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IMPACT OF THE NATURAL OLIGORIBONUCLEOTIDES IN COMPLEXES WITH D-MANNITOL ON TUMOR GROWTH AND EXPRESSION OF IMMUNE CELL MARKERS IN A MOUSE MELANOMA B16 MODEL

Ivanna Prylutska, Zenoviy Tkachuk

Cancer remains a leading cause of mortality worldwide, with chronic inflammation playing a critical role in tumour initiation and progression. Natural oligoribonucleotides in complexes with D-mannitol (ORN-D-M), derived from yeast RNA, have demonstrated anti-inflammatory and immunomodulatory effects in various disease models. Previous studies have shown their cytotoxicity against the B16 mouse melanoma cell line in vitro. This study ***aimed to*** *evaluate the antitumor properties of ORN-D-M in a B16 mouse melanoma model.*

Materials and methods. *The B16 melanoma model was established by transplantation of B16 cells into C57BL/6 mice. ORN-D-M was administered at varying doses via different routes (subcutaneous, oral, intraperitoneal), under prophylactic and therapeutic regimens, and simultaneously with transplantation of malignant cells. Tumour growth dynamics, survival, and gene expression of immune cell markers in peripheral blood (Cd3, Cd247, Cd4, Cd8, Cd68) using RT-qPCR were assessed.*

Results*. It was shown that simultaneous administration of ORN-D-M with tumour cell transplantation dose-dependently inhibited tumour formation and improved survival. However, neither therapeutic nor prophylactic administration after tumour transplantation showed significant effects. Additionally, ORN-D-M did not affect the mRNA expression of immune cell markers during the late stage of B16 melanoma.*

Conclusion*. ORN-D-M exhibits a dose-dependent cytotoxic effect when administered simultaneously with tumour cells but lacks efficacy in therapeutic or prophylactic regimens. Future studies should focus on optimizing targeted delivery systems to enhance drug stability and effectiveness in cancer therapy*

Keywords*: natural oligoribonucleotides, B16 melanoma, tumour formation, cytotoxicity, in vivo model, cancer*

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1. Introduction

Cancer remains a leading cause of mortality worldwide, claiming approximately 10 million lives annually. Chronic inflammation plays a significant role in cancer progression, regardless of its type and aetiology, contributing to tumour initiation, progression, and formation of the tumour microenvironment [1]. Therefore, in modern understanding, inflammation has been identified as one of the hallmarks of cancer and emerges as an initiating model of carcinogenesis [2, 3]. Toll-like receptors (TLR), a family of pattern recognition receptors, serve as critical linkers between the inflammatory response of innate immunity and the activation of adaptive immunity. These receptors play a pivotal role in modulating the expression of both pro-inflammatory and anti-inflammatory mediators, thereby orchestrating the response to pathogens and maintaining immune homeostasis [4, 5]. Due to their significant role in the regulation of inflammation, they emerge as a promising target for oncotherapy [6]. For instance, the Bacillus Calmette-Guérin (BCG) vaccine, which contains agonists for TLR 2/4, is approved by the United States Food and Drug Administration (FDA) and is currently used in bladder cancer treatment protocols. Its anticancer activity is explained by the accelerating maturation of antigen-presenting cells, which in turn leads to the secretion of pro-inflammatory cytokines (e.g., interleukin (IL) 2, tumour necrosis factor (TNF) α, interferon (INF) γ) and the subsequent activation of cytotoxic T-cells that infiltrate the tumour [7]. Additionally, the synthetic TLR3 agonist polyinosinic-polycytidylic acid (poly(I:C)), which mimics double-stranded RNA, inhibits the viability of various malignant breast cancer cell lines by increasing the expression of type I IFN and the p65 subunit of nuclear factor (NF) κB, leading to their caspase-dependent apoptosis [8]. However, the major drawback of therapeutic agents aimed at modulating inflammation is the increased risk of uncontrolled systemic inflammation (which leads to weakened immune response and sepsis), as well as heightened toxicity [9, 10].

One of the promising anti-inflammatory drugs, characterized by good tolerability, is ORN-D-M. Natural oligoribonucleotides (ORN) are a heterogeneous mixture of low-polymeric RNA fragments derived from yeast and purified from impurities of other biopolymers [11]. The high anti-inflammatory activity of yeast ORN has been demonstrated in a mouse paw carrageenan-induced oedema model, as it surpassed the anti-inflammatory capacity of aspirin [12]. A positive effect of the drug on the immune

defence mechanisms of the body has also been demonstrated, including an increase in CD4+ T lymphocytes in peripheral blood and normalization of the immunoregulatory CD4/CD8 ratio [13]. In previous studies, we demonstrated that ORN-D-M treatment exhibits dose- and time-dependent inhibition of the viability of various tumour cell lines, including mouse melanoma B16. At the same time, reduced cytotoxicity was observed toward non-tumor-derived cells, as evidenced by higher half-maximal inhibitory concentration (IC_{50}) compared to this for malignant cell lines [14]. It has also been established that the mechanism of action involves cell cycle arrest and the induction of apoptosis in the B16 cell population through the upregulation of genes encoding the inflammatory-related receptors Eif2ak, Tlr3, Tlr7, Tlr8 and their downstream pro-apoptotic target genes, as well as the modulation of inflammatory responses [15]. Therefore, our current study aimed to evaluate the antitumor properties of ORN-D-M in a mouse B16 melanoma model. We assessed the effects of different administration routes and regimens of the drug on tumour formation dynamics and animal survival. Additionally, we examined the impact of the drug on certain immunodeficiency markers in cancer by analyzing the relative expression of T-cell and general macrophage marker genes in the peripheral blood of mice.

2. Planning (methodology) of research

The study protocol describing the different stages of the research is presented in the following flow chart (Fig. 1).

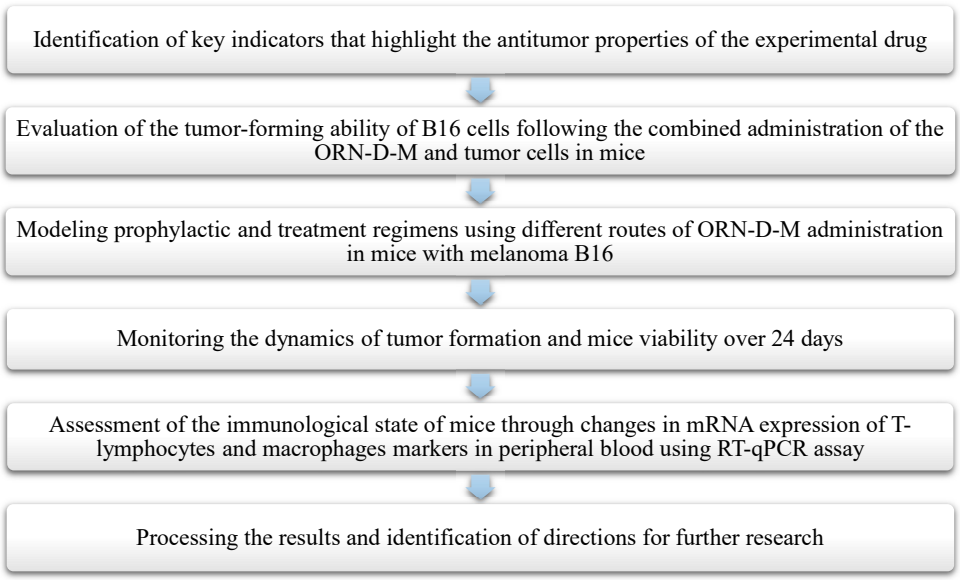

Fig. 1. Study protocol

3. Materials and methods

ORN-D-M is registered as the drug "Nuclex" (Valartin Pharma, Ukraine, registration certificate No. UA/5066/01/02), consisting of low-molecular-weight yeast-derived ORNs and D-M in a 2.5:1 ratio [11].

B16 cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich, USA) supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich, USA) and an antibiotic solution containing 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, USA). The cultivation was carried out using ventilated flasks (Orange Scientific, Belgium) in a CO_2 incubator (Thermo Scientific, USA) at 37 °C and 5 % CO_2. A solution of 0.05 mg/mL trypsin in EDTA (Gibco, USA) was used to detach cells from the substrate. Cultures in the exponential growth phase with less than 70 % confluency were utilized for the experiments.

B16 melanoma was modelled by transplanting B16 cells into mice. The study on animals was conducted following the Directive of the European Parliament and the Council of the EU 2010/63/EU of September 22, 2010, "On the protection of animals used for scientific purposes". The experimental design was reviewed and approved by the Bioethics Committee of the Institute of Molecular Biology and Genetics of NASU (protocol No. 41 of 06.02.2025). C57BL/6 female mice aged about 2 months and weighing 20±3 g, which were kept in the vivarium of the Institute of Molecular Biology and Genetics of NASU, were used for the study. For transplantation, the B16 cell culture was trypsinized, and cells were quickly transferred into ice-cold phosphate-buffered saline (PBS) (Gibco, USA) following centrifugation at 1500 rpm for 5 minutes at 4 °C. The supernatant was decanted, and the cells were resuspended in ice-cold PBS. The cell count was determined using a Goryaev chamber, and the concentration was adjusted to 2×10^5 cells/100 µL per animal. The cell suspension was injected subcutaneously into the right thigh of the mice using a syringe with a 27½-G needle. After injection, the mice were randomized into groups. A solid tumour became visible approximately 7–8 days after transplantation [16].

In the experiments, ORN-D-M was administered in a PBS solution. The concentrations of the drug were selected to ensure its dissolution in a volume that is the maximum permissible for the corresponding route of administration in mice [17].

The experiments included the following control groups:

– "control, B16": animals inoculated with B16 tumour cells without receiving the drug;

– "control, PBS": tumor-bearing animals were administered PBS solution without the drug;

– "control, ORN-D-M 70 (250) mg/kg": healthy animals were administered the corresponding concentration of ORN-D-M;

– "intact": healthy animals with no interventions.

Various factors were tested, based on which the animals were divided into the following groups:

1. Simultaneous administration of ORN-D-M with tumour cell transplantation.

2. ORN-D-M solution at concentrations of 17, 35, and 70 mg/kg was administered subcutaneously along with the tumour cell suspension for transplantation, mixing them immediately before injection.

3. Evaluation of different administration routes:

– peritumoral: ORN-D-M solution at concentrations of 35 and 70 mg/kg was administered subcutaneously around the tumour area;

– oral: ORN-D-M solution at concentrations of 125 and 250 mg/kg was delivered orally via intragastric gavage;

– intraperitoneal: ORN-D-M solution at concentrations of 125 and 250 mg/kg was injected into the peritoneal cavity.

4. Evaluation of different administration regimens:

– prophylactic: ORN-D-M solution was administered through the corresponding route 3 days before tumour cell transplantation, followed by injections every third day until day 24 of the experiment;

– therapeutic: ORN-D-M solution was administered through the corresponding route on day 8 (after the solid tumour became visible), followed by injections every third day until day 24 of the experiment.

The general number of animals in the experiment was 180. The experiment was concluded on day 24 when the tumour reached a critical volume [18]. During this time, animal survival in the groups was monitored, and Kaplan-Meier survival curves were created based on these data using GraphPad Prism 8.0.1 software.

Tumor volume data were collected every 4 days. A calliper was used to measure the length and width of the tumour. Tumor volume was calculated using the formula:

$$V = \frac{W^2 \times L}{2},$$

where V – tumour volume, W – width of the tumour at its narrowest point, L – length of the tumour at its longest point [19].

An analysis of changes in the relative mRNA expression of genes was performed using the RT-qPCR method. At the end of the experiment, approximately 1 mL of peripheral blood was collected from the animals following decapitation. Leukocyte mass was obtained by centrifugation at 1500 rpm and 4 °C, followed by repeated washing with PBS to remove erythrocytes and plasma. RT-qPCR technique was carried out as described previously [15]. Briefly, total RNA was extracted using Direct-zol™ RNA MiniPrep Plus kit (Zymo Research, Germany), followed by cDNA synthesis using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA). The reaction mixture for amplification included the following components: 0.054 U/μL HOT FIREPol DNA polymerase, 2 μL HOT FIREPol 10x B1 buffer, 2.5 mM MgCl2, 200 μM dNTP mix (all purchased from Solis BioDyne, Estonia), PCR dye (SSI "Institute for Single Crystals" of NASU, Ukraine) [20], 1.5 μg cDNA, 0.2 μM forward and reverse primers, and nuclease-free water (Zymo Research, Germany) to bring the final volume to 20 μl. The relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method, and normalization was performed relative to the 18S rRNA gene expression. The expression of the following genes was evaluated: Cd3, Cd247, Cd4, Cd8, Cd68. Primers were designed using NCBI Primer-BLAST and synthesized by Invitrogen, USA. The sequences are provided in Table 1.

Table 1

Sequences of Primers Used in the Study

Protein	Gene	Sequences of forward (F) and reverse (R) primers
CD3 Epsilon Subunit Of T-Cell Receptor Complex	*Cd3e*	F: 5′-TCGGTGGCCACAATTGTCAT-3′ R: 5′-GACTCCACAGCCTTACCCCT-3′
T-Cell Surface Glycoprotein CD3 Zeta Chain	*Cd247*	F: 5′-CCCAAGGTGACGAGAACACA-3′ R: 5′-ATCTGCTCGCCTTGTTTCCA3′
T-cell surface glycoprotein CD4	*Cd4*	F: 5′-CCAGCGTCTTCCCTTGAGTG-3′ R: 5′-TTCTATCTTCCTCCGCCCC TG-3′
T-cell surface glycoprotein CD8 alpha chain	*Cd8a*	F: 5′-GGAGTCTGCACTTTGTGCCT-3′ R: 5′-GATTTGGCTGCTCTTGGGGA-3′
Macrosialin	*Cd68*	F: 5′TTGTCATGGCTACAGGGCAG-3′ R: 5′GTGACGTGCTGGTTGGGAT-3′

Statistical analysis was performed using GraphPad Prism 8.0.1 software. Survival curves were generated using the Kaplan–Meier method. Statistical significance between groups was assessed using the log-rank (Mantel-Cox) test. The Shapiro-Wilk test was used to assess the normality of data distribution. One-way analysis of variance (ANOVA) was used to evaluate statistical significance for tumour volume, while two-way ANOVA was applied to analyze differences in gene expression between control and experimental groups, provided the data followed a normal distribution. Dunnett's post hoc test with correction for multiple comparisons was used to compare experimental groups with the control group.

4. Results

To investigate the effect of ORN-D-M on tumour cells in an animal model initially, we designed an experiment in which the drug was administered to mice at various concentrations simultaneously with the transplantation of tumour cells. This approach aimed to evaluate the impact of the drug on the tumour-forming ability of the cancer cells within the animal organism.

We found that at a high dose, specifically 70 mg/kg, the drug completely inhibited tumour formation (Fig. 2). Meanwhile, at a lower dose of 35 mg/kg, tumour development occurred more slowly, resulting in a nearly tenfold reduction in the average tumour volume compared to the tumour-bearing control. These results also influenced animal survival at the end of the experiment: in the groups treated with higher drug concentrations, no animal mortality was observed during the experiment. Thus, a statistically significant difference between the groups with ORN-D-M at doses 17 mg/kg and 70 mg/kg (χ^2=5.241, p=0.0221) was demonstrated (Fig. 2). The median survival was 19 days in the group treated with the lowest dose, while it was not reached (undefined) in the group receiving the highest dose, suggesting prolonged survival.

One of the key characteristics of the late stage of B16 melanoma is its ability to disrupt the hematopoietic balance by inhibiting the lymphoid lineage and promoting the production of agranulocytes, which subsequently leads to leukocytopenia [21, 22]. In addition, numerous studies have demonstrated that RNA-sensitive TLRs not only play a crucial role in cell-mediated immunity due to their presence in mature antigen-presenting cells (APCs) and lymphocytes but also have a direct impact on the regulation of hematopoiesis, as they are widely expressed in various types of hematopoietic progenitor cells [23–25]. Since previous studies have demonstrated the activation of TLR signalling under the influence of ORN-D-M in cell cultures and tissues [15], we hypothesized that the drug could potentially correct the immunosuppressive state in cancer by modulating hematopoietic balance. Therefore, we investigated whether the drug affects immune cell parameters in the peripheral blood of mice. For this purpose, we analyzed the mRNA expression of general markers of mature T-lymphocytes (CD3e, CD247), T-helpers (CD4), cytotoxic T-cells (CD8a), and general macrophages (CD68). As expected, tumour-bearing mice exhibited a dramatic decrease in the mRNA levels of all studied agranulocyte types, which may indicate a reduction in their cell numbers (Fig. 3). However, in animals that received a high dose of the drug simultaneously with tumour cells, which prevented tumour transplantation, the immune cell marker levels corresponded to those of healthy animals.

As the next step, we investigated the effects of ORN-D-M on tumour formation and survival under prophylactic and therapeutic regimens. Additionally, we tested different routes of drug administration, including peritumoral, oral, and intraperitoneal, to potentially identify the most effective route for the drug activity. However, despite the various administration methods, no statistically significant differences were observed between the control and experimental groups for any of the parameters studied (Fig. 4).

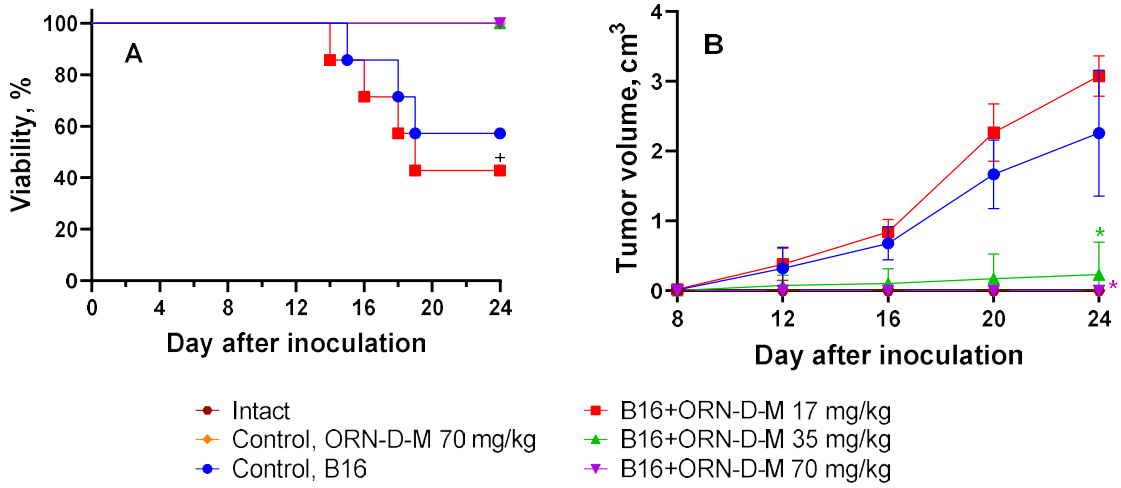

Fig. 2. Effect of ORN-D-M on tumor progression in the B16 melanoma model after the simultaneous transplantation of tumor cells with drug: A – Kaplan-Meier survival curves. + – $p \leq 0.05$, a significant difference compared to the B16+ORN-D-M 70 mg/kg group; B – tumor volume dynamics. Data are presented as mean±standard deviation (n=12); * – $p \leq 0.05$, a significant difference compared to the Control, B16 group

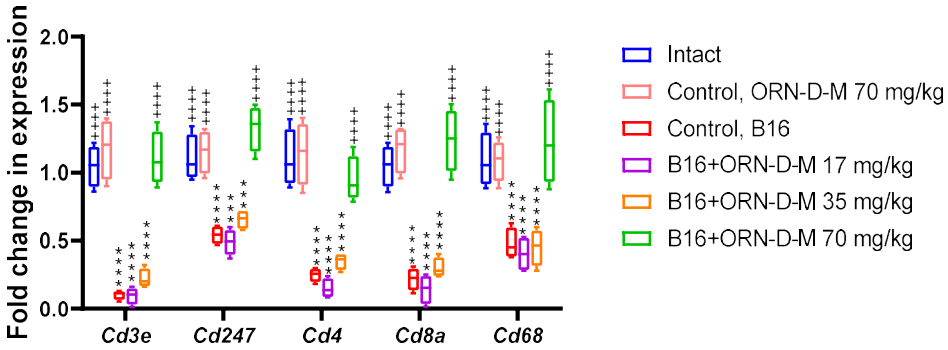

Fig. 3. Changes in mRNA expression of gene markers for specific types of leukocytes isolated from the peripheral blood of mice subjected to simultaneous administration of the drug and tumour cells. The relative gene expression changes were analyzed using the $2^{-\Delta\Delta CT}$ method, with normalization performed using the housekeeping gene 18S rRNA. Data are presented as box-and-whisker plots: the box extends from the 25th to the 75th percentile, with a horizontal line indicating the median and whiskers showing the minimum and maximum values ($n \geq 6$): *** – $p \leq 0.001$, **** – $p \leq 0.0001$, a significant difference compared to the Intact group; ++++ – $p \leq 0.0001$, a significant difference compared to the Control, B16 group

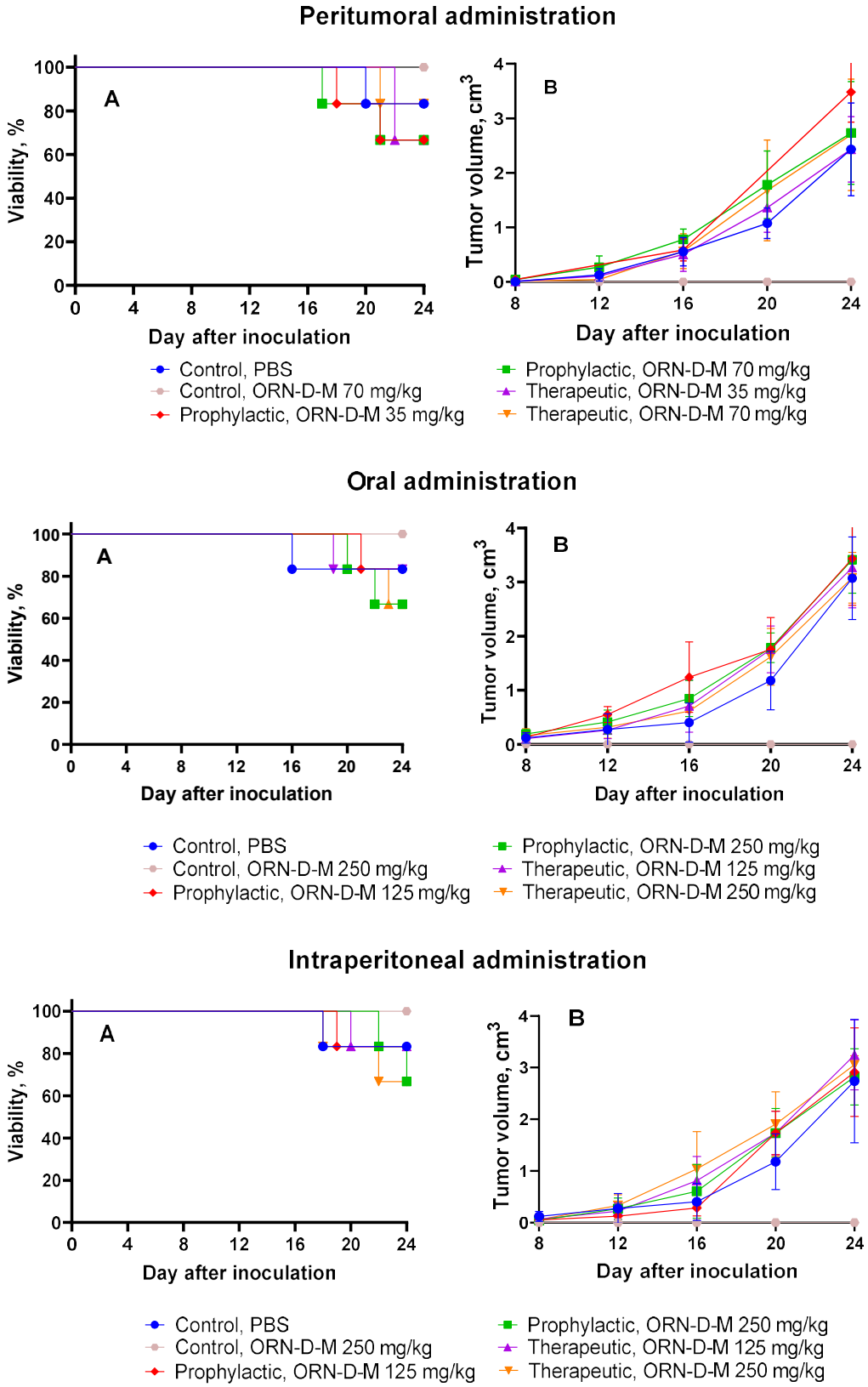

Fig. 4. Effect of ORN-D-M on tumor progression in the B16 melanoma model under different regimens and routes of drug administration: A – Kaplan-Meier survival curves; B – tumor volume dynamics. Data are presented as mean±standard deviation (n=6)

Additionally, an immune cell gene expression analysis was conducted on blood samples from mice subjected to prophylactic or therapeutic regimens with peritumoral administration of the drug. However, differences in the relative expression of these genes were observed only between tumour-bearing and healthy animals. The drug did not impact the immunodeficient state in the late stage of B16 melanoma (Fig. 5).

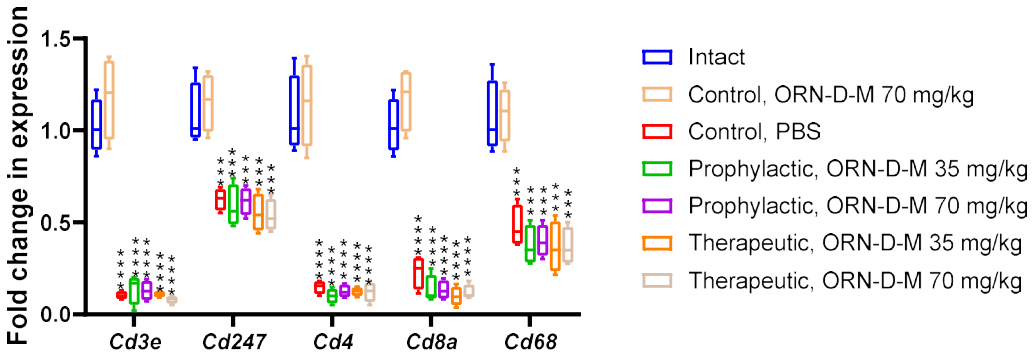

Fig. 5. Changes in mRNA expression of gene markers for specific types of leukocytes isolated from the peripheral blood of mice in the peritumoral drug administration groups. The relative gene expression changes were analyzed using the $2^{-\Delta\Delta CT}$ method, with normalization performed using the housekeeping gene 18S rRNA. Data are presented as box-and-whisker plots: the box extends from the 25th to the 75th percentile, with a horizontal line indicating the median and whiskers showing the minimum and maximum values (n=6): *** – $p \leq 0.001$, **** – $p \leq 0.0001$, a significant difference compared to the Intact group

5. Discussion

Targeting inflammatory pathways has emerged as a promising approach for anticancer treatment, driven by the increasing recognition of cancer-associated inflammation. In this context, TLR agonists, particularly those targeting nucleic-acid-sensitive receptors, are under extensive investigation and have shown significant results in cancer models. For instance, systemic administration of resiquimod, a synthetic nucleoside analogue and TLR7 agonist, reduces tumour growth in a mouse model of squamous cell carcinoma. Notably, it did not exhibit a direct inhibitory effect on this cell line, highlighting its role in enhancing anticancer immunity rather than exerting direct cytotoxicity on the tumour [26]. Synthetic short sequences of nucleic acids have also demonstrated antitumoral properties. Monotherapy with CMP-001, which contains synthetic unmethylated CG-rich oligodeoxynucleotides (CpG ODN), has been shown to reduce tumour size and improve survival in mice with A20 B lymphoma solid tumours. This compound activates TLR9, promoting T cell and dendritic cell infiltration into the tumour-associated draining lymph nodes and A20 tumours [27].

In previous studies of our research group, we found that ORN-D-M exhibits direct cytotoxic activity against tumour cell lines due to triggering the TLR-dependent molecular pathways [14, 15]. This effect was proved by administering the drug simultaneously with the inoculation of B16 cells in mice, which resulted in a dose-dependent reduction in their tumor-formation ability. However, in standard tumour prevention and treatment models, the drug showed no effect. Despite the variability in administration routes and regimens, no statistically significant differences were found between the tumour-bearing control group and the experimental groups in terms of tumour formation dynamics or the development of immunodeficiency.

The discrepancies in the effects of ORN-D-M observed between the cellular and animal models can be attributed to several factors. A key characteristic of the B16 mouse melanoma model is its low immunogenicity. These tumours exhibit insufficient antigen expression on the cell surface, leading to a low presence of antigen-specific T-lymphocytes infiltrating the tumour. Under the influence of high concentrations of protumorogenic cytokines, these T-lymphocytes not only lose their ability to mount an effective antitumor response but may also stimulate tumorigenesis [28, 29]. The consequence of this condition is lymphopenia associated with melanoma [21, 22]. Since ORN-D-M has previously been shown to have a normalizing effect on T-cell parameters in various diseases, particularly those characterized by heightened immunosuppression (e.g., AIDS) [13], we hypothesized that this drug, in addition to its direct inhibitory properties against the B16 cell line, would exhibit an immunomodulatory effect in an experimental cancer model, promoting tumour regression. However, likely due to the high ability of the studied tumour type to evade immune responses, the use of the drug in therapeutic and prophylactic regimens proved ineffective. On the other hand, the lability of nucleic acids and the lack of effective stabilizing delivery methods likely contributed to the absence of therapeutic effects, as it prevented the administration of an effective dose into the tumour site.

Comparing the antitumor effects of drugs not only across different cancer types but also across various models of one cancer type has always posed a significant challenge. In a publication by T. Voskoglou-Nomikos and colleagues, it was noted that data on the antitumor activity of cytostatic and cytotoxic drugs obtained specifically from *in vitro* cultures of solid tumours exhibit greater predictive accuracy for subsequent clinical studies compared to results derived from mouse allograft models. This is partly explained by the fact that mice possess a more efficient drug clearance system than humans, and the maximum tolerable dose of drugs is generally higher in these animals [30].

Practical Relevance. ORN-D-M shows potential for further research due to its direct cytotoxic activity against tumour cells and may be developed for the therapy of malignant tumour diseases.

Research limitations. The study was conducted on a cancer type characterized by low-immunogenic tu-

mours, which may limit the generalizability of the findings to other tumour types.

Prospects for further research. We believe that future research should investigate the antitumor properties of the drug in other cancer types, particularly immunogenic ones. Since this study demonstrated the inhibition of tumour formation by B16 cancer cells following treatment with ORN-D-M, it is essential to investigate its specific effects on the immune system in cancer patients further. This would help identify potentially sensitive cancer types. Additionally, methods for stabilizing and delivering the drug must be developed to ensure the administration of an effective dose at the tumour site. Such an approach, combined with the targeted delivery technology, could enhance the cytotoxic properties of ORN-D-M in the treatment of malignant tumours *in vivo.*

6. Conclusions

In this study, we investigated the antitumor effects of ORN-D-M in a B16 melanoma mouse model. Our findings demonstrated that when administered simultaneously with tumour cell transplantation, ORN-D-M exhibited a strong inhibitory effect on tumour formation at high concentrations (70 mg/kg), completely preventing tumour development. Lower concentrations (35 mg/kg) significantly delayed tumour growth and improved survival outcomes. Despite these promising effects under simultaneous administration conditions, ORN-D-M failed to exhibit significant therapeutic efficacy when applied in conventional prophylactic or treatment regimens. Across different administration routes – including peritumoral, oral, and intraperitoneal – no statistically significant differences were observed in tumour progression or survival rates compared to control groups. Additionally, immune cell marker analysis indicated that the drug has no influence on immunodeficiency at the late stage of melanoma.

Conflict of interest

Zenoviy Tkachuk has a patent, "Multiantivirus compound, composition and method for treatment of virus diseases", issued to Biocell Laboratories Inc (US8420617B2, 2013). Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The manuscript has no associated data.

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies when creating the current work.

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