### Mesyl phosphoramidate antisense oligonucleotides as an alternative to phosphorothioates with improved biochemical and biological properties

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Here we describe a DNA analog in which the mesyl (methanesulfonyl) phosphoramidate group is substituted for the natural phosphodiester group at each internucleotidic position. The oligomers show significant advantages over the often-used DNA phosphorothioates in RNA-binding affinity, nuclease stability, and specificity of their antisense action, which involves activation of cellular RNase H enzyme for hybridization-directed RNA cleavage. Biological activity of the oligonucleotide analog was demonstrated with respect to pro-oncogenic miR-21. A 22-nt anti-miR-21 mesyl phosphoramidate oligodeoxynucleotide specifically decreased the miR-21 level in melanoma B16 cells, induced apoptosis, reduced proliferation, and impeded migration of tumor cells, showing superiority over isosequential phosphorothioate oligodeoxynucleotide in the specificity of its biological effect. Lower overall toxicity compared with phosphorothioate and more efficient activation of RNase H are the key advantages of mesyl phosphoramidate oligonucleotides, which may represent a promising group of antisense therapeutic agents.

phosphorothioate DNA | oncogenic microRNA | miR-21

The antisense approach to posttranscriptional gene silencing was first demonstrated experimentally some 40 y ago (1) and recognized as a new therapeutic principle a decade later (2, 3). The early in vivo studies on antisense oligonucleotides (AONs) stressed the importance of protecting the internucleotidic phosphodiester (PO) bond against nuclease digestion (4). Phosphorothioate oligodeoxynucleotides (PS-ODNs), which substitute the sulfur atom for the oxygen atom in one of the nonbridging positions of the PO group (5), being relatively easy to synthesize (6) and showing a tolerable decrease in binding affinity to RNA (7), offer a respectable level of nuclease resistance (8) and thus are among the first antisense DNA analogs to show therapeutically relevant activity (9).

A significant advantage of PS-ODNs over some other AON types is the ability, similar to that of the natural PS-ODNs, to recruit cellular RNase H to cleave the RNA strand of the complementary heteroduplex (10, 11). Up to now, most Food and Drug Administration (FDA)-approved nucleic acid therapeutics have been recruited from PS oligonucleotides (PS-ONs) (12), with the latest, nusinersen (Spinraza), approved in 2016 (13). The therapeutic effectiveness of PS-AONs has been ascribed to their favorable pharmacokinetics (14), cellular uptake, and intracellular trafficking (15), which can be explained by interactions of the sulfur-containing ONs with cellular proteins (16, 17).

The toxicity of PS-AONs in vivo has been an important issue, however. Alicafoxes (ISIS 2302), a 20-nt PS ODN complementary to a part of the human intercellular adhesion molecule-1 mRNA (18), has been studied extensively as an enema-applied treatment for ulcerative colitis (19), inflammatory bowel disease (20), and pouchitis (21). Identified problems included immune stimulation (mice, 20 mg/kg) (22), increases in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in serum suggestive of hepatic damage (mice, 100 mg/kg) (23), lethargy, changes in blood clotting time and atrophic processes in kidneys (monkeys, 10 mg/kg) (24), and red blood cells in the renal lumen (monkeys, 50 mg/kg) (25, 26). Although the adverse effects gradually subside after discontinuation of AONs, some effects are evident even at 2–3 mg/kg. The main immunotoxicity has been ascribed to complement activation, which results in acute hemodynamic and hematologic alterations (25).

Another example is mipomersen (Kynamro; ISIS 301012), a 20-nt PS 2′-O-(2-methoxyethyl) (MOE) gapmer targeting apolipoprotein B-100, which was approved by FDA in 2013 to treat familial homozygous hypercholesterolemia (26). Mipomersen is administered by s.c. injection at a dose of 200 mg/kg on a weekly basis. Adverse effects during clinical trials included injection site reactions (in 76% of patients), flu-like symptoms (in 34%), increase in serum ALT and AST levels, and accumulation of fat in the liver (27–30). These effects appeared to be reversible after discontinuation of treatment but were severe enough to raise concerns about

**Significance**

Forty years of research have shown that antisense oligonucleotides have great potential to target mRNAs of disease-associated genes and noncoding RNAs. Among the vast number of oligonucleotide backbone modifications, phosphorothioate modification is the most widely used in research and the clinic. However, along with their merits are notable drawbacks of phosphorothioate oligonucleotides, including decreased binding affinity to RNA, reduced specificity, and increased toxicity. Here we report the synthesis and in vitro evaluation of the DNA analog mesyl phosphoramidate oligodeoxynucleotide. This oligonucleotide type recruits RNase H and shows significant advantages over phosphorothioate in RNA affinity, nuclease stability, and specificity in inhibiting key processes of carcinogenesis. Thus, mesyl phosphoramidate oligonucleotides may be an attractive alternative to phosphorothioates.
liver damage and potential long-term cardiovascular effects, which resulted in the repeated refusal of the European Medicines Agency to approve the drug for use in the European Union (31).

Therefore, new DNA analogs with favorable physicochemical and biological properties, such as strong and specific RNA binding, nuclease resistance, and low toxicity, would represent a welcome addition and potentially an alternative to PS-ODNs, and their design and development remains an important research task. Here we report on the synthesis and in vitro evaluation of a DNA analog that substitutes the mesyl (methanesulfonyl) phosphoramidate group (μ-modification) for the natural PO group in each internucleotidic position (Fig. 1A).

Results
Design and Synthesis of Mesyl Phosphoramidate ODNs. We recently evaluated DNA derivatives incorporating tosyl (p-toluenesulfonyl) phosphoramidate groups and found that their duplexes with RNA were destabilized compared with the natural DNA:RNA duplex (32), more so if the groups replace all of the phosphates in the ON chain. However, when one or two of less bulky mesyl phosphoramidate groups (μ-groups) have been incorporated into an oligodeoxynucleotide sequence, the thermal stability of the resulting duplexes with either DNA or RNA showed little difference from the corresponding native duplexes (33). This observation encouraged us to investigate mesyl phosphoramidate oligodeoxynucleotides (μ-ODNs) substituted at all internucleotidic positions with the μ-modification. This task was accomplished using our previously reported method (33) by replacing aqueous iodine oxidizer in an automated DNA synthesizer with 0.5 M mesyl azide in acetonitrile. The Staudinger reaction between the support-bound phosphite triester and mesyl azide was carried out for 15 min. After completion of the assembly, μ-ONs were cleaved from the solid phase and deprotected by conventional concentrated aqueous ammonia treatment at 55 °C. ONs were isolated by reverse-phase HPLC and, if necessary, purified to homogeneity by conventional PAGE under the same conditions as for unmodified ODNs or PS-ODNs. The structures of the μ-ONs thus obtained have been verified by ESI LC-MS or MALDI-TOF MS (Table 1).

Hybridization Properties of μ-ODNs Targeting miR-21. One of the most important goals of this study was to define the key biological properties of the μ-modification and to compare them with its nearest analog, PS modification of the phosphate backbone. To examine and compare the inhibitory potential of ONs, a pro-oncogenic microRNA (miRNA) miR-21 was selected as a target. As master regulators, miRNAs are involved in almost every cellular process in both normal and pathological conditions, and miRNA dysregulation is associated with tumor progression and spread. Among the numerous miRNAs already identified as regulators of tumorigenesis, miR-21 has emerged as a key miRNA that is deregulated and overexpressed in major types of tumors (34, 35). In this study, we compared the biological potency of miR-21 targeting ONs spanning the full length of miR-21 (22 nt) and its nearest analog, PS modification of the phosphate backbone. To examine and compare the inhibitory potential of PS-ODNs, or PS-miR-21-ODNs (Fig. 1B). It was revealed that the efficiency of duplex formation of μ-miR-21-ODN with miR-21 is almost equivalent to that of PS-miR-21-ODN. Quantitative binding of both ONs with miR-21 was observed even at 0.5 μM concentration (Fig. 1B). In contrast, PS modification resulted in a considerable decrease in hybridization efficiency. The degree of binding of PS-miR-21-ODN to miR-21 did not exceed 85% even at the highest concentration of the ODN (Fig. 1B).

RNase H Activation by μ-ODNs. Since irreversible degradation of miRNAs is more reliable and effective way of suppressing their functions than transient steric block, the RNase H-recruiting ability of a 22 nt μ-ON was tested and compared with the efficiency of miR-21 degradation in the duplexes with PS-miR-21-ODNs or PO-miR-21-ODNs by RNase H. It was revealed that the efficiency of duplex formation of μ-miR-21-ODN with miR-21 is almost equivalent to that of PS-miR-21-ODN. Quantitative binding of both ONs with miR-21 was observed even at 0.5 μM concentration (Fig. 1B). In contrast, PS modification resulted in a considerable decrease in hybridization efficiency. The degree of binding of PS-miR-21-ODN to miR-21 did not exceed 85% even at the highest concentration of the ODN (Fig. 1B).

Stability of μ-ODNs in Serum. We compared the nuclease resistance of μ-ODNs and PS-ODNs by examining their degradation profile in 10% FBS (SI Appendix, Fig. S1). Introduction of μ-modification to the nucleotide sequence led to a dramatic increase in ON stability.
22-mer μ-ODNs remained intact for 168 h (SI Appendix, Fig. S1). In contrast, 22-mer PS-ODNs demonstrated lower nuclease resistance with a degradation degree of ~58% after 168 h (SI Appendix, Fig. S1). These results indicate that the μ-modification is superior to PS in terms of stability in biological media and is extremely resistant to nuclease attack.

Effects of miRNA-Specific μ-ODNs and PS-ODNs on miRNA Levels in Tumor Cells. Functional potency of μ-miR-21-ODNs compared with PO-miR-21-ODNs and PS-miR-21-ODNs was studied using B16 melanoma cells. Stem-loop PCR analysis showed that none of the control ONs, PO-luc, PS-luc, and μ-luc (Table 1), had an impact on the level of miR-21. The effect of PS-miR-21-ODNs was investigated at a concentration range of 0.01–0.2 μM, as application of higher concentrations of PS-modified ODNs leads to severe unspecific cytotoxicity. Transfection of cells with PO-miR-21-ODNs and PS-miR-21-ODNs at a concentration of 0.01–1 μM resulted in only a 25–30% inhibitory effect after 24 h (Fig. 24). These data are in complete agreement with previously published experimental results suggesting that PO-ONs and PS-ONs exhibit very low levels of target miRNA inhibition (36, 37). The inhibitory effect of μ-miR-21-ODNs was much more significant. PCR results showed a dose-dependent decrease in miR-21 level, which reached 60% at 24 h after transfection (Fig. 24).

To establish whether the effect of μ-ODNs on miR-21 level is translatable to other miRNA targets, the potency of two other μ-ODNs targeted to miR-17 and miR-155 was studied. Transfection of B16 cells with μ-miR-17-ODNs and μ-miR-155-ODNs showed a relatively similar performance as with miR-21 (Fig. 24). The maximum inhibitory effect of these μ-ODNs was observed at a similar concentration range as for μ-miR-21-ODNs (50–100 nM) and reached 50%. Thus, the inhibitory effect of specific μ-ODNs was confirmed for several miRNA targets.

Effects of miRNA-Specific μ-ODNs and PS-ODNs on Tumor Cell Proliferation. OncomiR-21 participates in the processes involved in the maintenance of tumor cell proliferation and survival (34, 35). Thus, a decrease in miR-21 level should reverse the malignant behavior of tumor cells and in particular inhibit cell growth, induce apoptosis, and diminish the invasive and migratory properties of the cells. We examined the ability of B16 melanoma cells to proliferate after transfection with ONs (100 nM or 200 nM using Lipofectamine 2000) in real time using the xCELLigence cell analysis system (ACEA Biosciences). Treatment of cells with μ-miR-21-ODNs (100 nM) led to a significant inhibition of cell proliferation (Fig. 28, Left). At 72 h after transfection, the retardation of tumor cell growth amounted to 55% compared with the control cells (P = 0.0047) and 20% compared with control μ-luc-ODNs (P = 0.0161) (Fig. 28, Left), confirming that the suppression of proliferation is associated with μ-miR-21-ODN-mediated inhibition of miR-21. By 115 h, the antiproliferative effect of μ-miR-21-ODNs compared with μ-luc-ODNs rose to 32% (P = 0.0029). In the same period, PS-miR-21-ODNs caused a 65% inhibition of proliferation compared with the control cells (P = 0.0020) and only a 19% inhibition compared with control PS-luc-ODNs (P = 0.0046) (Fig. 2B, Left).

Taking into account that PS-ODNs cause only a 25% decrease in miR-21 level, these data raise the issue of considerable toxicity of this type of modified ON. With the rise of the concentration of ONs to 200 nM, the growth rate of cells transfected with nonspecific μ-luc-ODNs decreased only insignificantly (by 15% compared with a concentration of 100 nM) with the preservation of a specific effect of μ-miR-21-ODNs (average 30%), while the proliferative activity of cells incubated with PS-luc-ODNs dropped dramatically (by 85% compared with a concentration of 100 nM), with almost complete elimination of the specific effects of PS-miR-21-ODNs (Fig. 2B, Right).

Effects of miRNA-Specific μ-ODNs and PS-ODNs on Apoptosis Induction in Tumor Cells. To test whether μ-miR-21-ODNs inhibit cell proliferation through induction of apoptosis, the signs of apoptosis in B16 melanoma cells after treatment with ONs (100 nM) were assessed using Annexin V/flow cytometry analysis. Transfection of μ-miR-21-ODNs significantly increased the cell apoptosis rate; by 48 h after transfection, the percentage of apoptosis in B16 cells amounted to 17%, a threefold increase compared with intact cells, cells incubated with Lipofectamine 2000, and control μ-luc-ODNs (Fig. 2C). Thus, the specific inactivation of miR-21 mediated by μ-miR-21-ODNs triggers apoptosis in tumor cells. A study of the similar effects of PS-miR-21-ODNs showed that the increased proportion of apoptotic cells is mainly due to the nonspecific toxic effect of this chemical modification (PS-miR-21-ODNs, 27% apoptotic cells; control PS-luc-ODNs, 23% apoptotic cells) (Fig. 2C).

Influence of μ-miR-21-ODNs on the Migration Activity of Melanoma B16 Cells. The migration potential of B16 melanoma cells after treatment with μ-miR-21-ODNs and PS-miR-21-ODNs (100 nM) was evaluated by a scratch assay. As shown in Fig. 3, μ-miR-21-ODNs promoted a significant reduction in the migration activity of melanoma cells; approximately 1% of the initial scratch was filled with melanoma cells after 24 h, and even after 72 h, the healing of scratch area did not exceed 5%. The wound healing rate was reduced by 19-fold and by 6-fold compared with the control and μ-luc-ODNs, respectively, after 72 h (Fig. 3B). In contrast, PS-miR-21-ODNs inhibited cell migration to a lesser extent; the level of wound healing was 12% after 24 h and 21% after 72 h, and only fivefold and twofold decreases in the rate of migration were observed compared with control and PS-luc-ODNs, respectively (Fig.

### Table 1. ONs used in the study

| Name          | ON sequence (5'→3') | Calculated mass | Found mass |
|---------------|---------------------|-----------------|------------|
| PO-miR-21-ODN | TCACACATCGTCTGATAAGCTA | ~ | ~ |
| PO-luc-ODN    | TCACACATCGTCTGATAAGCTA | ~ | ~ |
| PS-miR-21-ODN | TCACACATCGTCTGATAAGCTA | 7,039.52 | 7,038.01 |
| PS-luc-ODN    | TCACACATCGTCTGATAAGCTA | 7,049.52 | 7,046.59 |
| μ-miR-21-ODN  | GGAACTCGAGCTGTTGATTAAGCTA | 8,321.57 | 8,323.58 |
| μ-mir-17-ODN  | GGAACTCGAGCTGTTGATTAAGCTA | 8,670.83 | 8,669.40 |
| μ-mir-155-ODN | GGAACTCGAGCTGTTGATTAAGCTA | 8,631.84 | 8,630.45 |
| μ-luc-ODN     | GGAACTCGAGCTGTTGATTAAGCTA | 8,331.57 | 8,332.68 |

3. PS linkage; 4, mesyl phosphoramidate linkage.

The luc sequence serves as a control and encloses a fragment of the firefly luciferase gene, which is not found in the mammalian genomes.
and SI Appendix, Fig. S2). It should be stressed that lower suppression of migration activity of B16 cells by PS-miR-21-ODNs can be linked to the general cytotoxic effects of PS-ONs.

Discussion

Antisense technology has been used as a highly specific technique for regulating gene expression since the 1970s. Recently, antisense technology has been applied to studying miRNAs and modulating the activity of some of them.

The most commonly used chemical technique for PS modification is the 2-cyanoethyl phosphoramidite method (38), in which the iodine oxidation of the intermediate phosphite triester to phosphotriester is replaced with the sulfurization reaction promoted by a range of sulfurizing reagents (39, 40). An alternative reaction that has been applied to produce P-modified ONs is the Staudinger reaction, which until recently was used only rarely for internucleotidic phosphate modification (41, 42). Several examples of ODNs replacing one or more phosphates with arenesulfonyl phosphoramidate groups have been published previously (43, 44). In those studies, the Staudinger reaction was successfully used in the solid phase between 2-cyanoethyl phosphite triester generated by phosphoramidite coupling and a corresponding arenesulfonyl azide. However, no attempt was made to assess the RNA affinity, nuclease stability, or RNase H-activating capacity of the ODNs obtained to ascertain their potential as antisense therapeutics targeting biologically important RNAs.

Here we describe a type of anti-miR ODNs with the mesyl (methanesulfonyl) phosphoramidate internucleotidic group, designated μ-ODNs. The biological properties of the oligomers were thoroughly investigated and compared with those of known PS-ODNs. We show that the μ-ODNs possess hybridization properties similar to those of the natural DNA, while the PS-ODNs demonstrate significantly less avid binding to the miRNA target. It was long assumed that fully modified phosphoramidate ODNs may have undesirable distortions in their duplexes with RNA and, consequently, lower binding efficiency than the natural ODNs (45). However, the μ-ODNs evaluated in this study possess nearly equal binding affinity to that of natural ODNs.

Another beneficial characteristic of AONs is their ability to recruit intracellular RNase H. Most of the chemically modified ODNs developed so far do not have RNase H-activating ability. For instance, RNA duplexes with fully modified 2′-OMe or 2′-MOE RNAs, or LNAs, are not RNase H substrates (46). Although some of these modifications, such as boranophosphate ODNs and 2′-fluoro-2′-deoxy-arabino ODNs, exhibit RNase H-activating ability...
(46, 47), only PS-ODNs have been applied to an appreciable degree. The RNase H-mediated degradation of miR-21 in a duplex with PS-ODNs occurs more efficiently than in a duplex with natural ODNs, consistent with previous studies (48). However, an essential finding of the present study is that a μ-ODN was able to elicit RNase H activity just as well. The initial amount of miR-21 degradation in a duplex with μ-ODNs is approximately 2.5-fold higher than that in duplexes with PO- or PS-ODNs. Currently, only the 5′-O-methylphosphonate modification appears to demonstrate comparable ability (49), although it has not yet been widely used in antisense studies.

One of the main incentives for chemical modification of AONs is the enhancement of their resistance to nuclease digestion. μ-ODNs have outstanding stability and remain unchanged in the presence of serum for at least 168 h. In comparison, 2′-OMe RNAs remained intact under the same conditions for no longer than 2 h (50). The introduction of 2′-deoxy-2′-fluoro-d-arabinonucleotides into the structure of a DNA aptamer led to an increase in $\tau_{1/2}$ of up to 9.5 h (50). Depending on the length and composition of the ON, the most active LNA mixmers containing alternating 2′-deoxy and LNA nucleotides have a $\tau_{1/2}$ of 15−17 h (51). PS modification is considered one of the most stable modifications to nuclease degradation (50). We found a $\tau_{1/2}$ of PS-ODNs of approximately 96 h, which is quite high but still significantly less than that of μ-ODNs.

We have shown that along with higher binding affinity, nuclease resistance, and RNase H recruitment, antisense μ-ODNs exhibit broad-ranging effects on tumor cells. We have determined that anti-miRNA μ-ODN suppresses cancer cell growth similarly to LNA or 2′-OMe RNA (52, 53). We also observed that μ-ODNs proved to be a potent inhibitor of migration of cancer cells that promoted a 19-fold decrease in their mobility. This is fourfold greater than the effect of the isosequential PS-ODNs and almost sevenfold greater than the activity of commercial chemically modified ONs targeted to miR-21, which cause an approximate twofold to threefold decrease in tumor cell migration (54). Along with efficient reduction of the proliferative and migration potential of melanoma cells, application of μ-ODNs led to a threefold increase in the proportion of apoptotic cancer cells, which is nearly equal to the activity of LNA and 2′-OMe RNA (53) but twofold lower than that of PS-ODNs. However, the effects of PS-ODN could be ascribed to the higher general cytotoxicity of this type of modification (22–30).

We have observed that the main drawbacks of PS-ODNs that limit their application in vivo, such as higher toxicity, reduced specificity, and lower target affinity, can be overcome in the μ-ODNs. Moreover, we found that μ-ODNs show greater nuclease resistance than PS-ODNs, as well as an ability to activate RNase H exceeding that of the natural DNA and very similar to that of PS-ODNs. Moreover, our preliminary data suggest that the toxic effects of μ-ODNs on liver and kidneys in mice could also be significantly lower than the toxic effects of the corresponding PS-ODNs. These results confirm the ability of μ-ODNs to inhibit key processes of carcinogenesis in cells.

Our data lead us to conclude that μ-ODNs can be an attractive alternative to PS-ODNs as a potent tool in antisense applications and in other cases to prevent the synthesis and expression of RNAs. We hypothesize that the μ-modification in the context of 2′-OMe or 2′-MOE RNA, LNA, or another non-RNase H-supporting backbone may be suitable for steric block antisense approaches, such as splice modulation. Furthermore, the mesyl phosphoramidate group could be potentially applicable for partial modification of synthetic guide RNAs in CRISPR/Cas9-based techniques to improve the efficiency and specificity of genome editing. However, unfolding the full potential of such DNA analogs will require a thorough and comprehensive study of μ-ODN efficiency and toxicity at the organism level.

Materials and Methods

**ONs.** MiR-21 5′-r(UAGCUAUAGACAGUGAUAUAU) was kindly provided by Maria Meschaninova, ICBFM SB RAS. For the synthesis, isolation, purification and analysis of anti-miR-21-μ-ODN 5′-d(TC\textsuperscript{3}CA\textsuperscript{3}CA\textsuperscript{3}G\textsuperscript{3}T\textsuperscript{3}C\textsuperscript{3}G\textsuperscript{3}A\textsuperscript{3}T\textsuperscript{3}A\textsuperscript{3}G\textsuperscript{3}C\textsuperscript{3}T\textsuperscript{3}P\textsuperscript{3}A) (μ-miR-21-ODN) and other μ-ODNs, anti-miR-21-PS-ODN 5′-d(TC\textsuperscript{3}CA\textsuperscript{3}CA\textsuperscript{3}A\textsuperscript{3}C\textsuperscript{3}G\textsuperscript{3}T\textsuperscript{3}C\textsuperscript{3}G\textsuperscript{3}A\textsuperscript{3}T\textsuperscript{3}A\textsuperscript{3}G\textsuperscript{3}C\textsuperscript{3}T\textsuperscript{3}P\textsuperscript{3}A) (PS-miR-21-ODN) and 5′-d(TC\textsuperscript{3}CA\textsuperscript{3}CA\textsuperscript{3}C\textsuperscript{3}G\textsuperscript{3}T\textsuperscript{3}C\textsuperscript{3}G\textsuperscript{3}A\textsuperscript{3}T\textsuperscript{3}A\textsuperscript{3}G\textsuperscript{3}C\textsuperscript{3}T\textsuperscript{3}P\textsuperscript{3}A) (PS-luc-ODN), where P represents the PS group, were used (Table 1 and SI Appendix, Materials and Methods).

**5′-RNA Labeling.** The 5′-end labeling of miR-21 was carried out as described previously (55) (SI Appendix, Materials and Methods).

**Transfection of Tumor Cells with ONs.** Mouse B16 melanoma cells were transfected with 0.01−1 μM ONs (Table 1) precomplexed with Lipofectamine 2000 (Invitrogen) in Opti-MEM medium according to the manufacturer’s instructions (SI Appendix, Materials and Methods).

**Quantitative PCR.** At 24 h after transfection, total RNA was isolated from the cells using TRizol reagent (Invitrogen) according to the manufacturer’s protocol. Expression of miRNA in B16 cells was analyzed using stem-loop PCR technology (56, 60, 61) (SI Appendix, Materials and Methods).

**xCELLigence Real-Time Analysis of Cell Proliferation and Cytotoxicity.** Proliferation experiments were performed using the xCELLigence real-time cell analysis system (ACEA Biosciences). Mouse B16 melanoma cells were transfected with ONs, and the cell index was monitored every 30 min for the 144-h duration of the experiment (SI Appendix, Materials and Methods).

**Annexin V-FITC/PI Apoptosis Assay.** The Annexin V-FITC Apoptosis Detection Kit (ab14085; Abcam) was used to evaluate the apoptosis of melanoma B16 cells after transfection with ONs (SI Appendix, Materials and Methods).

**Scratch Assay.** B16 melanoma cells were transfected with ONs followed by scratching of the wounds (SI Appendix, Materials and Methods). The migration area was estimated as the ratio of the area filled with cells to the initial scratch area after 24, 48 and 72 h. The migration rate of the cells was estimated as the degree of wound healing and calculated according to the formula $\omega = \frac{1}{x} \times 100\%$, where $x$ is the ratio of the scratch width at 24, 48, and 72 h to the scratch width at 0 h.

**Statistics.** The data obtained were statistically processed using one-way ANOVA and Tukey’s post hoc test ($P \leq 0.05$). The statistics package STAT- TICA version 10.0 was used for this analysis.

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