A Review of Recent Patents Concerning Therapy of Respiratory Diseases Using Gene Silencing by RNA*i* (RISC) and EGS (RNAse P)

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Abstract: This article will review recent developments in the field of gene silencing as a therapy for respiratory and related inflammatory and immunologic diseases. The respiratory epithelium offers an attractive target for therapies derived from nucleic acids since the respiratory epithelium contains endogenous lipids that can facilitate uptake of polar nucleic acids and related compounds. Both RNA*i* (RNA Interference) in which a messenger RNA (mRNA) is targeted by an endogenous enzyme complex termed RISC (RNA Interference Silencing Complex, also previously termed RNA Induced Silencing Complex in earlier references) and also gene silencing using EGS (External Guide Sequences) in which a messenger RNA (mRNA) is targeted by an endogenous RNA enzyme termed RNase P are summarized including selected patents. The strengths and limitations of these technologies such as problems of delivery to specific tissues and potential for non-specific inflammatory response and off-targeting are compared. The possibility of therapy designed exploit synergies between both RISC and RNAse P and therapeutic benefits of inhibiting either or both pathways are also considered.

Key Words: RNA*i*, EGS, RISC, RNAse P, gene expression, stem cells, mRNA, gene silencing.

INTRODUCTION

This review will review recent developments in the therapy of respiratory diseases with gene silencing using the new technologies of RNA*i* and External Guide Sequences (EGS). To facilitate the reader's understanding of the current state of this technology, a particular disease of clinical importance, influenza, is discussed in detail as a means of illustrating the current state of the art and problems remaining. In such a rapidly developing field it is impossible to refer to all relevant patents and references available. The approach of this review will be to utilize the selected patents as the basis of a discussion of two different methods of gene silencing RNA*i* and EGS applied to respiratory disease, rather than a comprehensive review of many rapidly expanding intellectual developments in this area.

The review will cite patents selected by the editors of this series as representative of recent patents in the subject area of gene silencing of respiratory diseases [1-10], as well as an additional patent obtained by the author of relevance to the subject [11]. Relevant respiratory diseases include inflammatory diseases such as asthma [1,3] as well as infectious diseases affecting the lung and upper respiratory tract such as influenza and other respiratory viruses [3,5]. Reference is made also to related therapy of other inflammatory diseases such as those of the eye [4] and malignancy [2] as well as therapy directed at altering the growth and properties of cells [7], viral infection [6] and the nature of lipid compounds enhancing the delivery of silencing constructs to tissues [10]. The special conditions and problems of therapy of respiratory diseases with gene silencing as well as advantages of this particular organ system as a target for gene silencing technology have been reviewed previously and will be summarized here [3,12].

The recent discovery of gene silencing double stranded RNA molecules termed RNA*i* (RNA interference or interfering RNA) that activate a host enzyme complex termed RISC (RNA Interference Silencing Complex also previously termed RNA Induced Silencing Complex) has generated tremendous scientific interest as well as meriting the Nobel prize [13-17]. RNA*i* can serve as a research tool and also may provide a new technology for translational research and therapy of human diseases such as influenza [18,19]. In gene silencing by RNA*i* a small double stranded RNA termed siRNA (Small interfering RNA) generated either by processing of a larger double stranded RNA transcript or introduced as a synthetic molecule into eukaryotic cells activates the endogenous RISC complex to cause cleavage or other transcriptional inactivation of a target mRNA similar or identical in sequence to the RNA*i*.

The concept of using endogenous enzyme complex to silence genes is not unique or novel to RNA*i* however, and in fact was first proposed as a strategy using the endogenous RNA enzyme RNAse P and small RNA sequences termed EGS (External Guide Sequences) as depicted in Fig. **1** [20,21]. Potential targets for EGS therapy involving the respiratory tract have included atopic diseases such as asthma [12] and influenza [22]. The discovery of RNase P, a molecule that is itself composed of RNA and thus termed an RNA enzyme also was rewarded with the Nobel Prize. Both the discovery of RNA*i* and RNAse P were in turn proceeded by studies of conventional anti-sense DNA for therapy of respiratory and other diseases, although this approach has

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been limited by the low potency and specificity of conventional anti-sense (a review of conventional antisense technology is beyond the scope of this article and the reader is directed to cited references) [12,23].

PRINCIPLES OF RNA*i* AND EGS THERAPY

The authors of pivotal studies in the discovery of RNA*i* and RISC demonstrated that double stranded RNA appears to silence genes through a mechanism distinct from conventional antisense [13]. Unexpectedly, while some decrease in gene expression of a target messenger RNA (mRNA) resulted from antisense single-stranded RNA binding to the target messenger RNA, much greater inhibition of gene expression of a target mRNA was evident with double stranded RNA containing both messenger RNA sequences and complementary sequences. This led to the subsequent discovery that an enzyme complex termed RISC and associated proteins imports and unwinds short mRNA sequences and their complementary sequences and uses the complementary sequence as a template to cleave multiple other copies of mRNA [17]. In invertebrates and plants other host proteins can amplify the double stranded RNA but RNA*i* amplification does not appear to occur in vertebrates [16].

In contrast to conventional antisense technology in which a small complementary RNA or DNA binds and inactivates mRNA consuming both the complementary RNA and the target mRNA, the RISC complex can cycle through many cleavage reactions of mRNA targets without being destroyed or consumed. Thus, RNA*i* is much more potent than conventional antisense technology. A single small double stranded RNA molecule introduced into a target eukaryotic cell could in theory destroy all complementary target mRNA or nearly complementary mRNA expressed in the cell for a prolonged period of time. Because of the potential for highly potent and specific gene inactivation a number of translational research studies and research programs were immediately initiated to capitalize on the potential of RNA*i*.

Virtually, any mRNA can be also be targeted to RNase P by designing an EGS complementary to the mRNA target and containing conserved transfer RNA loop sequences [20, 21] (Fig. **1**). The RNase P enzyme recognizes the structure of a transfer RNA in addition to certain highly conserved sequences in the transfer RNA loop regions. Like RNA*i*, small chemically modified EGS can be introduced into cells using lipids as a carrier to avoid the use of retroviral expression vectors [24]. As with RNA*i* the result is a catalytic inactivation of mRNA rather than a one-to-one inactivation typical of conventional antisense technology. RNA*i* and EGS technology are variations on a common theme of using an endogenous RNA processing pathway for a novel use namely catalytic degradation of a target mRNA sequence (Fig. **2**).

As with all nucleic acids including conventional antisense nucleotides, delivery of the highly charged double stranded RNA*i* molecules may be problematic. Therefore many initial translational therapies and patents involving RNA*i* have focused on topical applications for example therapy of respiratory diseases and diseases of the eye. All of these target organs are relatively exposed for topical therapy although in the future better delivery systems may permit delivery of RNA*i*, EGS and related small nucleotides to internal organs and tissues. In the case of respiratory tissues, the endogenous lipids of the lung, termed surfactant are capable of binding to nucleic acids such as RNA*i* and possibly permitting their spontaneous uptake into the respiratory epithelium and other tissues [12]. In other epithelial tissues or tissues of the eye, specialized lipids can be optimized for local delivery of the RNA*i* sequences although this is not a trivial problem [25].

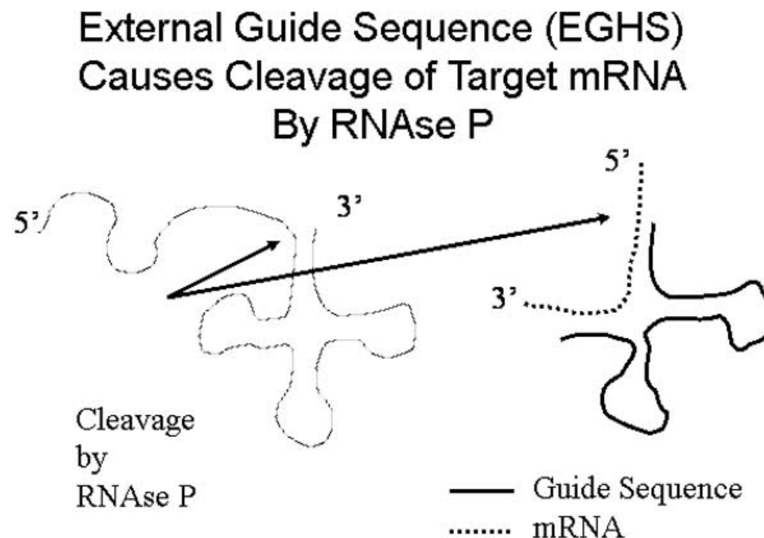

Fig. (1). As shown in the left side of the figure, the RNA enzyme RNAse P recognizes the folded structure of a tRNA molecule and certain conserved tRNA sequences in the tRNA t-loop. RNAse P then cleaves the precursor 5' tRNA leader sequence to generate an active tRNA shown in the conventional "cloverleaf" orientation. Cleavage of the 5' leader is required to generate mature tRNA. As shown in the right side of the figure, an External Guide Sequence (EGS) designed to bind to a target mRNA forms a structure resembling a tRNA precursor "cloverleaf" upon binding to a target mRNA and thus can cause site specific cleavage of the target mRNA by RNAse P. Following cleavage of the target mRNA the EGS can diffuse to another target mRNA permitting multiple enzymatic cleavage of target mRNA per EGS.

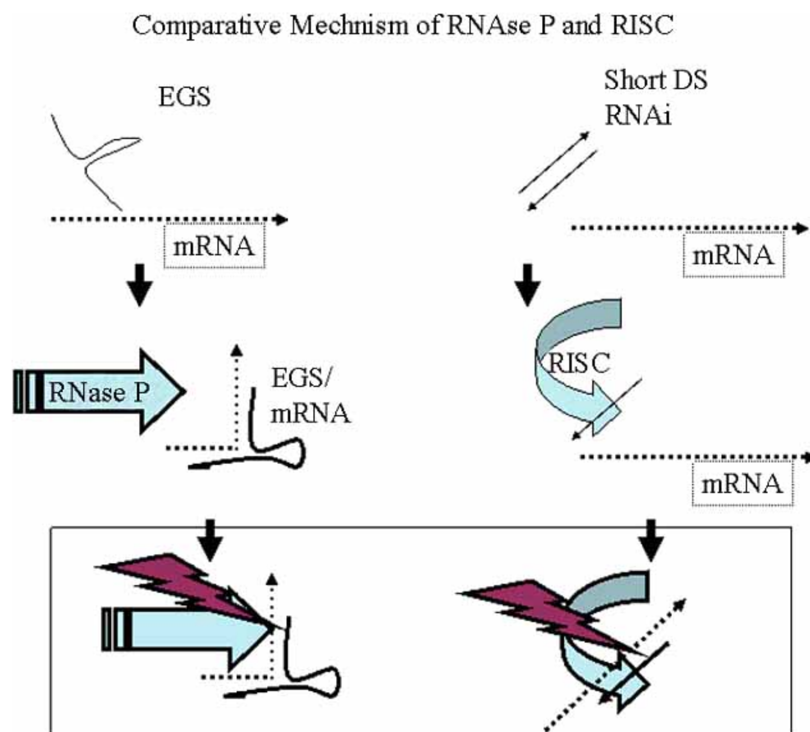

Fig. (2). In this figure cleavage of the target mRNA is illustrated by a jagged arrow with the steps for cleavage by EGS and RNAse P shown on the left and cleavage by RNA*i* and RISC on the right side of the figure respectively. The cleavage of target mRNA by RNAse P following binding of EGS to the target mRNA (left side) is analogous to targeted cleavage by the RISC complex of target mRNA following binding of the RNA*i* (right side). In both cases an endogenous RNA processing pathway RNAse P or RISC respectively is directed by a template sequence to cleave and inactivate a target mRNA through design of a complementary template sequence termed an EGS or RNA*i* respectively. As discussed in the text, in both cases the cleavage of mRNA targets can cycle through multiple cleavage reactions of the target mRNA since EGS and RNA*i* are not consumed or inactivated by cleavage of the target. Since EGS and RNA*i* utilize different enzymes and are active in different locations in the cell the potential exists for synergy or additive effects in target inactivation.

SIMILAR PROBLEMS OF RNA*i* AND EGS RELATED TO NON-SPECIFIC EFFECTS SUCH AS INTERFERON-MEDIATED INATE INFLAMMATORY RESPONSES TO OLIGONUCLEOTIDES AND OFF-TARGETING

Adding to the complexity of translational therapy with RNA*i*, and related therapies involving small nucleic acids, all eukaryotic cells contain receptors specific for small single and double stranded RNA termed Toll receptors. Toll receptors trigger an interferon-mediated inflammatory response as part of the innate immune system. Experimental observations have led to multiple at times conflicting observations regarding whether therapy with RNA*i* might associated with acceptable levels of nonspecific or inflammatory responses [26-30]. In the absence of data from clinical studies in humans these issues remain unresolved. Possibly, these concerns may be overcome by using a correct choice of RNA*i* molecule highly specific for the target mRNA and also limiting the dose of the RNA*i* as well as improving the chemistry of lipid or other carrier molecules, as noted in the introduction as a source of patents and potential issues of intellectual property. Clearly, the future of RNA*i* as a translational therapy will depend on both the ability to deliver the small double stranded RNA molecules to target cells effectively while limiting nonspecific and inflammatory effects of the therapy and compounds facilitating delivery of small nucleic acids have been described [10].

Conventional antisense therapies have been handicapped by relatively low potency relative to the required inactivation of target mRNA and nonspecific binding and irreversible effects or toxicity due to high concentrations of conventional antisense required to achieve measurable differences in target mRNA expression. The specificity of RNA*i* in terms of off-target effects is also complex however since slight or even significant mismatching between mRNA targets and other mRNA can still cause gene silencing through effects on protein translation of mRNA as well as degradation of mRNA by RISC [31-35].

These concerns are also relevant to the alternative technology of gene silencing using EGS that activate a host RNA processing complex termed RNase P [20,21]. Because both technologies use small nucleic acids complementary to host mRNA they are expected to have related problems related to tissue delivery and nonspecific inflammatory effects and off targeting. Without direct experimental evidence it is impossible to predict which technology, RNA*i* or EGS, will have less nonspecific inflammatory effects. Synthetic EGS contain different modified bases than nucleus

resistant RNA*i* [24]. In addition, upon introduction into the cell RNA*i* have a defined secondary structure as a double stranded RNA, whereas the structure of an individual EGS is not defined initially. EGS only assume a biologically defined structure upon combining with the target mRNA and exist prior to binding target mRNA as single stranded RNA molecule that may remain single-stranded or base pair to itself unpredictably [20]. EGS are slightly smaller than RNA*i* (approximately 30 nucleotides single stranded minimal EGS sequence) versus RNA*i* with a minimal size of approximately two 23 nucleotide sequences bound to each other (for a total of at least 46 nucleotides) and the size difference might, or might not, be important in terms of triggering endogenous innate immune receptors for nucleic acids.

Since RNase recognizes a target of approximately 11 complementary nucleotides [20] with some cleavage of target even with one or two mismatches in this region one might suppose a priori that EGS will have different patterns of target site mismatch and off targeting relative to RNA*i* that recognizes a target of 22 or 23 nucleotides in the target mRNA with also some tolerance of mismatches. However a number of factors such as target site accessibility and degree of mismatch tolerated preclude a conclusion without experimental evidence as to which technology will have less off targeting effects as well as less nonspecific inflammatory effects [20]. It is also likely that the method of delivery such as formation of lipid carriers will affect all of these variables in a manner that must be determined experimentally as noted previously.

Delivery to target tissues using viral expression vectors is possible with both RNA*i* and EGS [36] in contrast to conventional antisense thus allowing regulation of gene silencing to turn on and off genes as needed for various therapy applications. This could be a particularly important application of both of these technologies for example in design of stem cells induced to differentiate or and therapy of malignant tissues to permit very high levels of gene silencing RNA expression if toxic effects of RNA*i* or EGS expression can be limited. The optimism surrounding RNA*i* and EGS as a translational therapy, however, results from the very high potency of this approach to gene silencing and also the potential for reversible targeting of gene expression which may overcome the disappointments associated with conventional antisense therapy.

COMPARISON OF RNA*i* AND EGS AND POTENTIAL FOR SYNERGY IN THERAPY

There are important differences between the biology of the RISC complex required for RNA*i* and RNase P required for processing of transfer RNA. For example all cells are constantly making transfer RNA (tRNA) resulting in very high levels of RNase P present in all cells, in contrast to the RISC complex that is induced by inflammatory stimuli and may be present at low levels or not present in some cells. Thus it is possible that the RISC complex may be saturated or rapidly saturated with RNA*i* and thus unable to target high levels of mRNA or multiple targets as suggested in preliminary studies in some cell types [37]. In contrast it seems unlikely that RNase P would be saturated by multiple target mRNA.

Remarkably, even if the delivery problems to various tissues can be solved, the RISC complex is part of a much larger family of enzymes termed DDE recombinases including retroviral integrases and RNase H as well as the RAG (Recombination Activating Gene proteins) required for generation of immunoglobulin molecules [38]. All of these other DDE enzymes are either present in viral pathogens such as retroviruses or are components of the innate review response to viral pathogens that responds to the inflammatory cytokines termed interferons. Thus, RNA*i* cannot be viewed in isolation, but rather is part of a complex series of enzymes involved in innate immunity and interferon-mediated inflammatory responses. It is likely that activation of RISC to target mRNA will have complex effects on the innate immune system, and conversely therapy targeting the innate immune system or antiviral drugs may also interact with RNA*i* [38].

Differences in the biology of the two RNA processing pathways may also explain the observation that the onset of gene silencing by RNase P occurred within 24 hours of EGS expression versus 48 hours for RNA*i* in one comparative system where the two gene silencing technologies were compared directly using an identical mRNA target [39]. In this study both EGS and RNA*i* expressed from an identical retroviral vector were equally effective in decreasing target mRNA expression and were also both non-toxic to cells over the several day period studied.

A problem with direct comparisons of EGS and RNA*i* potency is that the target site size and consensus sequences of the two enzymes are very different, and thus the sites are not equivalent in terms of other factors such as accessibility to the targeting nucleotides and intra-target base pairing [40]. RISC also forms a complex with other proteins contributing other enzymatic capabilities such helicases that can provide the ability to unwind intra-molecular double stranded regions, while helicase activity is not present in RNAse P. Like RNA*i*, EGS can be optimized by selection protocols to increase potency and hence reduce non-specific effects [41-44].

A clear advantage of RNA*i* relative to EGS is that EGS can only target genes that are expressed in the cell nucleus where RNAse P resides, probably resulting in an inability to target some respiratory pathogens such as Respiratory Syncitial Virus (RSV) that do not enter the cell nucleus, while RNA*i*, active in the cell cytoplasm can target RSV mRNA. This problem could however be averted however by designing EGS specific for the viral receptors for pathogens such as RSV that are not accessible to RNAse P directly. Because gene silencing with RNA*i* is specifically designed for inactivation of viral pathogens and host genes RNA*i* might be more potent than gene silencing with RNase P as suggested by one study in which potency of RNA*i* was compared directly with RNase P [40]. It is likely that there may be some genes that are particularly accessible to silencing by RISC and other genes more accessible to silencing by RNase P related to unpredictable structural features of target mRNA, tissue expression and tissue specific binding factors as well as differing levels of the silencing complexes. Thus, comparative studies need to be repeated with other specific targets for any generalizations

can be made regarding comparison of the two technologies for a particular application to human disease.

Another interesting possibility is that since both RNA*i* and EGS can be expressed from viral vectors or introduced using similar lipids sequences or other delivery devices into cells, both forms of gene silencing could be delivered simultaneously [12]. In this way synergy between the two technologies, the possibly higher potency of RNA*i* versus the more rapid onset and higher levels of the enzyme complex of RNase p might become evident. One could imagine a scenario in which a mixture of RNA*i* and EGS were both introduced into the pulmonary epithelium for therapy of respiratory diseases such as influenza or asthma to maximize the benefits of both modalities.

STATE OF THE ART CURRENTLY WITH SPECIFIC REFERENCE TO THERAPY OF INFLUENZA USING RNA*i* AND EGS

As noted above, there are many theoretical observations suggesting that gene silencing with either RNA*i* or EGS may have advantages over conventional antisense or as a novel form of therapy in cases where existing small molecule therapies are limited by side effects or unavailable. It will be helpful to review the case of influenza as a respiratory disease targeted by both silencing technologies in experimental systems to illustrate the current state of the art and results of pre-clinical studies. Current therapy for influenza is limited to agents that either block the viral neuraminidase such as Oseltamivir (Tamiflu, Roche, Tamiflu.com) or amantidine and related compounds that block viral entry into cells. Available therapeutic agents for influenza are currently effective particularly for prophylaxis but have limitations such as the development of resistant flu strains, and side effects beyond the scope of this review. Vaccines for influenza are also beyond the scope of this review but are only effective against known strains of virus, not rapidly emerging strains and currently must be grown in eggs, a process requiring months. Most importantly, vaccines require 2 or more weeks for an immune response and often a second dose to achieve protective immunity, and thus would be ineffective for a rapidly emerging epidemic of a novel influenza strain.

Since influenza unlike many other respiratory viruses such as SARS or rhinovirus replicates in the cell nucleus it can be targeted by EGS or RNA*i* using these agents directly to block expression of viral proteins. Another possibility that has not been tested experimentally is that therapy of both influenza and related respiratory pathogens that are not directly targeted by EGS or not effectively targeted by RNA*i* could be targeted by eliminating the inflammatory response to the viral pathogen which in many cases resembles the cytokine profile of posts viral asthma [5, 12].

EGS targeting conserved viral proteins were first shown to block replication of the influenza virus using retroviral vectors to express EGS stably in cell lines [22]. Genes encoding polymerase binding protein (PB2) and nucleoprotein (NP) were chosen for targeting because these genes and the proteins they encode are highly conserved among many influenza strains in contrast to the rapidly changing hemaglutinin (HA) and neuraminidase (NA) genes and proteins targeted by vaccines and small molecule therapies. The authors showed that cell lines stably expressing EGS targeting either of these two proteins exhibited significantly decreased replication of the influenza virus and that cell lines expressing EGS targeting both proteins almost completely abolished viral replication. No adverse effects appeared on the cells relative to control. A limitation of the studies were that results were not extended to preclinical studies in animals or using nuclease resistant small EGS that do not require retroviral vectors [24].

Currently, Keren pharmaceutical has constructed retroviral vectors expressing EGS demonstrated to be effective against influenza replication in cell culture for further studies including preclinical studies in animals. These retroviral vectors are based on a lentivirus expression system that does not require replicating cells in contrast to the vectors used previously [22] and thus may more readily infect epithelial cells *in vivo*. EGS expressed from lentivirus might be considered as a prophylactic therapy in veterinary applications such as poultry, the natural host of influenza, if they are shown to be safe and effective. Also we anticipate that if EGS expressed from retroviruses are safe and effective in animal models such as mouse for prophylaxis and therapy of influenza then further studies would be justified using small nuclease resistant EGS in preclinical studies for eventual therapy in human subjects.

Following the discovery of RNA*i,* influenza was also targeted in a series of studies [18]. Two proteins were targeted [18] including the nucleoprotein (NP) and PA protein (alternative nomenclature for PB protein targeted in [22]). Following demonstration of RNA*i* efficacy and safety in preliminary studies in cell culture, RNA*i* were administered to mice both before and after viral challenge. Efficacy and safety of the therapy was confirmed against a variety of viral strains both as a prophylactic therapy and as a post-infection therapy in mice [1,18]. Therapeutic effects of the RNA*i* were also not related to nonspecific effects of interferon and appeared to be additive because targeting both proteins was more effective than targeting either protein singly. Unfortunately, the administration protocol in mice was complicated and included both hydrodynamic and nasal administration of small nuclease resistant RNA*i* constructs as well as a retroviral vector expressing the constructs thus it is not possible to determine which of these forms of therapy was effective.

As noted previously it would be desirable to use small nuclease resistant RNA*i* or EGS constructs administered directly to the respiratory tract for therapy in humans and it is unlikely that humans will tolerate either hydrodynamic therapy that involves injection of a large quantity of nucleotide and carrier under pressure intravascularly, or retroviral therapy with the possibility of permanent genetic alterations. Thus these results both for EGS and RNA*i* must be reviewed as preliminary although promising. These studies also both identify the mode of delivery of the EGS or RNA*i* therapy as a concern currently limiting applications in clinical settings. Another potential area of promise is the finding that RNA*i* can be expressed in the functional form

active against influenza in plants [19] for subsequent administration to animals or humans potentially as therapy. The potential for plant expression of EGS, potentially for veterinary applications is also under study as a therapy for influenza using EGS shown to be effective in cell culture[22].

POSSIBILITY FOR THERAPY DIRECTED AT INACTIVATION RATHER THAN ACTIVATION OF RNA*i* AND RNASE P

While a great deal of interest has been expressed in activation of the RISC complex by RNA*i* and activation of RNase P by EGS to silence mRNA, another possibility worthy of exploration is the inactivation of these enzyme complexes for therapeutic purposes. Clearly, both the RISC complex and RNase P are critical enzymes in the biology of cells. RISC also seems to play a critical role in developmental biology of multi-cellular organisms through its inactivation and silencing of host genes during embryonic development and tissue differentiation. A strategy for identifying and inactivating DDE enzymes based on a shared Magnesium binding site termed DDE has been also proposed and patented as a potential therapy for herpes virus replication and this could be extended to develop RISC inhibitors [11,38]. It is perhaps not unimaginable that silencing of the silencing complexes could have a therapeutic role as well for example in stem cell development or therapy of cancer in which altering pathways of cellular differentiation and growth may be critical.

In the case of RNase P, inhibitory analogues of the transfer RNA substrate would slow cell growth through the inability to make transfer RNA required for growth. In the case of RISC, small molecule and oligonucleotide or aptimer molecules can be based on existing inhibitors of other DDE enzymes related to RISC such as inhibitors of retroviral integrates or RNase H, currently in clinical development for therapy of HIV (Human Immunodeficiency Diseases). Since no experimental evidence is available currently regarding the feasibility of inhibition of RNAse P, or DDE enzymes such as RISC, and a more extensive review of this subject is beyond the scope of this article the reader is directed to a recent review [38] for additional references regarding the theoretical possibilities of this approach.

CURRENT & FUTURE DEVELOPMENTS

Regulated expression of gene silencing by either RNA*i* or RNase P will certainly have many applications in basic research as well as veterinary medicine and agriculture where concerns about non-specific or inflammatory effects are less of a concern than therapy of human diseases [41,45]. Delivery of these small RNA sequence using retroviral and other viral vectors for example to stem cells for regulation of gene expression during development are also not as much a concern in non-human applications as in therapy of human disease.

Initial applications of gene silencing with RISC and RNase P directly in human of human diseases will undoubtedly at first involve topical applications of target tissues such as the epithelium of the respiratory tract [1,3] and the eye [4] and therapy of infectious diseases infecting these tissues [3,5] because of the accessibility of these targets. Whether or not these therapies can be directed to internal tissues and specific target tissues such as malignancies [2] or other viral infection [6] as well as therapy directed at altering the growth and properties of cells [7] without triggering unanticipated or unpredictable inflammatory and off targeting responses [51] remains to be determined and involves a careful consideration of risks and benefits. In the case of diseases such as HIV and cancer for which curative therapies are limited the risk benefit analysis may favor early intervention [24,46-50], but perhaps therapy of non-lethal but more debilitating chronic diseases such hepatitis [51] as well as asthma and related atopic diseases, or preventative therapy of epidemic diseases such as influenza may also be possible if technical obstacles can be overcome.

DISCLOSURES

Dr. Dreyfus is the founder and Dr. Ghoda is the Scientific Director of Keren Pharmaceutical, a biotechnology company developing novel nucleotide based therapies of respiratory and immunologic diseases. Dr. Dreyfus is also a paid consul-tant for Medimmune, Inc. which makes vaccines for infl-uenza and other respiratory viruses.

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