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Oligoribonucleotides in complexes with D-mannitol alter cell cycle and cause apoptosis in murine melanoma B16 cells

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Aim. Natural oligoribonucleotides in complexes with D-mannitol (ORN-D-M) exhibit cytotoxic effects on various malignant cell lines in a dose- and time-dependent manner. This study aims to elucidate the potential mechanisms underlying the inhibition of mouse melanoma B16 cells viability by ORN-D-M. **Methods.** Flow cytometry, RT-qPCR. **Results.** Our findings reveal that ORN-D-M induce cell cycle arrest in the G_0/G_1 phase and elevate the level of fragmented (apoptotic) DNA. Additionally, we observed the upregulation of RNA receptors *Tlr3, Tlr7, Tlr8,* and *Eif2ak,* along with the inflammation-suppressive subunit of transcriptional factor *Nfkb1,* and the cytokines *Ifna* and *Ifnb,* known for their antitumor properties. As a result, the down-regulation of inflammatory cytokines *Tnfa* and *Il1b*, as well as the changes in apoptotic factors *Bcl2, Bax* and *Casp3* were observed. **Conclusions.** ORN-D-M induce the cell cycle arrest and apoptosis by promoting extensive production of the inflammation-suppressive subunit NFKB1 and cytokines IFN type I due to the triggering of RNA-sensitive receptors in the cell.

K e y w o r d s: ORN-D-M; RNA receptors; relative mRNA level; RT-qPCR; cell cycle.

Introduction

In the current understanding, inflammation is acknowledged as a model for carcinogenesis, due to its association with immunosuppression and damage of the genetic material [1]. Multiple studies have suggested that the use of anti-inflammatory agents reduces the risk of developing various types of cancer [2]. Furthermore, anti-inflammatory agents can directly impede the cancer cells through apoptosis, as evidenced, for instance, in the case of squamous cell carcinoma epithelial cell lines [3].

One of the promising anti-inflammatory agents are oligoribonucleotides with D-mannitol (ORN-D-M), which consists of highly purified fragmented yeast RNA, primarily low-molecular-weight oligoribonucleotides, complexed with the sugar alcohol D-mannitol [4].

This medication exhibits the antiviral properties against various infections, including respiratory infections, herpes *etc*. [5–7]. Moreover, it demonstrates the anti-inflammatory, antioxidant, and immunomodulatory effects [8–10]. A proposed molecular mechanism of action involves the inflammatory process inhibition by modulating the expression of specific ribonucleic acid receptors, such as the Toll-like receptors (TLR) types 3, 7, 8, RNA-dependent protein kinase EIF2AK (also known as PKR) and their downstream target nuclear factor κB (NF-κB), which are overexpressed in the inflammation processes. Additionally, the drug contributes to reducing the expression of pro-inflammatory cytokines, chemokines, and interferons [8,11]. In our prior research, we discovered that ORN-D-M also inhibit the viability of the malignant mouse melanoma B16 and human glioma U251 cell lines in a dose-dependent and time-dependent manner. Importantly, the effect of that drug on the non-malignant canine kidney MDCK cells is minimal [12].

This study aimed to elucidate the inhibitory mechanisms of ORN-D-M towards malignant cells, specifically examining the impact on the cell cycle and investigating the involvement of specific RNA-receptors and downstream signaling components in the mouse melanoma B16 cell line. We present the data indicating that ORN-D-M demonstrate a direct cytotoxic effect on the cancer cells.

Materials and Methods

Cell culture

The cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA), 1 % penicillin, and 1 % streptomycin (Sigma-Aldrich, USA) at 37 °C in a humidified incubator with 5 % CO_2. For cell detachment from the culture flask surface, they were washed with Versene solution (Sigma-Aldrich, USA) and incubated in a 0.05 mg/mL trypsin solution (Gibco, USA) for 10 minutes at room temperature.

Cell treatment

For cell treatment, we used ORN-D-M, patented [4] and registered as the pharmaceutical product "Nuclex" (Valartin Pharma, Ukraine). The cells were seeded on a 6-well plate at a density of 1.5×10^5 cells/well. After the cell attachment to the substrate, the culture medium was changed to incomplete nutrient medium for untreated control samples and to the one containing dissolved ORN-D-M (2.5 or 5 mg/mL) for experimental samples followed by incubation for 24 hours.

Flow cytometry

The treated cells were fixed in cold 70 % ethanol overnight, following the previously described technique [13]. Fixed cells were incubated in a propidium iodide solution with the RNAse — FxCycle PI/RNAse Solution (Invitrogen, USA) for 30 min in the dark at room temperature. The percentage distribution of the cell population in different phases of the cell cycle (Sub G_0, G_0/G_1, S, G_2/M) was assessed on the Attune NxT flow cytometer (Thermo Fisher, USA) based on the fluorescence intensity of cellular DNA upon binding with propidium iodide.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA from 1×10^6 treated and untreated cells was isolated using the Direct-zol™ RNA

MiniPrep Plus kit (Zymo Research, Germany). The quality of isolation was confirmed using the MCE-202/MultiNA electrophoresis system (Shimadzu, Germany). For each sample, 1.5 μg of total RNA were used to synthesize cDNA with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA). This cDNA was then utilized as a matrix for qPCR. The reaction mixture 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 (Institute for Single Crystals of NASU, Ukraine), 0.3 μL matrix, 0.2 μM forward and reverse primers, and nuclease-free water (Zymo Research, Germany) to bring the final volume to 20 μl. The qPCR analysis was carried out on the CFX96 Touch Real-Time PCR Detection System C1000 Touch (Bio-Rad, USA) following the standard protocol for reaction mixes with EvaGreen dye. The changes in the expression level of mRNA for different genes in the experimental sample relative to the untreated control were determined using the $2^{-\Delta\Delta CT}$ method [14], and normalization was performed relative to the *18S rRNA* gene. The primers for nucleic acid amplification were selected using NCBI Primer-BLAST (NIH, USA) and synthesized by Invitrogen, USA. The sequences are provided in Table 1.

Statistical analysis

Visualization and statistical analysis of the data were conducted using GraphPad Prism 8.0 software (GraphPad Software Inc, USA). The normality of data distribution was assessed using the Shapiro-Wilk test. To establish statistical significance, the non-parametric Mann-Whitney U-test was employed. Differences were considered statistically significant at $p \leq 0.05$.

Results and Discussion

In the previous studies, we have demonstrated that treatment with ORN-D-M reduced the viability of different malignant cell lines in a dose-dependent and time-dependent manner [12]. To elucidate the potential mechanisms of the cell growth inhibition induced by ORN-D-M, we conducted an analysis of the B16 cell population distribution in the cell cycle using flow cytometry with propidium iodide staining.

We revealed that 24-hour treatment with ORN-D-M led to dose-dependent changes in the cell cycle of the B16 population (Fig. 1). Notably, there was a substantial increase in the proportion of cells in the G_0/G_1 phase, with a 15 % rise at 2.5 mg/mL ORN-D-M and over 20 % at 5 mg/mL ORN-D-M. Simultaneously, a consistent decrease in the proportion of cells in the G_2/M phase, averaging 40 %, was observed. Furthermore, the compound induced a dose-dependent appearance of the Sub G_0 phase of fragmented DNA, indicative of apoptotic processes [15].

These results suggest that the inhibition of cell viability following ORN-D-M treatment is associated with the cell cycle arrest in the G_0/G_1 phase, coupled with the promotion of apoptosis within the cell population.

Whereas the drug ORN-D-M is a complex of ribonucleic acid with D-mannitol [4], its cellular impact is likely mediated through RNA receptors. Notably, prior research demonstrated the involvement of RNA receptors in the drug's mechanism in an influenza animal model [8].

Table 1. **Sequences of primers used in the study**

The protein encoded by the gene	Gene	Primer sequences
18S ribosomal RNA	*18S rRNA*	Forward 5′-CCA TCC AAT CGG TAG TAG C-3′ Reverse 5′- GTA ACC CGT TGA ACC CAT-3′
Toll-like receptor 3	*Tlr3*	Forward 5′-GCC CCC TCA GTC ATG GAT TC-3′ Reverse 5′-GAG GGA AGT GCT ATA GTT TGG GG-3′
Toll-like receptor 7	*Tlr7*	Forward 5′-ATC CTC TGA CCG CCA CAA TC-3′ Reverse 5′-TCA CAT GGG CCT CTG GGA TA-3′
Toll-like receptor 8	*Tlr8*	Forward 5′-GCC CCC TCA GTC ATG GAT TC-3′ Reverse 5′-GAG GGA AGT GCT ATA GTT TGG GG-3′
Eukaryotic translation initiation factor 2 alpha kinase 2	*Eif2ak2*	Forward 5’- CAC AGA GAT CTT AAG CCA GGT AAT-3’ Reverse 5’- AGA TGT CCA CTT CTT TTC CAT AGT-3’
Nuclear factor κB subunit 1	*Nfkb1*	Forward 5′-GGA CAT GGG ATT TCA GGA TAA CC-3′ Reverse 5′-AGA GGT GTC TGA TAC AGG TCA T-3′
RELA Protooncogene NF-κB Subunit	*Rela*	Forward 5′-GCC CAG ACA TCC AAA CCT GA-3′ Reverse 5′-GCG AGA GGA GCA CAG ATA CC-3′
NF-κB inhibitor alpha	*Nfkbia*	Forward 5′-GAG ACT CGT TCC TGC ACT TG-3′ Reverse 5′-AAG TGG AGT GGA GTC TGC TG-3′
Interferon alpha 2	*Ifna2*	Forward 5′-CTT ACT CAG CAG ACC TTG AAC C-3′ Reverse 5′-CTG CTG CAT CAG ACA GGT TT-3′
Interferon beta 1	*Ifnb1*	Forward 5′-GAT GCT CCA GAA TGT CTT TCT TGT-3′ Reverse 5′-CGA ATG ATG AGA AAG TTC CTG AAG-3′
Tumor necrosis factor alpha	*Tnfa*	Forward 5′-AAA GGG ATG AGA AGT TCC CAA AT-3′ Reverse 5′-ACT TGG TGG TTT GCT ACG AC-3′
Interleukin 1 beta	*Il1b*	Forward 5′-TTC ATC TTT GAA GAA GAG CCC AT-3′ Reverse 5′-TGG AGA ATA TCA CTT GTT GGT TGA-3′
BCL2 Apoptosis Regulator	*Bcl2*	Forward 5′-TCC AAT GTC CAG CCT TTG-3′ Reverse 5′-TTT GGG GCA GGC ATG TTG AC-3′
BCL2 Associated X Protein	*Bax*	Forward 5′-GCC CTT TTG CTT CAG GGT TTC-3′ Reverse 5′-TCC AAT GTC CAG CCT TTG-3′
Caspase 3	*Casp3*	Forward 5′-AAT TCA AGG GAC GGG TCA TG-3′ Reverse 5′-GCT TGT GCG CGT ACA GTT TC-3′

In mammalian cells, there is a set of receptors sensitive to exogenous RNA, including TLR types 3, 7, 8, and EIF2AK. Upon binding to an RNA ligand, TLR3, TLR7, and TLR8 initiate signaling pathways of the innate immune response and inflammatory reactions through IFN type I- and NF-κB-dependent pathways [16]. EIF2AK activates a series of events, leading to the translational arrest in cells followed by apoptosis. It also activates the transcription factor NF-κB, that, among other functions, regulates inflammatory and apoptotic signals [17]. In tumor cells, including B16, there is a high expression of TLR3, TLR7, and TLR8 [18,19], while

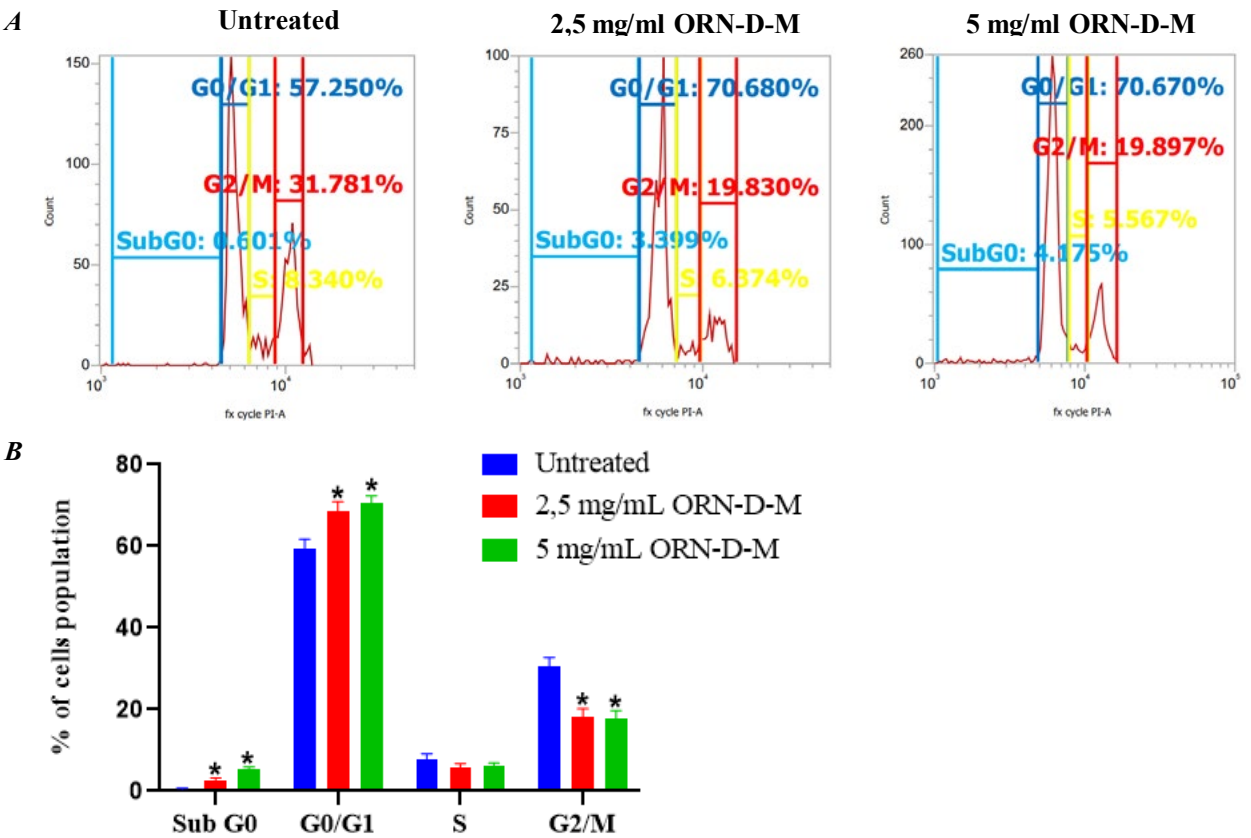

Fig. 1. Changes in the cell population distribution of mouse melanoma B16 cells under 24-hours treatment with ORN-D-M. *A* — Representative graphs illustrating the distribution of the cell population based on the amount of stained DNA. *B* — Diagram depicting the percentage distribution of the cell population in different phases of the cell cycle. Data are presented as the mean ±SD of three independent experiments. * — $p \leq 0.05$, indicating a significant difference compared to the untreated control group.

EIF2AK is constitutively expressed in all types of tissues [20]. After the treatment with ORN-D-M, a dose-dependent increase in the relative expression of genes encoding cellular RNA receptors was observed (Fig. 2). For instance, with the use of 5 mg/mL, the expression of *Tlr3, Tlr7,* and *Eif2ak* increased by more than twofold, while a slight increase in the *Tlr8* expression was also observed.

Whereas RNA-receptors activate a series of signaling pathways with two main objectives: regulating the synthesis of interferons type I (IFN type I) and pro-inflammatory cytokines by NF-κB-dependent pathway [20], we have further investigated the expression of these regulated genes.

IFN type I are the cytokines that induce the expression of hundreds of genes, mediating various cellular responses, including cell cycle arrest [21] and caspase-dependent apoptosis in tumor cells [22]. We observed a distinguished increase in the relative expression of IFN type I,

specifically *Ifna2* more than 4 folds and *Ifnb1* near to 2 folds (Fig. 2).

Another key downstream component of both TLR-dependent and EIF2AK-dependent signaling pathways is the dimeric nuclear transcription factor NF-κB [20], which regulates the transcription of genes associated with inflammation, innate immunity, and cell survival signals [23]. The NF-κB protein family consists of five subunits, but the RNA-sensitive receptors regulate the activity of three: RELA, c-REL, and NFKB1 [24]. The functions of NF-κB in dimeric form depend on the combination of these subunits — RELA and c-REL have a transcription activation domain in their structure, which is absent in NFKB1. Therefore, in the form of a homodimer, NFKB1 exerts transcriptional suppression [25, 26]. In our

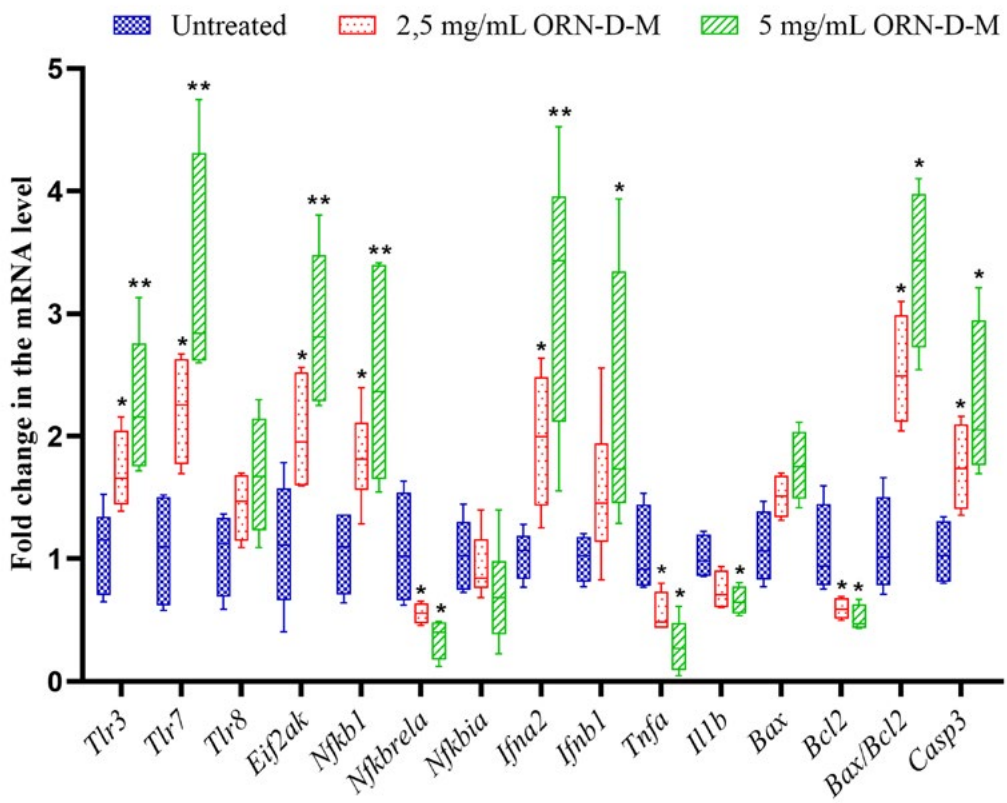

Fig. 2. Changes in the mRNA expression level of various genes in B16 mouse melanoma cells under the influence of ORN-D-M. After a 24-hour treatment with ORN-D-M, total cell RNA was extracted, and RT-qPCR was performed. Data analysis was conducted using the $2^{-\Delta\Delta CT}$ method, with normalization using the housekeeping gene *18S rRNA*. The data were obtained from at least three independent experiments ($n \leq 6$) and are presented in a box plot format: the box stretches from the 25th to the 75th percentile with a horizontal line at the median, whiskers extend from the smallest to the largest value. * — $p \leq 0.05$, ** — $p \leq 0.01$, indicating a significant difference compared to the untreated control group.

studies, following ORN-D-M stimulation, we observed an increase in suppressive *Nfkb1* and a decrease in *Rela* (Fig. 2). This suggests the potential formation of suppressive homodimers of NFKB1 at the protein stage. Due to the suppressive action of NFKB1 on the transcription of pro-inflammatory cytokines, a significant decrease ($p \leq 0.05$) in the expression of *Tnfa* and *Il1b*, pivotal pro-inflammatory cytokines, was observed.

As mentioned above, both NF-κB and IFN type 1 can trigger the apoptotic signals in tumor cells [21, 25]. Therefore, we observed a significant reduction ($p < 0.05$) in the level of the anti-apoptotic mitochondrial *Bcl2* (Fig. 2), which expression is directly controlled by NF-κB [27]. Additionally, there was a trend towards an increase in the expression of the apoptosis activator *Bax*, a product that competes with BCL2, contributing to the mitochondrial membrane damage followed by apoptosis (Fig. 2). The *Bax/Bcl2* ratio, an important indicator of apoptosis regulation in the tumor cells [28], significantly increased ($p < 0.05$) with an increase in the dose of the compound (Fig. 2). Another examined gene associated with apoptosis was *Casp3*, an effector caspase whose activation triggers an apoptotic pathway [15]. Under the influence of the drug, there was an increase in *Casp3* levels. These dose dependent changes in the mRNA expression of apoptotic factors correlate with the flow cytometry data, demonstrating a rise in the proportion of apoptotic cells with an elevated concentration of ORN-D-M (Fig. 1), as well as with our previous studies [12]. Taking into account the current results, it is important to explore the potential antitumor activity of ORN-D-M on the animal model of melanoma B16 in further research.

Conclusion

Our study shows that treatment of mouse melanoma B16 cells with ORN-D-M significantly diminishes their viability by inducing cell cycle arrest in the G0/G1 phase and activating apoptosis. This mechanism involves various cellular RNA receptors, triggering the TLR- and EIF2AK-dependent pathways, ultimately leading to the induction of IFN type I and the suppressive subunit of the transcription factor NFKB1, that results in the expression of apoptotic factors. Considering that ORN-D-M demonstrate not only direct activity against the tumor cells of mouse melanoma B16, but also exhibits anti-inflammatory and immunomodulatory effects, this research opens up opportunities for the development of ORN-D-M as a potential anticancer drug candidate.

Declaration of competing interest

Z.Yu.Tkachuk has patent “Multiantivirus compound, composition and method for treatment of virus diseases” issued to Biocell Laboratories Inc. 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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Олігорибонуклеотиди в комплексі з D-манітолом порушують клітинний цикл та спричиняють апоптоз у клітин меланоми миші B16

І. М. Прилуцька, З .Ю. Ткачук

Мета. Природні олігорибонуклеотиди у комплексі з D-манітолом проявляють цитотоксичність на різних злоякісних клітинних лініях у дозозалежний та часозалежний спосіб. Поточна робота спрямована на визначення можливих механізмів інгібування клітин меланоми миші B16 під впливом ORN-D-M. **Методи.** Проточна цитометрія, ЗТ-кПЛР. **Результати.** Показано, що ORN-D-M призводить до зупинки клітинного циклу в фазі G_0/G_1 та збільшення рівня фрагментованої (апоптотичної) ДНК. Ми відзначили підвищення експресії мРНК рецепторів *Tlr3, Tlr7, Tlr8* та *Eif2ak*; субодиниці *Nfkb1,* яка супресує транскрипцію запальних генів у клітині; та цитокінів *Ifna, Ifnb,* що відомі протипухлинними властивостями. Внаслідок цього спостерігалася знижена експресія запальних цитокінів *Tnfa* та *Il1b,* а також зміни в апоптотичних факторах *Bcl2, Bax* і *Casp3*. **Висновки.** Отже, ORN-D-M спричиняє порушення клітинного циклу та апоптоз клітин B16 шляхом індукції експресії супресивної транскрипційної субодиниці NFKB1 та IFN типу I через вплив на РНК-чутливі рецептори в клітині.

Ключові слова: ОРН-D-M; РНК-рецептори; відносний рівень мРНК; ЗТ-кПЛР; клітинний цикл.

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