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Engineered microRNA therapeutics

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ABSTRACT Targeting of microRNAs that are overexpressed or replacement of microRNAs whose expression is lost are two distinct and novel approaches to treat disease(s) driven by microRNA dysregulation. This can be achieved by chemical modification of either a single stranded oligonucleotide called an anti-miR or a double stranded nucleic acid molecule termed a microRNA mimic. With hundreds of microRNAs identified and knowledge of their role in disease becoming clearer there is the prospect, over the coming years, to harness engineered microRNA therapeutics to revolutionise the way diseases are treated. Both types of engineered microRNA therapeutics have advanced into clinical development with human proof of concept achieved with an anti-miR targeting miR-122 (one of the most abundant microRNAs in human hepatocytes that is utilised by the hepatitis C virus to enable its function and replication). Rather than targeting individual proteins or enzymes involved in human disease, an opportunity now exists to modulate multiple different proteins/enzymes which act in concert in the progression of disease.

KEYWORDS anti-miR therapeutics, engineered microRNA therapeutics, delivery, microRNA mimics, oligonucleotides

DECLARATION OF INTERESTS Dr Gibson is an employee of Regulus Therapeutics Inc.

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INTRODUCTION

MicroRNAs are small noncoding RNAs usually 22 nucleotides in length. They regulate expression of multiple target messenger RNAs (mRNAs) through sequence specific hybridisation to mostly the 3' untranslated region (UTR) of mRNAs, blocking the translation of the mRNA or facilitating its degradation.[1] MicroRNAs are conserved across species, indicating their evolutionary importance as modulators of critical biological pathways and processes. MicroRNAs recognise mRNA targets through the first 6-8 nucleotides in the 5' portion of the microRNA (the 'seed'). Consequently, a single microRNA is able to regulate multiple mRNAs that share the same sequence complementary to the microRNA 'seed'. Although the effect of a microRNA on individual mRNA targets is in general subtle (change in expression level of twofold or less), the combined effect of a single microRNA on multiple mRNAs is significant and produces measurable phenotypic alterations.[2] The ability of microRNAs to influence an entire network of genes involved in common cellular processes provides tremendous therapeutic potential and differs from the specificity of today's drugs which act on individual cellular targets.

Key questions are being addressed in order that engineered microRNA therapeutics can emerge as new drug candidates, including the following:

1. Can microRNA therapeutics be engineered to have minimal off-target effects and be safe and well tolerated in humans?
2. Is modulation of microRNAs a valid approach to treat human disease?
3. Can engineered microRNA therapeutics be delivered to the relevant target tissues and cell types in sufficient amount to ensure efficacy?

SAFETY AND TOLERABILITY OF ENGINEERED MICRORNA THERAPEUTICS

Engineered microRNA therapeutics are either single stranded oligonucleotides (anti-miRs) or double stranded nucleic acid species (microRNA mimics) with multiple chemical modifications including modified bases, modified ribose(s) and a modified RNA backbone.[3,4] These modifications improve affinity to the target microRNA, increase protein binding, decrease susceptibility to nuclease degradation thus influencing potency, pharmacokinetics and tissue distribution. In any given

20-nucleotide long anti-miR that includes four different chemically modified riboses there are over a billion possible combinations representing the multiple ways that each distinct modified nucleotide can be incorporated into that exact specific sequence. Thus a library of anti-miR therapeutics of identical sequence, but different pattern of modifications, can be synthesised and tested for on-target potency, metabolic stability, protein binding, and off-target effects. At this time the size of screening libraries being evaluated is in the thousands rather than billions. As a result we have limited understanding of the rules that may drive structure activity relationships (SAR) associated with both on-target activity as well as off-target activity.

As with the discovery of small molecules the integration of computer modeling and bioinformatics is enhancing the efficiency in which novel engineered microRNA therapeutics can be discovered and subsequently developed. A series of algorithms has recently been described that can help predict those chemically modified oligonucleotides with increased potential to induce liver injury.[5] A class associated off-target effect of oligonucleotides is to stimulate an immune response that may represent activation of membrane-associated and/or cytosolic nucleic acid sensors such as the toll-like receptors and retinoic acid-inducible gene 1, respectively. Binding of oligonucleotides to these sensors leads to the stimulation of an interferon response.[6-8] Judicious placement of the chemically modified nucleotides within the engineered microRNA therapeutic can either enhance or silence these off-target activities without significantly impacting on-target potency.[7,8] Recent work has suggested that quantum mechanical studies of oligonucleotides can model the molecular structure and electrostatic potential associated with oligonucleotides.[9] Subtle changes in chemical structure or modification pattern can induce a significant change in molecular structure and, by inference, strongly influence the ability to bind to cellular proteins. Such differences may explain why oligonucleotides that have the same sequence but different pattern of modification vary in their ability to trigger off-target effects. Understanding the structural rules (i.e. SAR) by which oligonucleotides can induce immune stimulation or induce liver damage will enhance our ability to design novel libraries of anti-miR therapeutics or microRNA mimics devoid of those effects.

Given the growing interest in microRNA over the past few years, there are now a number of 'tool' anti-miRs readily available and a large number of investigators have used these 'off-the-shelf' reagents in their publications. Unfortunately, it is an under-appreciated fact that many of these reagents have not been optimised and are likely to produce unintended non-specific effects in the systems being investigated.[10] Thus published findings could be misleading unless it is clearly shown that the anti-miR therapeutic being tested is devoid of off-target effects. The problem also extends to the use of mismatch controls. The introduction of a mismatched nucleotide will alter many properties of the engineered microRNA therapeutic in addition to the intended disruption of the Watson-Crick base pairing. To be an appropriate control a mismatched oligonucleotide would need to retain the same biophysical and chemical properties of the active agent including but not limited to protein binding, metabolic stability, level of off-target activity, pharmacokinetics and tissue distribution. It is unlikely that many of the mismatch controls used in the literature have been appropriately designed and thus the conclusions or interpretation of such work, especially those studies reporting in vivo efficacy, should be viewed with caution.

MICRORNAS AS VALID TARGETS IN HUMAN DISEASE

A growing body of literature supports the concept that microRNAs are valid therapeutic targets.[11-24] A number of distinct human disease states could be treated with engineered microRNA therapeutics including multiple liver diseases where hepatocyte function has been dysregulated,[3,19,22] cardiac ischemia and other related complications in the heart,[11,13,16] fibrosis in multiple tissue types,[17,18,25] inflammatory disease states such as rheumatoid arthritis and other inflammatory conditions with macrophage involvement,[15] and in various cancers.[14,20,23,26] For the sake of brevity, miR-122 and miR-21 will be used as examples of validated microRNA targets. In both these examples, the pharmacological efficacy observed with the engineered microRNA therapeutic was consistent with the genetic studies performed in support of target validation.

miR-122 and HCV

miR-122, a microRNA abundantly and specifically expressed in hepatocytes, is a critical host factor for hepatitis C virus (HCV) accumulation.[27,28] miR-122 interacts with HCV by binding to two target sequences in the 5' non-coding region, resulting in stabilisation, replication and translation of the virus.[25,29] Importantly, the miR-122 binding sites are completely conserved in all genotypes and subtypes of HCV.[30] When such microRNA binding sites are mutated, replication of HCV in Huh7.5 cells is eliminated regardless of genotype.[31] These data provide compelling genetic validation that inhibition of miR-122 with chemically modified anti-miR therapeutics could eliminate viral replication. Preclinical and clinical proof of concept has been achieved with miravirsen, a chemically modified anti-miR therapeutic.[32,33] Miravirsen reduced viral titer in chronically HCV-infected chimpanzees[32] and in clinical trials in healthy volunteers miravirsen was well-tolerated.[19] In treatment naïve HCV-infected patients, miravirsen showed up to

a three log reduction in viral load when dosed once a week (for a total of five weeks) at a dose of 7 mg/kg.[19] This pivotal clinical study established proof of the concept that targeting microRNAs in humans could provide a substantial and meaningful therapeutic benefit.

miR-21 and renal fibrosis

A second example of a validated microRNA target is miR-21 in fibrosis. Mice deficient in miR-21 develop far less interstitial fibrosis in response to kidney injury when compared to wild type mice.[25] The genotypic effect observed in miR-21 knockout mice was phenocopied in wild type mice treated with miR-21 anti-miRs.[25] These results suggest that inhibition of miR-21 is a viable therapeutic approach for the treatment of conditions that result in renal fibrosis. In mice, mutations in Col4A3 and Col4A4 have been shown to lead to renal fibrosis, a decline in renal function and premature death with disease pathogenesis which parallels that observed in patients with Alport syndrome.[34,35] miR-21 expression in kidneys is increased early in the progression of renal disease in both these variants of the Col4A3 mutant mouse. In addition to the increased miR-21 expression the Col4A3 mouse also shows significant glomerular damage, increased cell death of the proximal tubular epithelial cells, increased infiltrating macrophages, and increased interstitial fibrosis that may contribute to the decline in renal function over time.[17,18] As a result of these kidney complications the Col4A3 mice die early in life. Inhibition of miR-21 by an optimised miR-21 anti-miR in the Col4A3 mutant mouse has been shown to impact all aforementioned endpoints in a dose responsive manner, including an increase in the lifespan of the Col4A3 mutant mice.[18]

DELIVERY OF ENGINEERED MICRORNA THERAPEUTICS

A challenge that has limited the success of engineered microRNA therapeutics in general has been the ability to deliver a sufficient drug concentration to the right tissues and cells to ensure target engagement. Engineered microRNA therapeutics can be formulated or delivered through a number of different approaches designed to ensure efficient delivery including the following:

1. Administration of engineered microRNA therapeutics in saline.
2. Direct conjugation of the engineered microRNA therapeutic to a ligand that when bound to a cell specific receptor can enrich delivery to that specific cell type.
3. Formulation of the engineered microRNA therapeutic in nanoparticles (lipid and lipid like).

Saline formulation

Single-strand anti-miR therapeutics, but not double-stranded microRNA mimics, can be formulated in saline, administered systemically (either subcutaneously or intravenously) and achieve inhibition of a given microRNA target in liver, kidney, macrophages, adipose tissue and muscle. As a result the majority of the pre-clinical and human data generated to date with anti-miR therapeutics have been with this type of approach and target disease states associated with those organs or cell types. Although this delivery approach has shown success, its application may be limited. One challenge associated with the systemic administration of oligonucleotides formulated in saline is their ability to accumulate and induce histological changes in the kidneys.[36,37] It is not clear at this time whether the histological changes observed after chronic administration of engineered microRNA therapeutics will be associated with loss of renal function in humans. Injection site reactions and the histological changes induced by oligonucleotides in the kidney may be driven in part by immune stimulation[37] and, if so, the ability to design new chemically modified engineered therapeutics with minimal pro-inflammatory potential may limit both of these concerns.

Direct conjugation

To enrich delivery to a specific target cell type, engineered microRNA therapeutics can be directly conjugated to ligands that are internalised upon binding to cell surface receptors.[38,39] The use of a modified galactosamine-conjugated siRNA therapeutic (GalNAc conjugate) that can bind to the asialoglycoprotein receptor on hepatocytes has shown efficient delivery of the nucleic acid therapeutic to hepatocytes.[38] An miR-122 anti-miR, conjugated to GalNAc, can inhibit miR-122 and reduce HCV viral load in a 'humanised' mouse model of HCV infection.[22] GalNAc conjugates are considered to be safe and well tolerated with acceptable therapeutic indices. This opens up the opportunity to target other liver diseases in which hepatocyte microRNAs are dysregulated. In addition, direct conjugation of engineered microRNA therapeutics to other ligands whose receptors show cell type or tissue specific expression may lead to new therapeutic opportunities. For example CD22, a B-cell specific receptor, has a known carbohydrate ligand that is rapidly internalised upon receptor engagement[26,40] and might be harnessed to facilitate specific uptake of anti-miRs or microRNA mimics into lymphocytes. The identification of additional ligands that are rapidly internalised by cell specific receptors has the potential to expand the number of disease states that can be targeted by engineered microRNA therapeutics.

Nanoparticle formulation

Engineered microRNA therapeutics are being formulated in a variety of sophisticated nanoparticle

formulations – lipid and lipid like.[41-43] While these formulations have been shown to enhance the potency of the nucleic acid therapeutics they have also shown significant liabilities such as cytokine induction that limits their safety and tolerability.[41-43] Recent work, however, suggests that the therapeutic index associated with such formulations can be increased and new chemical rules are emerging that will help the design of biodegradable nanoparticles that can show target engagement at significantly lower doses.[41,42]

CONCLUSIONS

Engineered microRNA therapeutics represent a novel mechanism to control pathways or gene networks. This is distinct from the standard approach taken with a variety of therapeutic modalities that target individual proteins or enzymes. The first engineered microRNA therapeutics have already entered into clinical development and in the case of an anti-miR therapeutic targeting miR-122, human proof of concept has been achieved. New and improved engineered microRNA therapeutics are also showing excellent efficacy in pre-clinical models and it is likely that the number of human diseases being targeted through the control of biological networks will continue to expand. Such expansion will be stimulated by recent progress in the design of new formulation and delivery approaches. Engineered microRNA therapeutics have a tremendous opportunity to revolutionise the way in which human diseases can be treated and managed.

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