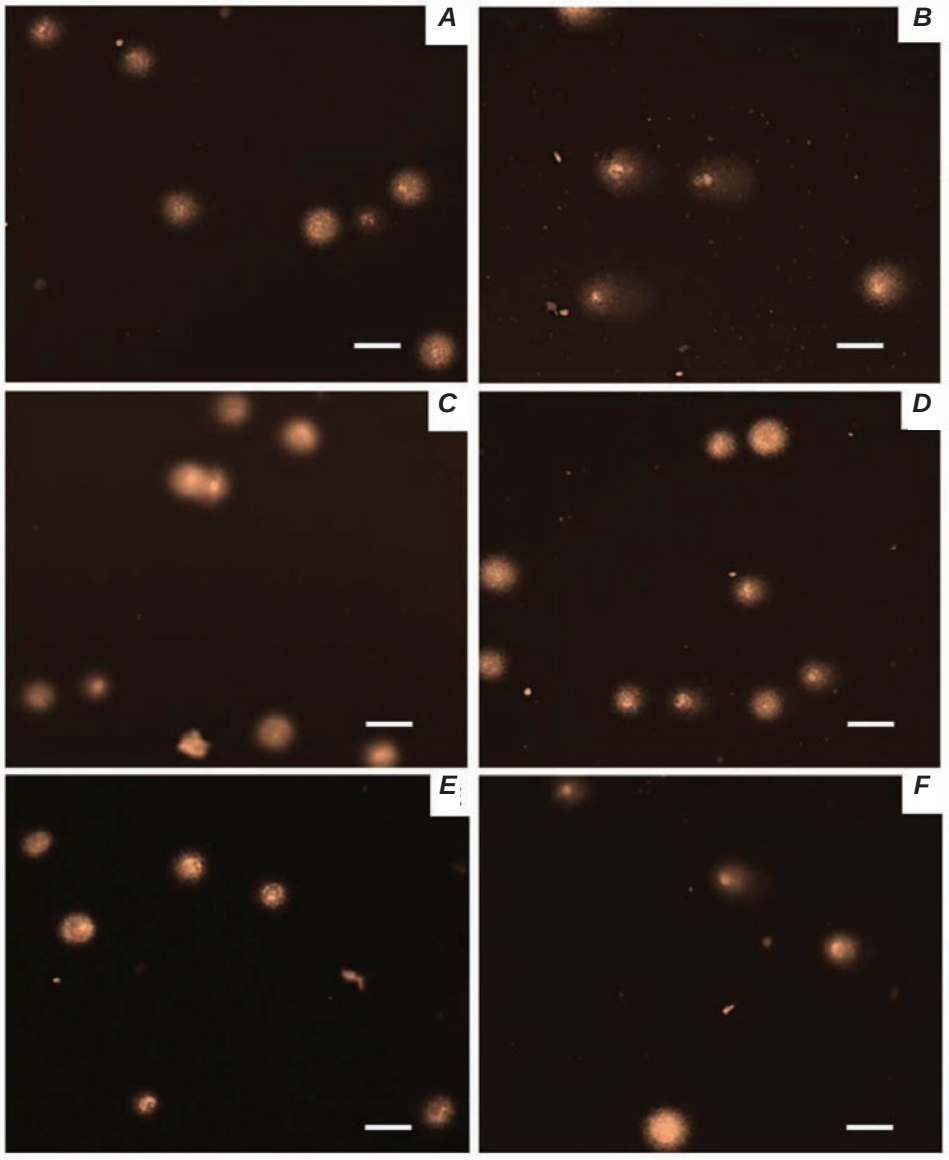

*Fig. 5. The photographs of the DNA comets of isolated PBMCs cells treated for 24 h with the compound Les-6287 at 10 µM (**C**) and 100 µM (**D**); Les-6294 at 10 µM (**E**) and 100 µM (**F**); and doxorubicin at 10 µM (**B**); and untreated (control) cells (**A**). Bar equals 40 µm*

produced a mutagenic index exceeding 1.6 in either *S. typhimurium* strain TA98 or TA100, in the presence or absence of metabolic activation (S9 fraction). These values fall within the non-mutagenic range as defined by OECD guidelines [17], indicating that the tested compounds are incapable of inducing either frameshift or base-pair substitution mutations under the tested conditions. The validity of the assay was confirmed by the robust mutagenic response elicited by the positive controls, with mutagenic activity values of 3.7 to 4.9. These findings are consistent with previously reported data for structurally related thiazolidinone derivatives, which similarly demonstrated an absence of mutagenic activity in the bacterial reverse mutation assay. The obtained results correlate with data of Buzun et al. (2022) who reported that (2-{5-[(Z,2Z)-2-chloro-3-(4-nitrophenyl)-2-propenylidene]-4-oxo-2-thioxothiazolidin-3-yl}-3-methylbutanoic acid) potent cytotoxicity against breast cancer lines while remaining Ames-negative [7]. Its lack of mutagenicity, despite containing an aromatic nitro group, is attributed to the steric hindrance provided by its bulky 3-methylbutanoic acid moiety, which prevents enzymatic reduction by bac-

terial nitroreductases into mutagenic intermediates. Furthermore, specific 4-thiazolidinone derivatives have demonstrated antigenotoxic properties, effectively protecting DNA against exogenous chemical mutagens. For instance, compound C4 has shown a 47.9% inhibition rate of DNA damage induced by the mutagen 9-aminoacridine, suggesting a potential role as a protective chemotherapeutic adjuvant [25]. It was reported that pyrazole derivatives generally exhibit a robust safety profile in standard genotoxicity screens. This is largely because they primarily function through non-covalent inhibition of specific kinase domains rather than direct DNA binding. Pyrazolo[3,4-b]pyridines, in particular, benefit from a thermodynamically stable 1H-tautomer that minimizes conformational fluctuations and off-target risks. Studies on thiophene-substituted pyrazole Schiff bases (3a, 3b) have confirmed them to be entirely non-mutagenic in the Ames test [26]. The lack of mutagenicity in both the presence and absence of S9 suggests that neither the parent compounds nor their Phase I metabolites possess direct or S9-activation-dependent mutagenic properties under the conditions tested. However, as the S9 mix represents only a first-tier approximation of Phase I–mediated metabolic activation, this result should be interpreted as a partial rather than exhaustive assessment of the compounds' full metabolic activation and detoxification profile *in vivo.*

The *A. cepa* ana-telophase assay provided complementary evidence regarding the cytogenotoxic profile of the test compounds at the chromosomal level. Treatment with the compounds Les-6287 and Les-6294 did not produce a statistically significant increase in chromosomal aberration frequency relative to the negative control (CA = 2.8%), with observed values ranging from 3.3% to 4.3% across all tested concentrations. Furthermore, no significant alterations in the mitotic index were recorded, suggesting the absence of notable antiproliferative effects on meristematic cells at the concentrations examined. The *A. cepa* assay is a well-validated plant-based genotoxicity model that has been widely applied in the assessment of pharmaceutical compounds and environmental genotoxicants owing to its sensitivity, reproducibility, and cost-effectiveness [27]. Based on the obtained results, we suggest that Les-6287 and Les-6294 show no statistically significant clastogenic effect under the conditions tested. However, this concentration-dependent trend should be further evaluated in future studies using larger sample sizes or extended concentration ranges to confirm the absence of a genuine, biologically relevant mutagenic potential.

Assessment of primary DNA strand break induction by means of the alkaline comet assay revealed that exposure to the 4-thiazolidinone-based compounds did not result in a statistically significant increase in DNA damage relative to untreated control cells, as reflected by comparable levels of percent DNA in the tail 1.3-1.9% for treated cells. The alkaline comet assay is recognised as a highly sensitive method for the detection of single- and double-strand DNA breaks, alkali-labile sites, and incomplete excision repair events at the level of individual cells [28]. The absence of significant DNA-damaging activity in this assay indicates that the tested compounds do not compromise genomic integrity through direct DNA strand scission or the generation of alkali-labile lesions. Thus, PBMC data indicate the general safety profile of the studied Les-6287 and Les-6294 towards normal cells.

The (E)-1-(2-hydroxyphenyl)-3-(4-methylphenyl)-prop-2-en-1-one likewise showed no increase in His+ bacterial revertant frequency in the Ames test and no elevation in micronucleus frequency or comet assay DNA strand breaks, supporting an overall absence of its genotoxic potential [29]. Structurally related ten 4-thiazolidinone derivatives (C1-C10) tested by the Ames *Salmonella*/microsome mutagenicity assay (0.2-1.0 mM/plate) similarly showed no mutagenic activity across three *S. typhimurium* strains up to the highest concentration tested [25]. However, Mamilla et al. (2023) reported that 4-fluorophenyl) thiazolidin-4-one at 7.5 μM significantly elevated chromosomal aberration frequency and micronucleus formation, together with disturbances in mitotic activity in treated CHO-K1 cells [30].

In addition to direct DNA interaction, oxidative stress and mitochondrial dysfunction represent plausible underlying mechanisms for the genotoxic effects observed with the tested hybrid molecules. Thiazolidinone and related heterocyclic scaffolds have been shown to disrupt mitochondrial membrane potential and electron transport chain activity, thereby promoting reactive oxygen species (ROS) generation; elevated ROS levels, in turn, can cause oxidative DNA lesions as well as single- and double-strand breaks [31, 32]. It was reported that the hybrid molecule containing a thiazolidinone and a (2Z)-2-chloro-3-(4-nitrophenyl)prop-2-ene structural fragment, which has been shown to selectively in-

duce apoptosis in tumor cells without causing non-specific genotoxic damage in normal cellular models [7].

Limitations of the study. Metabolic activation was assessed using the standard rodent S9 fraction, which reflects the hepatic Phase I biotransformation; thus, while neither the parent compounds nor their Phase I metabolites showed mutagenic activity under the tested conditions, this represents a partial rather than exhaustive assessment of their metabolic and detoxification profile in vivo. Future studies with larger sample sizes or extended concentration ranges are warranted. The longer-duration or repeated-exposure protocols would be needed to detect potential delayed genotoxic effects and adaptive cellular responses.

Conclusion. Taken together, the results obtained across three mechanistically distinct genotoxicity endpoints – bacterial mutagenicity, chromosomal aberration induction, and primary DNA damage – provide primary data that the investigated pyrrolidinedione-thiazolidinone hybrid molecules do not pose a genotoxic risk under the tested experimental concentrations of 10 and 100 μM. This potential genotoxic safety profile is of particular significance in the context of anticancer drug development, where the inherent cytotoxic selectivity of candidate compounds must be balanced against the risk of off-target genotoxic effects in non-malignant tissues. The present findings support the need for further dedicated safety studies before advancing these compounds towards preclinical evaluation as potential antitumor agents. Longer or repeated-exposure protocols (e.g., 48-72 h, or multi-day repeated dosing) would be needed to capture delayed genotoxicity, as well as to assess potential adaptive or compensatory cellular responses.

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ВПЛИВ ГІБРИДНИХ МОЛЕКУЛ ПІРОЛІДИНДІОН-ТІАЗОЛІДИНОНУ НА ГЕНОТОКСИЧНІСТЬ *IN VITRO*

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Вступ. Гібридні молекули піролідиндіон-тіазолідину є перспективним класом потенційних протипухлинних сполук; однак всебічна оцінка їхнього генотоксичного та мутагенного профілю безпеки є необхідною умовою перед проведенням подальших доклінічних досліджень. **Мета.** Дослідити генотоксичний потенціал гібридних похідних піролідиндіон-тіазолідину (Les-6287 та Les-6294), які виявляють протипухлинну активність. **Методи.** Мутагенний потенціал досліджуваних сполук оцінювали за допомогою тесту Еймса на зворотних мутаціях із використанням штамів *Salmonella typhimurium* TA98 та TA100 у присутності та за відсутності метаболічної активації (фракція S9). Вплив на хромосомний рівень оцінювали за допомогою ана-телофазного тесту з використанням *Allium cepa*. Індукцію первинних розривів ланцюгів ДНК визначали методом лужного гель-електрофорезу одиночних клітин (комет-аналіз у лужних умовах). **Результати.** Сполуки Les-6287 та Les-6294 у концентраціях 10 та 100 мкМ/л не виявили мутагенної активності, що перевищує значення 1,6, у жодному зі штамів *S. typhimurium* TA98 або TA100 – як за наявності, так і за відсутності метаболічної активації, опосередкованої S9. У ана-телофазному тесті

на *A. cepa* обробка сполуками Les-6287 та Les-6294 не призводила до статистично значущого підвищення частоти хромосомних аберацій порівняно з негативним контролем (2,8%); отримані значення становили від 3,3 до 4,3% у всіх досліджених концентраціях. Статистично значущих змін мітотичного індексу зафіксовано не було. Лужний комет-аналіз не виявив значущого підвищення рівня первинних пошкоджень ДНК: вміст ДНК у хвості комети в оброблених мононуклеарних клітинах периферичної крові становив 1,3-1,9%. **Висновки.** Гібридні похідні піролідиндіон-тіазолідинону Les-6287 та Les-6294 у концентрації до 100 мкМ/л не виявляють генотоксичного або мутагенного ризику за досліджених експериментальних умов. Перш ніж переходити до доклінічної оцінки сполук Les-6287 та Les-6294 як потенційних протипухлинних агентів, необхідно провести подальші цілеспрямовані дослідження їхньої безпеки.

Ключові слова: гібридні молекули піролідиндіон-тіазолідинону; генотоксичність; тест Еймса; ана-телофазний тест; лужний комет-аналіз.

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