Article

Stereochemistry Enhances Potency, Efficacy, and Durability of *Malat1* Antisense Oligonucleotides In Vitro and In Vivo in Multiple Species

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Purpose: Antisense oligonucleotides have been under investigation as potential therapeutics for many diseases, including inherited retinal diseases. Chemical modifications, such as chiral phosphorothioate (PS) backbone modification, are often used to improve stability and pharmacokinetic properties of these molecules. We aimed to generate a stereopure *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) antisense oligonucleotide as a tool to assess the impact stereochemistry has on potency, efficacy, and durability of oligonucleotide activity when delivered by intravitreal injection to eye.

Methods: We generated a stereopure oligonucleotide (MALAT1-200) and assessed the potency, efficacy, and durability of its *MALAT1* RNA-depleting activity compared with a stereorandom mixture, MALAT1-181, and other controls in in vitro assays, in vivo mouse and nonhuman primate (NHP) eyes, and ex vivo human retina cultures.

Results: The activity of the stereopure oligonucleotide is superior to its stereorandom mixture counterpart with the same sequence and chemical modification pattern in in vitro assays, in vivo mouse and NHP eyes, and ex vivo human retina cultures. Findings in NHPs showed durable activity of the stereopure oligonucleotide in the retina, with nearly 95% reduction of *MALAT1* RNA maintained for 4 months postinjection.

Conclusions: An optimized, stereopure antisense oligonucleotide shows enhanced potency, efficacy, and durability of *MALAT1* RNA depletion in the eye compared with its stereorandom counterpart in multiple preclinical models.

Translational Relevance: As novel therapeutics, stereopure oligonucleotides have the potential to enable infrequent administration and low-dose regimens for patients with genetic diseases of the eye.

Introduction

Antisense oligonucleotides can bind to RNA via complementary base pairing and promote RNase H–mediated degradation of a target RNA, thereby reducing protein expression.[1] Because unmodified oligonucleotides have limited in vitro and in vivo stability and poor pharmacokinetic (PK) properties due to sensitivity to enzymatic degradation, chemical modifications are often used to produce oligonucleotides suitable for therapeutic use.[1–3] Oligonucleotides are amenable to chemical modification at multiple locations (e.g., nucleobase, phosphodiester backbone, sugar),[1] which can improve the stability, biodistribution, and cellular uptake of oligonucleotides.[1,4,5]

Modifications to the phosphodiester backbone often create chiral centers.[6] Phosphorothioate (PS) modification of the backbone was one of the earliest and remains one of the most common chiral backbone modifications used in oligonucleotide therapeutics.[3–5] PS modification can improve the in vitro

and in vivo stability of oligonucleotides by making them less susceptible to enzymatic (endonuclease or exonuclease) degradation.[3] However, in PS-modified oligonucleotides, one of the nonbridging oxygen (O) atoms is replaced with a sulfur (S) atom at each linkage of the backbone,[5] adopting either an "*R*p" (R) or "*S*p" (S) orientation (Supplementary Fig. S1A).[4] Traditional oligonucleotide synthesis does not control for *R* or *S* integration at each backbone linkage, yielding a mixture of molecules with a random distribution of backbone stereochemistry (stereorandom, Supplementary Figs. S1B, S1C).[6] By contrast, stereopure oligonucleotides are predominated by a single stereoisomer with precisely controlled chirality of each internucleotide backbone linkage (Supplementary Figs. S1B, S1C).[6] We have previously reported that a 3′-SpSpRp-5′ motif (SSR) in the backbone of a stereopure PS-modified oligonucleotide can enhance RNase H activity in vitro and in vivo.[6,7]

Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1,* denoted *Malat1* in rodents) is a ubiquitously expressed (including expression in eye tissues, e.g., cornea, iris, and retina[8–11]) long noncoding RNA that has been used as a surrogate target to evaluate the impact of chemical modifications to oligonucleotides acting through an RNase H mechanism.[12] Antisense oligonucleotides with constrained ethyl (cEt) modifications that target *MALAT1* were potent in reducing *Malat1* RNA in the mouse eye, although specific tissues within the eye were not evaluated.[12] *MALAT1* sequence is conserved among mammals, so it is possible to identify an oligonucleotide, with no changes to its sequence that can be evaluated across multiple species. *MALAT1* is primarily localized in the nucleus[13]; therefore, reduction of *MALAT1* RNA provides confirmation that an antisense oligonucleotide entered the cell, then entered the nucleus and executed its activity by the expected mechanism of action.[12] For these reasons, we opted to use *MALAT1* as a surrogate ocular target to evaluate whether stereopure oligonucleotide technologies provide benefits over traditional stereorandom technologies in the eye. *MALAT1* has recently emerged as a potential therapeutic target for the treatment of diabetic retinopathy, as depletion of *MALAT1* in diabetic animal models rescued the retina from hyperglycemia-induced degeneration.[10,11]

There are significant unmet needs in many ocular diseases, including difficulty with drug delivery, toxicity, and a lack of understanding of the mechanisms of various diseases.[14] While intravitreal (IVT) injections are an established and effective route of administration,[15] patients with ocular disease and their caregivers have reported considerable treatment burden from multiple IVT injections; thus, reducing injection frequency could help lower the burden of therapy and enhance disease management by improving compliance.[16] Advances in antisense oligonucleotides (e.g., IVT fomivirsen was approved by US Food and Drug Administration in 1998 for treatment of cytomegalovirus retinitis)[17] may offer the opportunity to treat chronic ocular diseases with infrequent IVT injections while minimizing toxicity.[14,18] Especially in inherited retinal diseases (IRDs), antisense oligonucleotide administration via IVT injection could be a strong approach to correct the consequences of various mutations at the pre-mRNA level.[19]

Here we applied our previously reported SSR motif[6] to generate a stereopure oligonucleotide that can promote RNase H–mediated degradation of *MALAT1* (MALAT1-200). We aimed to assess the in vitro and in vivo potency and efficacy as well as in vivo durability of the stereopure oligonucleotide in comparison to a stereorandom control to evaluate the potential application of stereopure oligonucleotides in ophthalmologic diseases, especially in IRDs.

Methods

Detailed methods are available as Supplementary Materials.

Results

Stereopure Oligonucleotides Targeting *Malat1* Were More Potent Than Stereorandom Oligonucleotides In Vitro

Structures of stereorandom (MALAT1-181) and stereopure (MALAT1-200) oligonucleotides used in this study are shown in Figure 1A. Although the design of stereorandom and stereopure oligonucleotides has been described previously,[6] we provide a brief recap in Supplementary Figure S1. The stereopure oligonucleotide targeting *Malat1* possesses the same sequence and 2′-ribose modifications as the stereorandom oligonucleotide but differs in backbone stereochemistry. Because of this difference, the stereorandom oligonucleotide is a mixture of over 65,000 stereoisomers, and the stereopure oligonucleotide is predominately a single isomer (Supplementary Fig. S1). The initial velocity (V_0) for RNase H observed with the stereopure oligonucleotide (16.3 nM/s) was approximately twofold higher than that observed with the stereorandom oligonucleotide (8.3 nM/s),
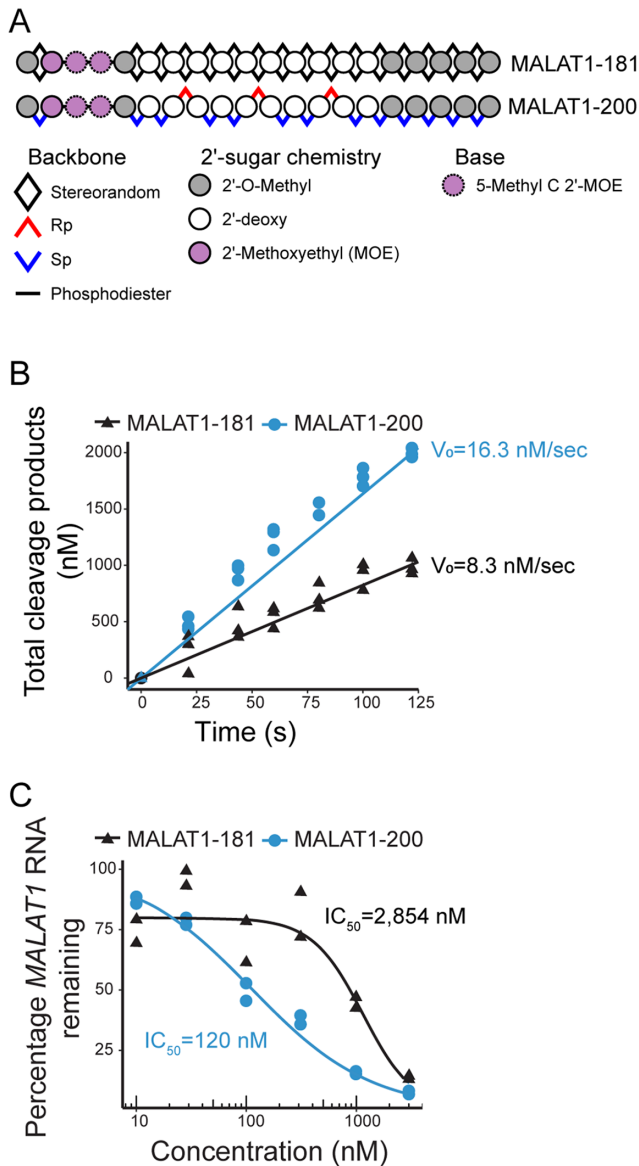
A

B

C

Figure 1. Stereopure oligonucleotide (MALAT1-200) is more potent than a sequence- and chemistry-matched stereorandom oligonucleotide control in vitro. (A) Schematics of stereorandom (MALAT1-181) and stereopure (MALAT1-200) oligonucleotides are shown. The backbone stereochemistry differs, but the sequences and the chemical modifications are identical. MALAT1-181 is a mixture of over 65,000 stereoisomers (Supplementary Fig. S1 and Supplementary Table S1). (B) Time-dependent activity of RNase H1 in vitro on heteroduplexes formed between a complementary *Malat1* RNA and the stereopure (MALAT1-200) or stereorandom (MALAT1-181) oligonucleotide. Initial velocities (V_0) were calculated from the slopes of the lines ($n = 3$ per time point). (C) Relative expression of *MALAT1* in iCell neurons after treatment with increasing concentrations of stereorandom or stereopure oligonucleotide. Half-maximal inhibitory concentrations (IC_{50}s) were calculated from the best-fit curves ($n = 2$ per treatment concentration).

indicating that the stereopure oligonucleotide generated more cleavage products at any given time and more total cleavage products over time than the stereorandom oligonucleotide (Fig. 1B). For in vitro evaluation, we used iCell neurons. This testing system allows for gymnotic (free uptake) delivery of oligonucleotides to neurons, which provides a better representation of what would happen in vivo than testing systems that require a delivery agent. Typically, in vitro nanomolar half-maximal inhibitory concentration (IC_{50}) ranges provide in vivo–active molecules. The IC_{50} in iCell neurons was determined by assessing the percentage of remaining *MALAT1* RNA as a function of the concentration of stereorandom or stereopure oligonucleotides under gymnotic conditions. The observed IC_{50} for RNase H–mediated *MALAT1* RNA degradation with the stereopure oligonucleotide (120 nM) was ~24-fold lower than that observed with the stereorandom oligonucleotide (2864 nM), indicating a potency shift for the stereopure oligonucleotide (Fig. 1C).

Potency and Efficacy Improvements Observed In Vivo in Mouse Eye

To further evaluate the relative activities of stereopure and stereorandom oligonucleotides in the eye, we assessed their activities in the mouse eye 1 week after a single IVT injection (Fig. 2A). Mice received phosphate-buffered saline (PBS) or different doses (5 µg, 15 µg, and 50 µg) of nontargeting control (NTC), stereorandom oligonucleotide, or stereopure oligonucleotide, and the expression of *Malat1* RNA in the anterior portion (containing cornea, lens, and iris) or posterior portion (containing retina, choroid, and sclera) of mouse eye was assessed (Fig. 2A).

Overall, we detected an effect of treatment independent of dose ($P < 0.001$), an effect of dose ($P < 0.001$), and an effect of treatment at the same dose ($P < 0.001$, three-way analysis of variance [ANOVA]). In addition, we also detected an effect on portion of the eye ($P < 0.001$), with oligonucleotides being more active in the posterior portion than the anterior portion. Both stereopure and stereorandom oligonucleotides significantly decreased *Malat1* RNA levels compared with NTC in the anterior (MALAT1-181: 5 µg, $P < 0.05$; 15 µg, 50 µg, $P < 0.001$; MALAT1-200: 5 µg, 15 µg, 50 µg, $P < 0.001$, three-way ANOVA with Tukey's multiple comparison test) and posterior (MALAT1-181: 5 µg, 15 µg, 50 µg, $P < 0.01$; MALAT1-200: 5 µg, 15 µg, 50 µg, $P < 0.001$, three-way ANOVA with Tukey's multiple comparison test) portions of the mouse eye 1 week after a single IVT injection at all doses tested.

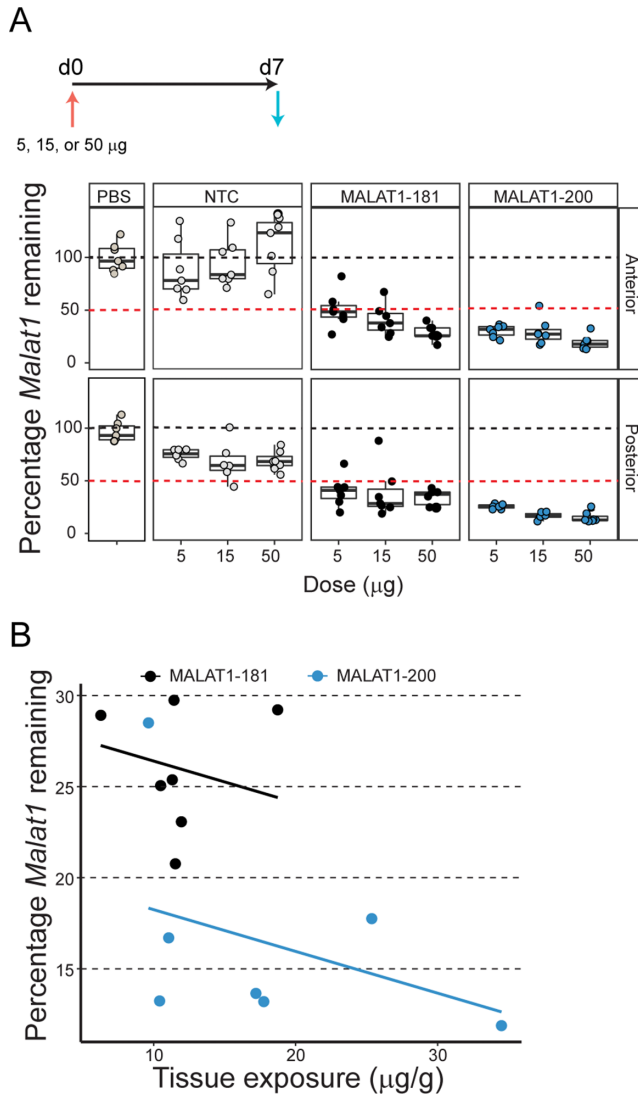At all doses tested, the stereopure oligonucleotide led to a lower percentage of *Malat1* RNA expression

A

B

Figure 2. Stereopure oligonucleotide (MALAT1-200) is more efficacious and more potent than the stereorandom control in vivo in mouse eye. (A) Dosing regimen for 1-week mouse study, with mice receiving a single IVT injection on day 0 (d0, *red arrow*) and with sample collection 1 week later (d7, *blue arrow*). In vivo expression of *Malat1* in anterior (*top row*) or posterior (*bottom row*) portions of mouse eyes 1 week after treatment with the indicated oligonucleotide (*top* of graph, dose indicated on x-axis) or PBS. $n \leq 7$ depending on treatment; each point represents one treated eye. The *red dotted line* demarcates 50% *Malat1* expression. (B) PK-PD relationships for stereorandom (50 µg) and stereopure (50 µg) oligonucleotides in the posterior portion of the eye at 1 week are shown. The percentage of *Malat1* remaining is plotted with respect to the concentration of oligonucleotide detected in the tissue ($n = 7$); each point represents data from one treated eye.

compared with the stereorandom oligonucleotide in both the anterior (5 µg: 30.1% and 50.4%; 15 µg: 29% and 40.7%; 50 µg: 19.3% and 28.5%, respectively) and posterior (5 µg: 25.6% and 40.2%; 15 µg: 16.8% and 38.7%; 50 µg: 15.3% and 34.3%, respectively) portions of the mouse eye, indicating that the stereop-

ure oligonucleotide was more efficacious than the stereorandom oligonucleotide. Additionally, knockdown achieved with 15 µg stereopure oligonucleotide was significantly greater than that with 50 µg stereorandom oligonucleotide ($P < 0.01$), suggesting the stereopure oligonucleotide is threefold or more more potent in vivo 1 week after IVT injection. Therefore, the stereopure oligonucleotide is more efficacious and potent than the stereorandom oligonucleotide, particularly in the posterior portions of mouse eye.

We also evaluated the PK-pharmacodynamic (PD) relationships (i.e., the relationship between the concentration of oligonucleotide detected in the posterior portion and the impact on *Malat1* RNA expression) for 50-µg doses (Fig. 2B). The stereopure oligonucleotide was more active than the stereorandom oligonucleotide (treatment effect, $P < 0.05$; analysis of covariance [ANCOVA]). We also observed greater tissue exposure with the stereopure oligonucleotide than with the stereorandom (exposure effect, $P < 0.05$; ANCOVA). Moreover, greater knockdown of *Malat1* RNA was observed even at lower tissue exposures with stereopure than with stereorandom oligonucleotides, indicating that the increased activity of the stereopure oligonucleotide is driven predominately by an efficacy gain rather than an increase in tissue exposure.

Stereopure Oligonucleotide Yielded Durability Benefit in Mouse Eye

After establishing the potency and efficacy of the stereopure oligonucleotide in the mouse eye, we evaluated the durability of *Malat1* RNA knockdown induced by these oligonucleotides. Because data generated 1 week postdose provided evidence of a potency benefit for stereopure oligonucleotide over stereorandom oligonucleotide, we chose to only evaluate the high dose (50 µg) for stereorandom oligonucleotide. Additionally, because 5 µg of stereopure oligonucleotide was as active as 50 µg of stereorandom oligonucleotide, we chose to include a dose an order of magnitude lower (0.5 µg) in the duration of effect experiment. PBS, NTC (50 µg), stereorandom oligonucleotide (50 µg), or stereopure oligonucleotide (0.5 µg, 5 µg, and 50 µg) was administered by single IVT injection. *Malat1* RNA levels were evaluated in the anterior and posterior portions of mouse eyes on days 8, 15, 29, 56, 85, and 168 (Fig. 3A).

If we assume an allele-selective approach, then the goal of an antisense oligonucleotide treatment would be a 50% reduction of the target RNA. Using 50% remaining as a benchmark, 50 µg of stereorandom oligonucleotide and all doses of stereopure oligonucleotide led to more than 50% knockdown of *Malat1*
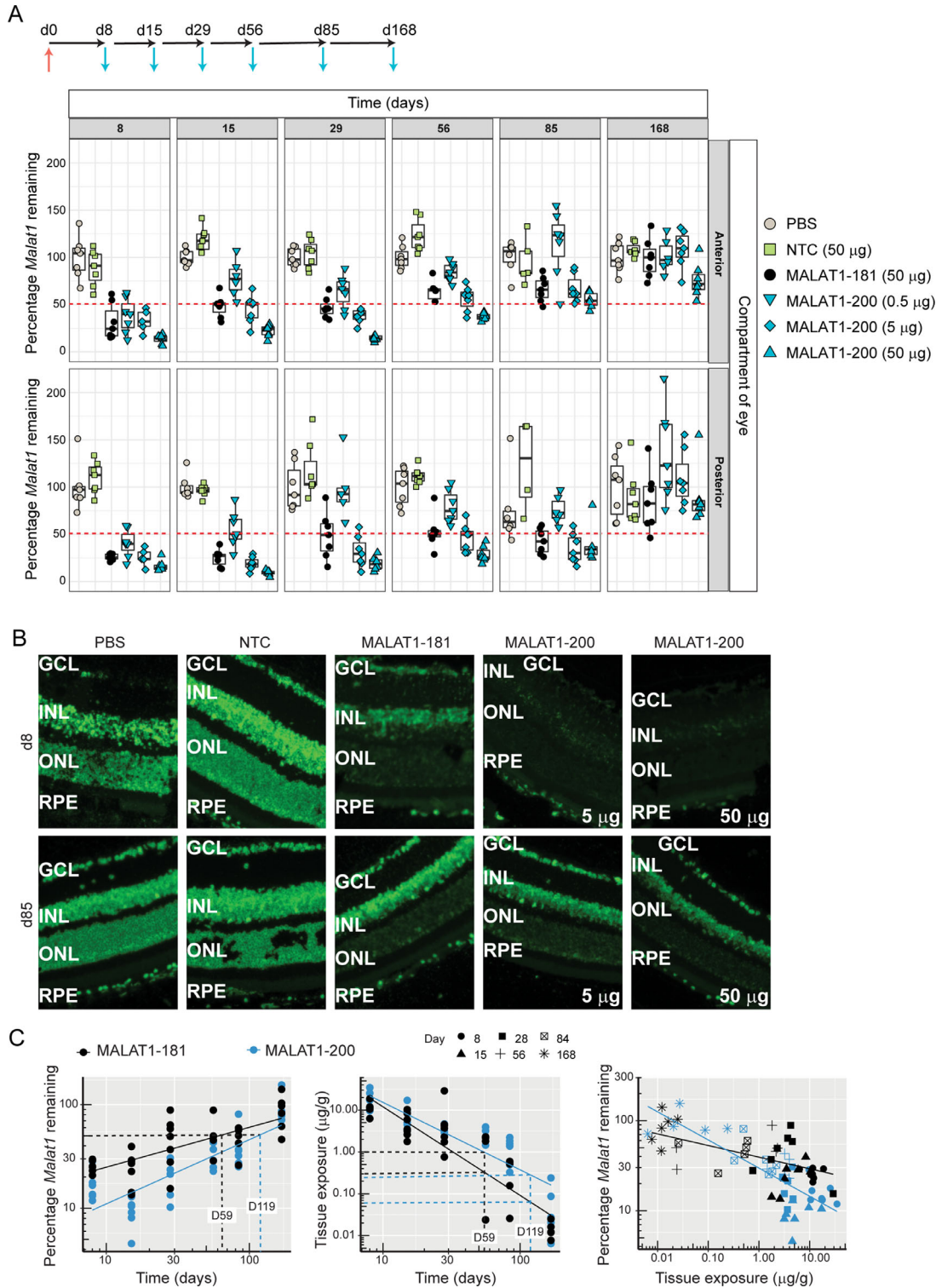
Figure 3. Stereopure oligonucleotide (MALAT1-200) leads to more durable *Malat1* knockdown in the mouse eye than a stereorandom control. (A) Schematic representation of dosing regimen for longer-term mouse study. Mice were dosed on day 0 (d0, *red arrow*) and were evaluated on days 8 (d8, 1 week), 15 (d15, 2 weeks), 29 (d29, 1 month), 56 (d56, 2 months), 85 (Dd85, 3 months), and 168 (d168, 6 months, *blue arrows*). The percentage of remaining *Malat1* RNA after treatment at days 8, 15, 29, 56, 85, and 168 post-IVT injection (time points indicated across *top* of graph). From *left* to *right* within each time point, data for treatment with PBS (*beige*), 50 μg NTC (*green*), 50 μg stereorandom MALAT1-181 (*black*), or stereopure MALAT1-200 at 0.5 μg (*blue inverted triangle*), 5 μg (*blue diamond*), and 50 μg (*blue triangle*) are shown (*n* = 7). Each point represents one treated eye. (B) Visualization of *Malat1* RNA expression (*green*) after treatment with PBS, NTC, 50 μg stereorandom oligonucleotide (MALAT1-181), or stereopure oligonucleotide (MALAT1-200, 5 μg or 50 μg) at days 8 and 85 in

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mouse retina. Treatment is indicated across the *top* of the images; the time points are indicated to the left. (C) Efficacy comparisons between 50 µg stereopure (MALAT1-200) and stereorandom (MALAT1-181) oligonucleotides in posterior portion of eye. *Left*: the percentage *Malat1* remaining over time. Days 59 (*black*) and 119 (*blue*), when MALAT1-181- or MALAT1-200-treated samples return to 50% expression, respectively, are indicated by *dotted lines*. *Center*: oligonucleotide tissue exposure over time, with days 59 and 119 indicated by *dotted lines*. *Right*: the percentage of *Malat1* remaining with respect to oligonucleotide tissue exposure. Data for stereorandom (black) and stereopure (blue) oligonucleotides are shown for all time points.

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at day 8 (∼1 week postinjection) (Fig. 3A). *Malat1* expression in eyes treated with 50 µg of stereorandom oligonucleotide recovered to ∼50% levels by 29 days (∼1 month postinjection) in the anterior and posterior portions of the eye. *Malat1* expression in eyes treated with 0.5 µg of stereopure oligonucleotide recovered to ∼50% levels by 15 days (∼2 weeks postinjection) in the posterior portion of the eye, although recovery was faster in the anterior portion. *Malat1* expression in eyes treated with 5 µg of stereopure oligonucleotide recovered to ∼50% by day 56 (∼2 months postinjection) in the anterior portion and day 85 (∼3 months postinjection) in the posterior portion of the eye. *Malat1* expression in eyes treated with 50 µg of stereopure oligonucleotide recovered to ∼50% by day 85 (∼3 months postinjection) in the anterior portion and >85 days in the posterior portion of the eye (Fig. 3A). Thus, 5-µg and 50-µg doses of stereopure oligonucleotide led to more durable knockdown of *Malat1* RNA than 50 µg of stereorandom oligonucleotide, particularly in the posterior portion of the eye.

Malat1 RNA expression was visualized after treatment with PBS, NTC, stereorandom oligonucleotide (50 µg), or stereopure oligonucleotide (5 µg and 50 µg) at days 8 and 84 postdose in mouse retina. As shown in Figure 3B, *Malat1* is expressed in the ganglion cell layer (GCL), the inner nuclear layer (INL), the outer nuclear layer (ONL), and the retinal pigmented epithelium (RPE). IVT injection of NTC resulted in no qualitative change in *Malat1* RNA expression through 12 weeks postdose compared with PBS-treated samples. By 1 week postdose, 50 µg of stereorandom treatment decreased *Malat1* RNA expression in GCL, INL, and ONL relative to control eyes. Stereopure treatment with either 5 µg or 50 µg resulted in starker reduction of expression in all retinal layers compared with the stereorandom treatment. The 50-µg stereopure treatment also reduced *Malat1* RNA expression in the RPE. At ∼12 weeks postdose, *Malat1* RNA expression in the GCL and INL appeared to recover to control levels, whereas ONL continued to exhibit reduced *Malat1* RNA expression for eyes treated with stereorandom oligonucleotide. The 5-µg stereopure treatment resulted in a similar expression pattern to the 50-µg stereorandom treatment (Fig. 3B), which is consis-

tent with our findings with quantitative polymerase chain reaction (qPCR) (Fig. 3A, day 85). In samples treated with 50 µg of stereopure oligonucleotide, by day 85, *Malat1* RNA expression in GCL and INL recovered; however, *Malat1* expression in ONL remained low relative to other layers within this treatment, as well as across all other treatments. Therefore, the enhanced efficacy and durability of stereopure oligonucleotide may result from sustained activity in the ONL.

When directly comparing efficacy, the 50-µg dose of stereopure oligonucleotide has a longer half-life (20 days) than stereorandom oligonucleotide (15 days) in the posterior portion of the eye. Using 50% *Malat1* remaining as a benchmark, stereorandom oligonucleotide MALAT1-181 lost activity at day 59, while stereopure oligonucleotide maintained at least 50% reduction to day 119 (Fig. 3C, left). Moreover, at day 59, stereopure oligonucleotide had four times greater tissue exposure than stereorandom oligonucleotide (1.08 vs. 0.27 µg/g, respectively). At day 119, the difference was 6.5 times (0.39 vs. 0.06 µg/g, respectively) (Fig. 3C, middle). Considering the PK/PD relationship of both oligonucleotides over time, as shown in Fig. 3C (right), the slope for the stereopure oligonucleotide is steeper than that for stereorandom oligonucleotide, suggesting that the stereopure oligonucleotide achieves greater reduction of *Malat1* RNA across tissue exposures than the stereorandom oligonucleotide over the course of the study.

Potent and Durable Activity Was Observed In Vivo in Nonhuman Primate Eyes

We further tested the potency and durability of stereopure oligonucleotide in nonhuman primate (NHP) eyes following a single IVT injection of 45, 150, or 450 µg. Because our mouse studies analyzed the retina, choroid, and sclera as a single sample, we chose not to do a retinal dissection for this study but rather to process the retina, choroid, and sclera together as in the mouse. The iris and cornea were also collected for analysis. A dose-dependent decrease in *MALAT1* RNA expression in all portions tested of the NHP eye was observed 1 week (8 days) after injection
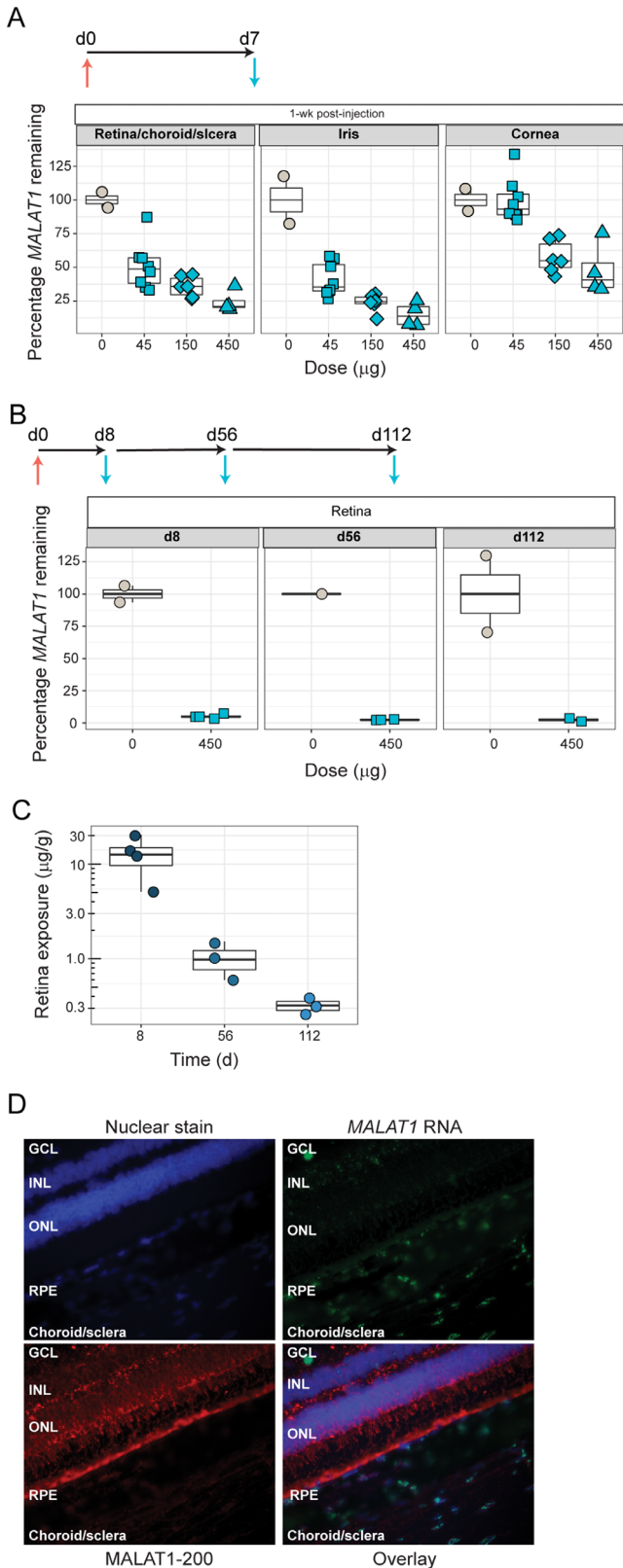
(Fig. 4A). To evaluate the durability of a single 450-µg dose of stereopure oligonucleotide, we measured the *MALAT1* RNA levels at 1 week, 2 months (56 days), and 4 months (112 days) after IVT injection. For this study, we isolated the retina from the choroid and sclera for analysis. At 1 week, 2 months, and 4 months postinjection, *MALAT1* RNA levels in the treated retina were decreased by ~95% compared with PBS-treated control (Fig. 4B). Tissue exposure for the stereopure oligonucleotide was examined at 1 week, 2 months, and 4 months postdose. Retinal exposure was highest (12.7 µg/g) 1 week after IVT injection, declining to 1.03 µg/g at 2 months and 0.39 µg/g by 4 months (Fig. 4C). These PK data suggest that minimal exposure at later time points was sufficient to maintain a durable reduction of *MALAT1* RNA expression. Moreover, we visualized the *MALAT1* RNA expression and stereopure oligonucleotide distribution in retina. At 4 months after the 450-µg injection, stereopure oligonucleotide was detected throughout the retina, including the GCL, INL, ONL, and RPE (Fig. 4D). *MALAT1* RNA was detected at very low levels in the INL, with little to no signal in the GCL or ONL. The choroid and sclera regions showed detectable *MALAT1* RNA levels throughout. The overlaid image (Fig. 4D, last panel) indicated no to very low expression of *MALAT1* RNA across the retina where oligonucleotides were detected. This visualization supports the qPCR data and suggests that the durable reduction of *MALAT1* RNA levels in the retina results from stable distribution of oligonucleotide in the tissue.

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expression at 1 week after treatment with PBS (*beige*, 0 µg) or 45 µg (*blue squares*), 150 µg (*blue diamonds*), or 450 µg (*blue triangle*, dose indicated at the *bottom* of graph) stereopure oligonucleotide in retina/sclera/choroid, iris, or cornea (tissue indicated at the *top* of graph). PBS: *n* = 1 treated eye with technical replicates. Oligonucleotide: 45 µg, *n* = 4 treated eyes with technical replicates for each eye; 150 µg, *n* = 3 treated eyes with technical replicates for each eye; 450 µg, *n* = 2 treated eyes with technical replicates for each eye. (B) Schematic representation of longer-term study in NHPs; animals were dosed (*red arrow*) with a single 450-µg IVT injection on day 0 (d0), and samples were evaluated on days 8 (d8, 1 week later, *blue arrows*), 56 (d56, 2 months later), and 112 (d112, 4 months later). *MALAT1* expression at d8, d56, and d112 after treatment with PBS (*beige*, 0 µg) or stereopure oligonucleotide (*blue*, MALAT1-200) in NHP retina. PBS: *n* = 1 treated eye with technical replicates. Oligonucleotide: 450 µg, *n* = 2 treated eyes with technical replicates for each eye at 1 week and 2 months; *n* = 1 eye with technical replicates at 4 months. (C) From the same longer-term experiment depicted in panel B, MALAT1-200 was quantified in the retina (retina exposure) at days 8, 56, and 112. (D) Visualization of *MALAT1* RNA (*green*) and MALAT1-200 (*red*) at d112 (4 months) in NHP retina after injection of 450 µg stereopure oligonucleotide (as shown in panel B). Nuclear DNA (*blue*) visualized by Hoechst staining.

Figure 4. Stereopure oligonucleotide (MALAT1-200) leads to durable knockdown of *MALAT1* in NHP retina. (A) Schematic representation of 1-week experiment in NHP; animals were dosed (*red arrow*) with a single IVT injection on day 0 (d0) and samples were evaluated on day 7 (d7, *blue arrow*). Percentage *MALAT1* RNA
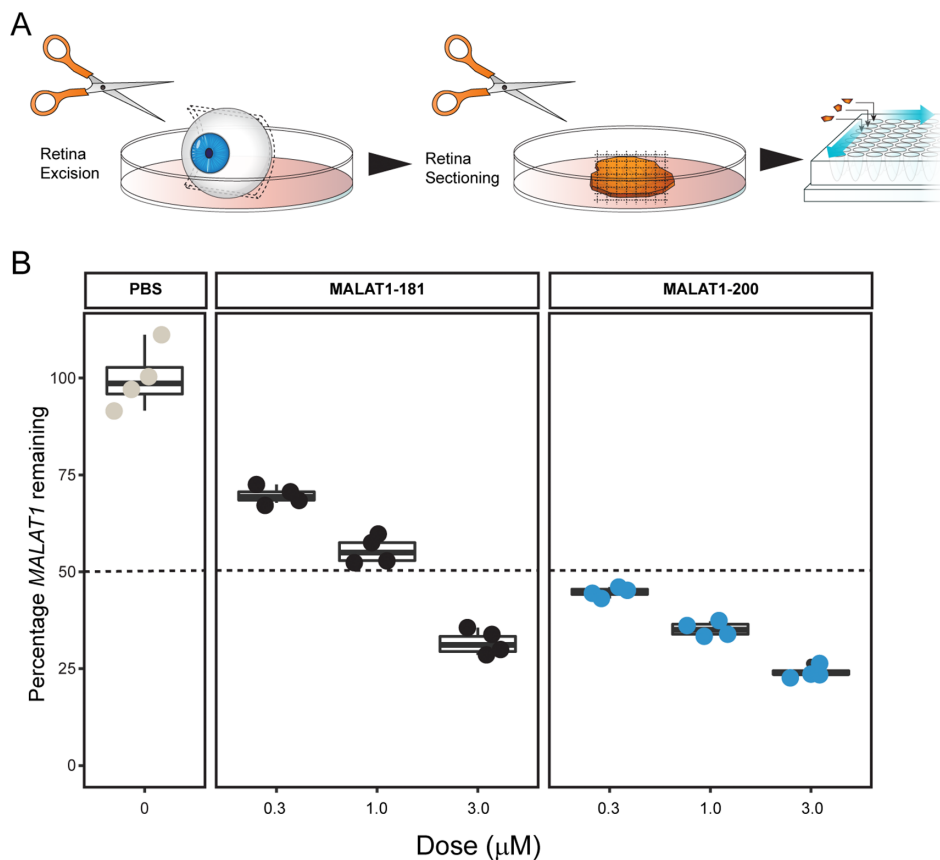
Figure 5. Stereopure oligonucleotide (MALAT1-200) is more efficacious and more potent than a stereorandom control ex vivo in human retinal tissue. (A) Schematic representation of ex vivo cultures generated with human retinal tissue from human donor eyes, showing excision of retina, sectioning of tissue, culture, and treatment under free-uptake conditions. (B) Percentage *MALAT1* expression 48 hours after treatment with PBS (*beige*), stereorandom oligonucleotide (*black*, MALAT1-181), or stereopure oligonucleotide (*blue*, MALAT1-200) in human retina tissue ex vivo ($n = 4$). Treatment is indicated at *top* of graph; concentration of oligonucleotide is indicated at *bottom*.

Stereopure Oligonucleotide Showed Increased Efficacy and Potency in Human Retinal Tissue

After validating the efficacy and durability of the stereopure oligonucleotide in NHP eyes, we tested its activity in ex vivo cultures of human retinal tissue from donor eyes (Fig. 5A). Retinal tissue samples were treated with vehicle, stereorandom MALAT1-181 (0.3, 1, and 3 µM), or stereopure oligonucleotide MALAT1-200 (0.3, 1, and 3 µM) under gymnotic conditions for 48 hours. Overall, there was an effect of treatment independent of dose ($P < 0.001$), an effect of dose independent of treatment ($P < 0.001$), and an effect of treatment at each dose ($P < 0.001$, three-way ANOVA). At 0.3- and 1-µM doses, stereopure oligonucleotide was more active than stereorandom oligonucleotide, leading to a significantly larger decrease in the percentage of remaining *MALAT1* RNA expression (0.3 µM: 44.7% vs. 60.7%, $P < 0.001$; 1 µM: 35.3% vs. 55.4%, $P < 0.001$). In addition, 0.3 µM of stereopure oligonucleotide led to a significantly larger decrease in the percentage of *MALAT1* RNA expression versus 1 µM of stereorandom oligonucleotide ($P < 0.05$). At 1 µM, stereopure activity was not different from that of 3 µM of stereorandom oligonucleotide ($P > 0.05$), indicating the potency and efficacy benefit with stereopure oligonucleotide detected in mice may translate to humans (Fig. 5B).

Discussion

Significant advances in antisense oligonucleotide therapeutics have been made in recent decades, with the development of chemical modifications to improve stability, activate RNase H, increase specificity, and decrease toxicity.[20] Antisense oligonucleotides can be administered systemically (e.g., intravenously and subcutaneously) or locally (e.g., IVT injections for eye diseases or intrathecal injections for central nervous

system diseases),[20] offering flexibility to deliver therapy for multiple disease types and making antisense oligonucleotides ideal candidates as targeted therapies. Here, we assessed the potency, efficacy, and durability of an optimized stereopure oligonucleotide (MALAT1-200) and a more traditional stereorandom oligonucleotide mixture (MALAT1-181) in mouse and NHP eyes via IVT injections. Our results demonstrate a benefit in activity of optimized, stereopure oligonucleotide over the stereorandom oligonucleotide in in vitro assays, in vivo assays in mouse and NHP, and in ex vivo retinal cutures from human eyes. Benefits in potency, efficacy, and durability were noted in the posterior portion (retina, choroid, and sclera) and anterior portion (cornea, lens, and iris) of the mouse eye and in retina, iris, and cornea of NHPs. Slight differences in the degree of activity existed between the posterior and anterior portions of the mouse eye as well as between the retina, iris, and cornea of NHPs. These differences can result from the IVT injection, which is an injection to the back of the eye. Because MALAT1-200 shares the same sequence and chemical modification pattern as MALAT1-181, it represents one stereoisomer of the more than 65,000 stereoisomers that comprise MALAT1-181. These results provide proof of concept, consistent with our prior work,[2] that the identification of stereoisomers with desirable activity profiles can yield benefits over mixtures of randomly generated stereoisomers. Although assessment of the activity of the more than 65,000 stereoisomers that comprise MALAT1-181 is beyond the scope of this work, it is likely that many stereoisomers in that mixture are inactive or poorly active or have undesirable pharmacologic profiles. The elimination of these inactive molecules and the enrichment for a stereoisomer with desirable activity likely explain the in vitro and in vivo activity gains observed with MALAT1-200. Because optimized stereopure oligonucleotides can exhibit improved potency, efficacy, and durability compared with stereorandom mixtures, we believe this technology is potentially suitable for the treatment of genetic diseases, including IRDs.

Considerable treatment burden has been reported from multiple IVT injections by patients with ocular diseases and their caregivers.[16] In our study, the durable activity observed in the posterior portion of the mouse eye suggests the possibility of once-per-quarter treatment. Findings in NHP retina showed even more enhanced durability of activity in retina, with durable knockdown ~95% at 4 months postinjection, suggesting that one or two injections per year may be feasible for stereopure oligonucleotides in the retina. Infrequent IVT injec-tions could reduce the burden of therapy and enhance compliance.[16]

Despite study limitations, including the small sample size for NHP studies and the resulting lack of NTC and stereorandom oligonucleotide control in NHP eyes, as well as the small number of human eyes, the improved activity of the stereopure oligonucleotide observed in vivo in the mouse eye, NHP eye, and ex vivo human retinal cultures provides proof of concept that the stereopure technology offers the opportunity to develop treatments for multiple genetic diseases, especially for IRDs. Antisense oligonucleotides have already been explored in multiple IRD models, including those targeting *CEP290* for Leber congenital amaurosis,[21] *OPA1* for inherited optic neuropathies,[22] *P23H rhodopsin* for retinitis pigmentosa,[23] and *USH2A* for Usher syndrome type 2A.[24] Incorporating the stereopure technology into antisense oligonucleotides targeting these genes may provide enhanced and durable therapeutic benefit.

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