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Preliminary study of a novel transfection modality for *in vivo* siRNA delivery to vocal fold fibroblasts

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Abstract

Objective—An obstacle to clinical use of RNA-based gene suppression is instability and inefficiency of current delivery modalities. Nanoparticle delivery likely holds great promise, but the kinetics and transfection conditions must be optimized prior to *in vivo* utility. We investigated a RNA nanoparticle complex incorporating a “lipitoid” transfection reagent in comparison to a commercially-available reagent.

Study Design—In vitro

Methods—We investigated which variables influence transfection efficiency of lipitoid oligomers and a commercially-available reagent across species, *in vitro*. These variables included duration, dose, and number of administrations, as well as serum and media conditions. The target gene was Smad3, a signaling protein in the Transforming Growth Factor (TGF)- β cascade implicated in fibroplasia in the vocal folds and other tissues.

Results—The two reagents suppressed Smad3 mRNA for up to 96 hours; Lipitoid performed favorably and comparably. Both compounds yielded 60–80% mRNA knockdown in rat, rabbit, and human vocal fold fibroblasts ($p < 0.05$ relative to control). Dose and number of administrations played a significant role in gene suppression ($p < 0.05$). Suppression was more dose-sensitive with Lipitoid; at a constant siRNA concentration, a 50% decrease in gene expression was observed in response to a 5-fold increase in Lipitoid concentration. Increased number of administrations enhanced gene suppression; ~45% decrease between one and four administrations. Neither serum nor media type altered efficiency.

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Conclusion—Lipitoid effectively knocked down Smad3 expression across multiple transfection conditions. These preliminary data are encouraging and Lipitoid warrants further investigation with the ultimate goal of clinical utility.

Level of Evidence—N/A

Keywords

siRNA; transfection; lipitoid; lipofectamine; Smad3; vocal fold; voice; fibroblast

Introduction

siRNA are a family of small double-stranded RNA molecules between 20–25 base pairs in length that associate with complementary nucleotides within target mRNA species, yielding degradation of mRNA and diminished translation.¹ The gene silencing effects of siRNA can modulate a variety of biochemical pathways and, although transient, the process holds significant therapeutic promise across disease states. Beginning with the discovery of RNA interference in mammals,^{2,3} interest has evolved regarding the utility of RNA-based therapeutics.^{4–6} siRNA has several ideal characteristics for localized delivery and gene silencing particularly relevant to laryngeal pathologies; it is temporary, genes are targeted directly, and off-target effects are minimized while high concentrations of siRNA can be maintained at the relevant site.

Although administration of siRNA via peripheral circulation has shown promise for systemic diseases, such administration may also induce significant gene silencing in major non-target organs such as the lung, liver, and spleen, resulting in serious morbidity.⁷ Furthermore, systemic administration could be limited by rapid degradation of siRNAs by nucleases prior to uptake at the target organ.⁸ Confounds of systemic therapy also include the loss of siRNA via urinary output and insufficient penetration into cells in the absence of liposome transfection or electroporation.^{9,10} These factors result in significantly increased costs of systemic siRNA therapy, as exceedingly high concentrations of siRNA are required. Consequently, optimal modalities for direct siRNA treatment and focused delivery are high priorities for treatment of localized disease.

The issue of delivery is particularly germane as a recent review reported that nearly 50% of current, commercially-funded clinical trials using RNA-based therapeutics do not employ any delivery media, and as such, are referred to as “naked” delivery of siRNA.⁴ This approach is problematic, considering that siRNA is vulnerable to rapid hydrolytic cleavage. In addition, siRNA oligonucleotides are resistant to uptake by most cells due to their large molecular weight and anionic charge.¹¹ However, carrier molecules can facilitate movement of siRNA through the cell membrane, thereby increasing transfection efficiency and reducing the effective concentration of siRNA required for therapeutic benefit.^{10,12–17} Therefore, the development of novel transfection reagents to enhance the efficacy of siRNA-based therapeutics has the potential to directly impact patient care paradigms.

Cationic lipids are attractive delivery molecules for nucleic acids as they undergo electrostatic association with polyanionic siRNA oligonucleotides and facilitate

compatibility with the membrane lipid bilayer.^{18–21} This process is similar to lipofection by virtue of a facilitated nucleic acid transport across the hydrophobic cell membrane as part of a nanoparticulate complex. Lipofectamine®, a popular *in vitro* transfection reagent composed of cationic lipid subunits, similarly delivers siRNA via liposome formation resulting in lipid compatibility of the sequestered nucleic acids for delivery across the cell membrane.²² Yet, these carrier molecules are limited to a few commercial reagents, which may have limited clinical utility due to cytotoxicity and an undetermined capacity to effectively deliver siRNA *in vivo*.⁴

To address these issues, our group exploited a class of peptidomimetic oligomers called peptoids. Sequence-specific peptoids displaying cationic side chain groups and conjugated to a phospholipid tail with hydrophobic compatibility are referred to as lipitoids.²³ Lipitoids were initially developed for intracellular plasmid DNA delivery as an alternative to potentially infectious methods of DNA delivery through viral encapsulation,^{24–26} as well as non-viral DNA delivery vehicles typically associated with increased cytotoxicity and decreased transfection efficiency.⁴ Early reports identified a peptoid sequence, subsequently referred to as **Lipitoid**, with particularly favorable properties including high transfection efficiency, resistance to proteolytic degradation and limited cytotoxicity.^{23–27}

Lipitoid features trimeric repeats of cationic and aromatic side chain groups connected to a phosphatidyl moiety presenting myristic acyl C₁₄ chains, all of which enhance interactions with nucleic acids, facilitate intercellular uptake, and reduce non-specific cell adhesion. In contrast to Lipofectamine®, Lipitoid generally does not assemble into liposomes to encapsulate oligonucleotide cargo which may provide more effective siRNA delivery.²⁸ Recently, critical parameters impacting the size and morphology of the siRNA nanometer-scale complexes have been determined for optimal transfection mediated by Lipitoid.²⁸ Lipitoid has been successfully used as a transfection reagent for both plasmid DNA and siRNA, across many types of mammalian cells including primary cell types that have proven to be refractory to traditional transfection methods.²⁹

Ultimately, we seek to effectuate siRNA-based therapies *in vivo* to alter wound healing in the upper aerodigestive tract. In the current study, we sought to provide preliminary, foundational data regarding the effectiveness of Lipitoid *in vitro*. Our laboratory recently described Smad3 as a key biochemical switch underlying the fibrotic phenotype and a potential target for siRNA-based therapeutics.^{30,31} Smad3 is critical to Transforming Growth Factor (TGF)- β signaling which is fundamental to aberrant wound healing in the vocal folds and other tissues. We hypothesized that Lipitoid would provide increased transfection efficiency across multiple species in both primary and immortalized vocal fold fibroblasts. Due to differing mechanisms underlying the delivery of nucleic acids, we sought to evaluate critical differences in dose-sensitivity and number of administrations in order to identify optimal transfection conditions.

Methods

Lipitoid Synthesis

Manual solid phase peptoid synthesis of Lipitoid was conducted according to previously described procedures and the product was purified by high performance liquid chromatography.²³

Cell Lines

Several cell types were employed including an immortalized human vocal fold fibroblast cell line developed in our laboratory and referred to as HVOX.³² Primary rat and rabbit vocal fold fibroblasts were also employed.

Standard Transfection

Cells were grown in 6-well plates to 80% confluency. siRNA and transfection reagent (Lipitoid or Lipofectamine®) were dissolved in 500µL Opti-MEM® (Life Technologies, Carlsbad CA); Opti-MEM is recommended for use with cationic lipid transfection reagents. For standard transfection, siRNA at a concentration of 5µM was combined with 1.00mg/mL Lipofectamine® or 1.07mg/mL Lipitoid. siRNA/lipid solution in reduced serum media (Opti-MEM®) was incubated at room temperature for 20 minutes and 500µL was added to each well containing 1.5mL of Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad CA) with 10% Fetal Bovine Serum (FBS; Life Technologies, Carlsbad CA). The media was then changed to 10% FBS, 1% antibiotic DMEM after six hours. RNA was then harvested at the determined experimental endpoint. Continuous Transfection. Cells and transfection reagents were prepared as described for Standard Transfection. Cells were then treated with transfection media through the duration of the experiment until the determined experimental endpoint (6, 24, 48, and 72 hours). In both the standard and continuous transfection conditions, cells in the control condition were not transfected, but subjected to media changes.

RNA Extraction and Quantification

At the appropriate experimental endpoint, RNA was extracted employing the Qiagen RNeasy Kit (Qiagen, Valencia CA). RNA was quantified using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE) according to the manufacturer's protocol.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

The Taqman RNA-to-Ct 1-Step kit (Applied Biosystems, Grand Island, NY) was used to perform quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Sequences for Smad3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were obtained in the form of Taqman gene expression assays (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) was run on the Applied Biosystems ViiA7 Real-Time PCR System, as recommended by the manufacturer. The Ct method was employed with GAPDH as the housekeeping gene.

Statistical Analyses

All experiments were performed in triplicate, at minimum. Data are presented descriptively as means $\pm$ standard error of the mean (SEM). The dependent variable of interest was subjected to a one way analysis of variance. If the main effect was significant, post hoc comparisons were performed via the Scheffé method. Statistical significance was defined as $p < 0.05$ using StatView 5.0 (SAS Institute, Berkeley, CA).

Results

Transfection Efficiency

Two transfection methods were compared; one in which there was a single administration of reagents for 6 hours (standard transfection), and another in which there was extended period throughout which the cells were exposed to prolonged transfection (continuous transfection). In our human vocal fold fibroblast cell line, standard transfection methods with Lipofectamine® significantly decreased Smad3 expression at 6, 24, 48 and 72 hours following transfection (Figure 1A; $p=0.0001$, 0.0001 , 0.0001 , 0.0092 , respectively). With Lipitoid, Smad3 expression decreased significantly at 6, 24, 48 and 72 hours ($p=0.0001$, 0.0001 , 0.0001 , and 0.0469 , respectively). At 24 hours, Lipitoid yielded enhanced Smad3 suppression when compared to Lipofectamine® ($p=0.0003$).

Under continuous transfection, Smad3 expression significantly decreased with Lipofectamine® at 6, 24, and 48 hours ($p=0.0271$, 0.0001 , and 0.0001 , respectively). Continuous transfection with Lipitoid decreased Smad3 expression at 24 and 48 hours ($p < 0.0001$ for all; Figure 1B). Similar to standard transfection conditions, Lipitoid outperformed Lipofectamine® at 24 and 48 hours ($p=0.0003$ and 0.0058 respectively) under continuous transfection.

Dose Response and Multiple Administrations

siRNA concentration was kept constant, but concentrations of Lipofectamine® and Lipitoid were varied to determine optimal transfection conditions. Continuous transfection for 24 hours decreased Smad3 mRNA expression as a function of increasing concentration (Figure 2A). Lipofectamine® decreased Smad3 expression relative to control at 0.5, 1.0, 1.5, 2.0, and 2.5 $\mu\text{g/mL}$ ($p < 0.0001$ for all). Continuous transfection with Lipitoid yielded decreased Smad3 expression relative to control at 1.0, 1.5, 2.0, and 2.5 $\mu\text{g/mL}$ ($p < 0.0001$ for all).

In order to observe the effects of multiple administrations of transfection reagents, HVOX were treated with 2.5 $\mu\text{g/mL}$ of Lipofectamine® or **Lipitoid** siRNA complex every 24 hours for 96 hours (Figure 2B) under continuous transfection. Multiple administrations yielded increased gene suppression across both reagents. At 96 hours, Smad3 expression decreased to 20% and 27% of control for Lipofectamine® and Lipitoid, respectively. In contrast, with repeated transfections every 24 hours, Smad3 expression decreased at 96 hours to 12% and 9% of control, respectively ($p < 0.0001$ for all).

Optimal Transfection Conditions

The effects of serum content (0% and 10% FBS) and media type (DMEM and Opti-MEM) were investigated with regard to transfection efficiency (Figure 2C and D). Under continuous transfection with Lipofectamine® for 24 hours in DMEM and Opti-MEM, Smad3 mRNA expression decreased to 18% and 27% control, respectively. For continuous transfection for 24 hours in DMEM with 10% FBS and 0% FBS, Smad3 mRNA expression decreased to 14% and 17% of control, respectively ($p < 0.0001$ for all). In DMEM and Opti-MEM, Smad3 expression decreased to 18% and 21%, respectively, and with 10% FBS and 0% FBS, Smad3 expression decreased to 8% and 11%, respectively ($p < 0.0001$ for all). No significant differences were observed when comparing Lipofectamine® with Lipitoid under these conditions. Lipofectamine with Opti-MEM performed significantly better than DMEM ($p = 0.0081$). No differences were observed when comparing the media types with Lipitoid transfection.

Multiple Species Analysis

In addition to experiments conducted in a human cell line, transfection efficiency was quantified in both rat and rabbit primary vocal fold fibroblasts. In rat VFF (Figure 3A), Smad3 mRNA decreased to 40% and 43% of control with Lipofectamine® and Lipitoid, respectively ($p = 0.0003, 0.0002, 0.0077, 0.0006$ for Lipofectamine® at 6–72 hours, respectively; $p = 0.0003, 0.0043, 0.0076, 0.0009$ for Lipitoid at 6–72 hours, respectively). Under continuous transfection (Figure 3B), Smad3 expression decreased to 51% and 45% of control for Lipofectamine® and Lipitoid, respectively, at all time points ($p = 0.0001, 0.0267, 0.0014$ for Lipofectamine® at 24–72 hours, respectively; $p = 0.0145, 0.0001, 0.0015, 0.0001$ for Lipitoid at 6–72 hours, respectively). No differences were observed between Lipofectamine® and Lipitoid in rat VFF.

In rabbit VFFs, Smad3 expression decreased with Lipofectamine® transfection to 31% and 24% of control under standard and continuous transfection, (Figures 3C and D) respectively, at all time points ($p = 0.0001, 0.0001, 0.0001, 0.0082$ under standard transfection at 6–72 hours respectively; $p < 0.0001$ for all time points under continuous transfection). With Lipitoid, Smad3 decreased to 40% and 24% for standard and continuous transfection, respectively ($p = 0.0025, 0.0001, 0.0001$ for standard transfection at 6–48 hours respectively and $p < 0.0001$ at all time points for continuous transfection). Similarly, Lipofectamine® yielded Smad3 suppression in rabbit VFFs under both standard ($p = 0.0047, 0.0025, 0.0001$ for 6–48 hours, respectively) and continuous transfection ($p = 0.0008, 0.0003, 0.0468$ for 6–48 hours, respectively).

Discussion

Vocal fold injury and the complex reparative response often results in clinically significant pathology. Current therapies to alter this tissue response are limited. Globally, we hypothesize that siRNA-based therapeutics hold significant promise in this regard. However, as outlined previously, issues of delivery remain problematic and warrant further investigation. Specifically, we hypothesize that lipid-compatible transfection reagents can facilitate highly-efficient siRNA therapeutics. Transfection reagents are likely to overcome

limitations in delivery of uncomplexed siRNA in a therapeutic setting. However, current transfection reagents are not optimized for *in vivo* use and further investigation is needed to both develop novel reagents and optimize their efficiency in models of disease. To that end, we investigated the transfection efficiency of a peptidomimetic reagent, a lipitoid, in vocal fold fibroblasts across species to provide a foundation for future investigation.

We sought to quantify the efficiency of this lipitoid agent across transfection conditions, specifically, dose, time, and number of applications in both an immortalized human vocal fold fibroblast cell line as well as primary vocal fold fibroblasts from two non-human sources. Ideally, primary human vocal fold fibroblasts would also be employed in this type of preliminary, foundational investigation. However, primary human vocal fold fibroblasts are exceedingly rare given that their acquisition could be associated with significant architectural alteration to the vocal fold. As such, we investigated primary cells from species commonly employed for the acquisition of pre-clinical data regarding both injury and repair in the vocal folds. However, the lack of investigation employing primary human vocal fold fibroblasts may be considered a limitation to overall generalizability. Regardless, two transfection protocols were employed. In the first, referred to as 'standard transfection', the transfection media was changed after six hours and replaced with standard cell culture media. In the second, termed 'continuous transfection', the transfection media was left throughout the duration of experimentation, in some cases, up to 96 hours. These two protocols were selected to observe the duration of gene suppression and to provide context for the potentially-fleeting nature of siRNA therapeutics *in vivo*. Under both standard and continuous transfection conditions, Smad3 expression decreased initially in the presence of either Lipitoid or Lipofectamine®. This effect, however, did not persist with standard transfection regardless of transfection reagent. Continuous transfection yielded persistent Smad3 suppression. These differences in gene expression relative to duration of transfection suggest that both reagents may be continuously active for at least 72 hours, at least in the contrived cell culture environment.

Variability was noted with regard to the Smad3 suppression across species and cell type. The mechanisms underlying these differences are unclear and were not specifically investigated, but may be related to varying metabolic environments across cell types. In rat vocal fold fibroblasts, transfection yielded significant knockdown of Smad3 at 6–24 hours consistent with standard knockdown potential of the two compounds. Interestingly, Smad3 suppression was enhanced in rabbit vocal fold fibroblasts compared to other species. Furthermore, in rabbit cells, Lipofectamine® was more effective at Smad3 knockdown; statistically-significant differences were noted between Lipofectamine® and Lipitoid. This effect was particularly pronounced during continuous transfection. In the human and rat vocal fold fibroblasts, both Lipitoid and Lipofectamine® were equally effective at Smad3 knockdown.

The concentration of Lipofectamine® and Lipitoid in the context of constant siRNA concentrations was correlated with diminished Smad3 suppression. As the relative concentration of transfection reagents was increased, Smad3 expression decreased. However, Lipitoid appeared more responsive to altered dose. These results were consistent with previous transfection studies in which cell viability and target gene suppression were observed to be dependent on the charge ratio between the applied cationic transfection

reagent and the anionic oligonucleotide; optimal results were obtained when this positive/negative charge ratio was maintained at about 3:1.³³ Prior work from the Kirshenbaum laboratory found increased cytotoxicity beyond the 3:1 positive/negative charge ratio, suggesting that Smad3 suppression in this context may be related altered cell health.²⁸

Lipitoid was also more responsive to multiple administrations as a means to maintain an extended time course of Smad3 suppression. These differences may be indicative of differing kinetics between the two reagents, in addition to variable molecular mechanisms of siRNA delivery.²⁸ In order to facilitate the practical application of these compounds *in vivo*, media type and serum concentration were varied. Neither FBS nor media type altered Smad3 suppression. These data may suggest that conditions specific to the chosen cell culture environment are not required for effective transfection.

These data are encouraging with regard to the utility of Lipitoid as a means to effectively deliver siRNA, and suggest that under many conditions, Lipitoid can outperform Lipofectamine® for *in vitro* siRNA transfection. However, optimal suppression of protein expression *in vivo* may necessitate extended exposure to the transfection reagent or repeated transfections, as performed in the current study. In both cases, limited toxicity as well as biocompatibility of the transfection reagent are critical. Although toxicity was not the focus of the current study, we previously demonstrated minimal toxicity in cell culture in response to Lipitoid, in contrast to the observed toxicity of Lipofectamine®.^{27,34,35} These data suggest that Lipitoid may be better suited for sustained dosing. Furthermore, the modular solid phase synthesis of chemically-diverse Lipitoid oligomer sequences facilitate the identification of particular sequence variants that enable optimized siRNA delivery to particular disease tissue types, including the vocal fold. Based on these findings, Lipitoid is likely to prove quite beneficial for siRNA delivery, particularly as the evolution to clinical applications will likely include more demanding protocols and a greater focus on both efficacy and safety at the cellular and organism level.

Conclusion

Delivery of effective concentrations of siRNA to adequately treat pathological processes *in vivo* remains problematic. Transfection efficiency was quite high with Lipitoid as a function of transfection conditions and cell species. Cumulatively, these data are encouraging with regard to the potential utility of this nanoparticle for *in vivo* siRNA delivery. The modular oligomer sequence composition of lipitoids may facilitate variations in physicochemical properties of the transfection reagent to optimize pharmacological attributes and address critical challenges for introduction of siRNA mediated gene silencing to the clinical setting.

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Figure 1.

Smad3 expression as a function of standard transfection where cells were exposed to the transfection media for 6 hours (A) and continuous transfection where cells were exposed to the transfection media for the entire duration at the times indicated (B) methods in human vocal fold fibroblasts (N/S—random siRNA segments; * $p < 0.05$ relative to control/not transfected. # $p < 0.05$ relative to Lipofectamine®/Lipitoid)

Figure 2.

Smad3 expression under 24 hours continuous transfection as a function of lipid concentration (siRNA concentration remains constant) (A), number of dose administrations, (B) presence of FBS (C) and media type (D) (N/S—random siRNA oligonucleotide; * $p < 0.05$ relative to control/not transfected. # $p < 0.05$ Opti-MEM relative to DMEM)

Figure 3. Smad3 expression following siRNA administration under standard (A) and continuous (B) transfection methods in rat Vocal Fold Fibroblasts (VFF) and standard (C) and continuous (D) transfection methods in rabbit VFF. (N/S—random siRNA segments; * $p < 0.05$ relative to control/not transfected. # $p < 0.05$ relative to Lipofectamine®/Lipitoid)