<u>**Review Article**</u>

RNA BASED THERAPEUTICS

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ABSTRACT

Astounding progress has been made in the identification of genes responsible for cell growth, development, and neoplastic transformation. With this knowledge has come a natural desire to "translate" this information into new therapeutic strategies for many of the common maladies that afflict humankind. These include in particular cardiovascular, gastrointestinal, neurological, infectious, and neoplastic diseases. Traditionally, therapies for most of the diseases are chemical/herbal drugs. Recent advances in molecular biology have made nucleic acid-based therapies feasible and while still relatively unproven, they represent one of the most exciting new areas in biomedical research. Attempts at inserting genes into cells that either replace, or counter the effects of, disease-causing genes has been one of the primary ways in which scientists have tried to exploit this new knowledge. This technically complex, as yet largely unrealized endeavor is what most individuals think of when the terms "gene therapy" or "molecular medicine" are discussed. Nevertheless, alternative strategies for treating diseases at the gene level are being developed. The common goals of these various strategies, which are turning out to be as technically demanding as more traditional gene therapy, is to identify disease related genes and target them for "silencing." Because the numbers of maladies that might be treated by this approach are genuinely enormous, this is clearly a most important field of endeavor. In this review the main scientific and technical aspects of RNA interference are introduced and some of its potential clinical applications are discussed.

Keywords: RNA based therapeutics

INTRODUCTION

"Recent advances in our understanding of RNA biology continue to reveal that the functions of RNA are more complex than was once thought. In addition to its fundamental function encoding genetic information as ribonucleic acid, other forms of RNA have been found to have catalytic properties the RNA enzymes or ribozymes the substrates for which are other RNA molecules. Specific secondary structure of RNA molecules that can sense regulatory signal from cellular environment can regulate gene expression. These sensing RNA motifs (riboswitch) prevent gene expression either at transcriptional or post-transcriptional level. Mimics of the regulatory response element on genes (RNA decoy) are also showing great promise as therapeutic agent. More recently, an intrinsic cellular gene silencing mechanism centered on RNA the RNA interference (RNAi) pathway has been uncovered in organisms from worm to fly to mouse and man. Such discoveries have opened up the field of RNA therapeutics and offer the prospect of RNA-based gene silencing technologies being developed as therapies for human disease"

RNA interference

RNA interference (RNAi) is a natural mechanism by which small interfering RNAs (siRNAs) operate to specifically and potently down-regulate the expression of a target gene. This down-regulation has been demonstrated by targeting siRNAs to the mRNA (post transcriptional gene silencing) as well as to the gene promoter, regulating gene expression epigenetically by transcriptional gene silencing. These observations significantly broaden the role RNA plays in the cell and suggest that siRNAs could prove to be a potent future therapeutic for the treatment of diseases such as viral infections, cancer and inflammation. The specificity and simplicity of design and the ability to express siRNAs from mammalian promoters make the use of siRNAs to target and suppress virtually any gene or gene promoter of interest a soon-to-be-realized technology. However, the delivery and stable expression of siRNAs to target cells remain an enigma that could be surmounted.

Mechanism of RNA interference

RNAi is induced within the cytoplasm when long, double-stranded RNA (dsRNA) is recognized by Dicer, a multidomain RNase III enzyme. Dicer processes dsRNA into short [21–25 nucleotide (nt)] duplexes that are termed siRNAs. Like products of other RNase III enzymes, siRNA duplexes contain 5' phosphate and 3' ☐hydroxyl termini, and two single stranded nucleotide overhangs on their ends 3' These structural features are important for the entry of siRNAs into the RNAi pathway because blunt-ended siRNAs or those that lack a 5' phosphate group are ineffective in triggering gene silencing. The generated siRNA associates with a multiprotein complex, the RNA-induced silencing protein complex (RISC), which becomes activated on ATP-dependent unwinding of the siRNA duplex. One of the two siRNA strands is retained within the complex and confers sequence specificity in targeting of the mRNA by Watson–Crick base pairing. A perfectly homologous mRNA is cleaved at a single site in the center of the duplex region formed with the guide siRNA, 10 nt from the 5 ☐ end of the latter. Finally, RISC is released and the cleaved mRNA is further degraded by cellular exonucleases. The specific degradation of mRNA in turn leads to decreased synthesis of the respective protein and eventually to a loss of protein function. Concentrations of only a few siRNA molecules per cell can lead to a pronounced silencing effect, demonstrating the catalytic action of RISC. Generally, although greatly diminished, residual mRNA levels can be detected. Hence, the RNAi-mediated silencing of a particular gene is generally referred to as a 'knockdown' rather than a 'knockout'.[1] Sense and antisense strands are either produced from two independent promoters and anneal within the cell[2], or more commonly the two strands are linked by a 9 base pair spacer leading to the expression of a stem loop structure termed short hairpin RNA (shRNA). The hairpin is subsequently cleaved by Dicer to generate effective siRNA molecules.[2,3]

RNA interference in mammalian cells

Originally, the RNAi pathway was thought to be nonfunctional in mammalian cells, where dsRNA longer than 30 base pairs induces a nonspecific antiviral response[4]. This so-called interferon response is characterized by the activation of the RNA-dependent protein kinase[5], leading to phosphorylations of the translation initiation factor eIF-2 and thereby to a nonspecific arrest in translation and induction of apoptosis[6]. The breakthrough for the use of RNAi in mammalian cells came when Elbashir and coworkers[7] and Caplen and colleagues[8] showed that siRNA, when directly introduced into mammalian cells, does not trigger the RNA-dependent protein kinase response but effectively elicits RNAi, presumably by directly associating with RISC. Recently, evaluation of target gene specificity on a genome-wide level by applying gene expression profiling led to conflicting results. In two studies[9] no effects on non target genes were observed, although high concentrations (100 nmol/l) of siRNA were shown to induce stress-response and apoptosis-related genes. In contrast, Jackson and coworkers[10] challenged the idea of perfect sequence specificity of siRNA; they detected silencing of non targeted genes with limited sequence.
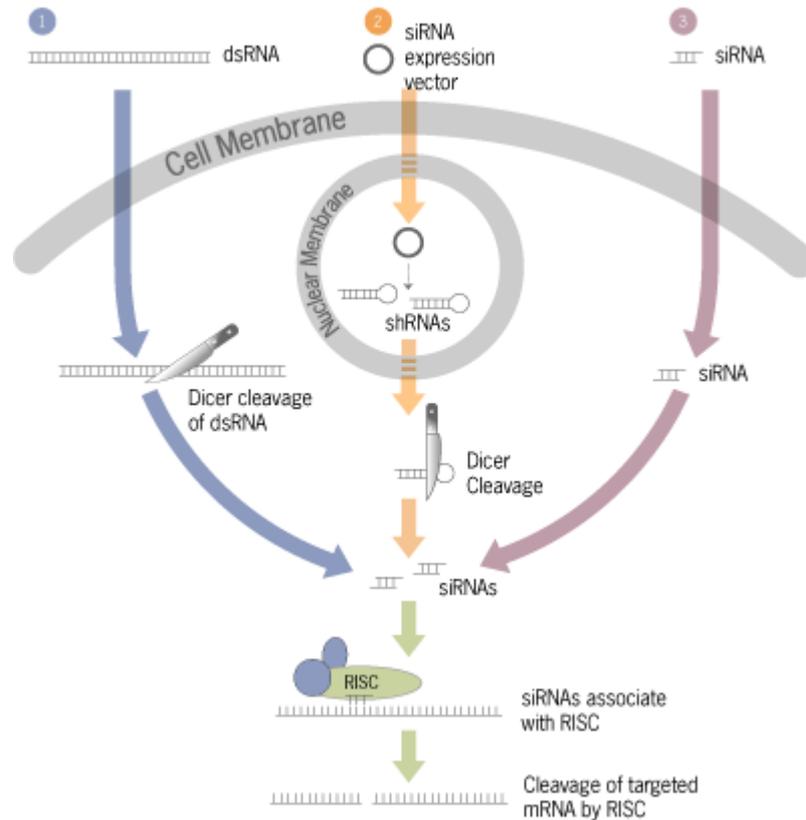
Fig. 1: The RNA Interference Pathway.

Long double-stranded RNA (dsRNA) or small hairpin RNA (si RNA) is processed by Dicer to form a small interfereing RNA (si RNA), which associates with RNA-induced silencing protein complex (RISC) and mediates target sequence specified specificity for subsequent m RNA cleavage.[5]

Table 1: Guidelines for siRNA design[12]

Guidelines for siRNA design	
General guidelines for siRNA design	Select 23-nt long sequences from the mRNA conforming to the consensus 5' AA [N19] UU3' or 5'NA[N19]NN3' (where N is any nucleotide)
	Avoid targeting the regions that are likely to bind regulatory proteins, such as 5' UTR, 3'-UTR and region to the start sites
	Choose sequences with GC content between 30% and 70%
	Avoid highly G rich sequences
	Design sense and antisense N 19 sequences, add 2-deoxythymidine residue to the 3' ends
	Perform blast search to exclude potential homology to other genes
Additional consideration for vector based siRNA expression	Avoid more than three consecutive As or Ts in the targeting sequence
	U6 promoter requires a G at position +1
	H1 promoters prefers A at position +1
	Design oligonucleotide containing N19 targeting sequence (often 5' TTCAAGAGA-3'), followed by the reverse complementary targeting sequence and five to six consecutive thymidine residues for termination of transcription
SiRNA design tool on internet	Add respective restriction site for cloning
	http:/www.ambion.com
	http:/www.qiagen.com/siRNA
	http:/www.jura.wi.mit.edu/bio
	http:/www.dharmacon.com
	http:/www.sinc.sunysb.edu/stu/shilin/rnai.html

Modes of application and routes into the mammalian cell

For induction of RNAi in mammalian cells, chemically synthesized siRNA and shRNA expression plasmids can be delivered to cells using standard transfection methods. Synthetic siRNA can be generated by chemical synthesis, by *in vitro* transcription using a T7 polymerase [12] or by Dicer digestion of long dsRNA. Synthesized siRNA induces potent silencing at concentrations of 1–10 nmol/l [13]. siRNA expression vectors utilize mostly U6-snRNA or H1 (RNase P) promoters, both of which are members of the RNA polymerase III promoter family, which lack downstream transcriptional elements and produce a transcript without a cap or poly-A tail. Transcription is terminated at a stretch of five to six thymidine residues, leading to the incorporation of two to three uracil residues at the 3' end, which is compatible with the two or three nucleotide overhangs that are found to be indispensable for silencing activity in natural siRNAs.

Gene silencing occurs very rapidly after the transfection of an efficient siRNA. Although the kinetics may vary depending on the gene of interest, usually target mRNA levels will be diminished after 48 hours, reaching a minimum at 72 hours after transfection. A knockdown efficiency of 90–95% reduction in the amount of target

mRNA can be achieved. However, the major drawback of the method is its transient gene silencing effect. The duration of the knockdown using synthetic siRNA is generally in the range of 3–5 days. Protein levels will return to normal 5–7 days after transfection[13]. The longevity of silencing depends on factors such as the abundance of target mRNA and protein, the stability of target protein, transcriptional feedback loops, and the number of cell divisions diluting the siRNA, rather than on the degradation of the siRNA itself. [1]

Thereby, the efficiency mainly depends on the type of cell that is targeted. Because of their small size, transfection of synthetic siRNAs is usually very efficient, even in primary mammalian cells. A number of cationic lipid-based or liposome-based transfection reagents optimized for the transfection of oligonucleotides are commercially available. In cells that are more resistant to chemical transfection methods (e.g. suspension cells), electroporation may achieve an efficient induction of RNAi. Transduction rates with siRNA of up to 80–90% have been reported for some haematopoietic cell lines and primary cells[15]. Optimized for the transfection of primary human cells with siRNA, NucleofectionTM technology (Amaxa biosystems, Cologne, Germany) appears to be a very efficient and convenient approach[16].

Viral gene delivery systems are perfectly suited for efficient transduction of primary cells and some of them have the inherent ability to integrate into the host cell genome, thereby leading to stable transgene expression. Several adenoviral[17], onco-retroviral[18] and lentiviral[19] vectors have been utilized for the efficient delivery of shRNA expression cassettes. Adenoviral infection is transient whereas onco-retroviral vectors, based on the Maloney Murine Leukaemia virus or the murine stem cell virus, integrate into the host cell genome, leading to a prolonged silencing effect. Lentiviral vectors based on HIV-1 bear the additional advantage of efficiently transducing both dividing as well as nondividing cells, such as stem cells and terminally differentiated cells. Moreover, they are resistant to developmental silencing after integration of the provirus, and therefore they can be used to generate transgenic animals. Several groups have reported the use of lentiviral systems for the silencing of genes in a variety of cultured as well as primary cells, such as human and murine T cells[20], haematopoietic stem cells[20] and mouse dendritic cells[20].

In a recent issue of Nature Biotechnology, Song et al1 showed that small interfering RNAs (siRNAs) can be delivered into a target cell population through antibody-mediated endocytosis[21].
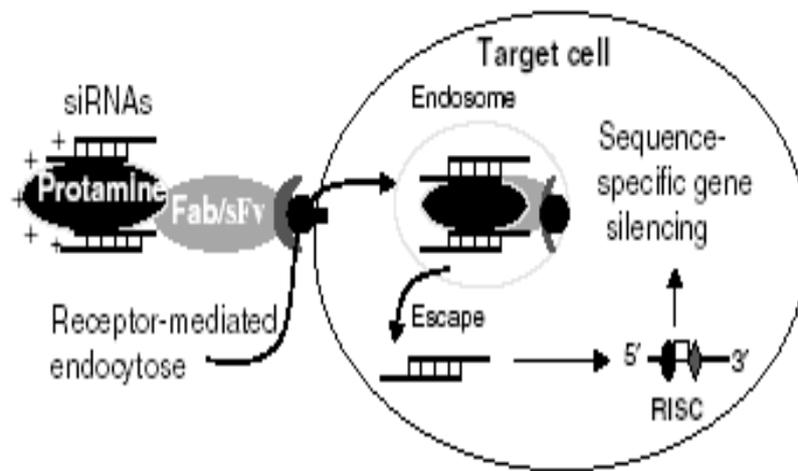
Fig. 2: The antibody recognizes its antigen receptor expressed on the cell surface, and then the complex of antibody and siRNA is internalized via endocytosis. Subsequent to endosomal escape, the siRNA is released into the cytoplasm and then incorporated into the RNAi targeting complex known as RNA-induced silencing complex (RISC) that uses the antisense siRNA strand to recognize and cleave the targeted mRNA.[12]

In vivo application of RNA interference in mammals

A number of potential candidate genes, especially in viral infection, cancer, inflammation and inherited genetic disorders has been defined and successfully targeted in vitro. Consistent with its natural function as an antiviral defense mechanism, siRNA was found to inhibit *in vitro* replication of several viruses effectively, including HIV, hepatitis C virus and influenza virus, by interfering with various stages of the virus life cycles [22]. Lentiviral siRNA vectors have been used to generate stable transgenic 'knockdown' animals by infection of fertilized eggs[23]. In another study, Rubinson and co-workers[24] used lentiviral vectors expressing green fluorescent protein as a selection marker and an siRNA targeting CD8 for embryo infection. Between 25% and 50% of the resulting mice were transgenic and expressed both green fluorescent protein and siRNA in all tissues tested. Transgenic mice exhibited a reduction in CD8 expression of about 90%; however, the percentage of cells affected by gene silencing varied among individual mice and correlated with the number of integrated viruses per genome. Recently it has been demonstrated that dendritic cells, transfected with an siRNA against the immunomodulatory cytokine interleukin-12 are transformed in mammals treatments using RNAi

AIDS

A number of previous approaches using either synthetic siRNAs or plasmid expressed constructs have successfully targeted viral transcripts and achieved effective viral inhibition. Of these, some anti-HIV-1 siRNAs, such as siRNAs against tat, tat-rev had been introduced into lentiviral vectors and their efficacy was demonstrated both in cell lines and primary T cells and macrophages.

In addition to targeting viral transcripts, many studies including ours also investigated the efficacy of siRNAs in down regulating host cell molecules necessary for HIV-1 infection .An advantage in targeting cellular molecules is that efficacy will be more broad spectrum against all the clan of the virus and the frequency of escape mutants will be lower. Down regulation of the primary cell surface receptor CD4 and consequent inhibition of HIV-1 infection was shown using synthetic siRNAs. However, since CD4 is an essential cell surface molecule for immunological function, it is not a practical target for HIV gene therapy. Chemokine receptors CCR5 and CXCR4 play critical roles as coreceptors for viral entry during infection with macrophage tropic R5 and T cell tropic X4 HIV-1 viral strains respectively. In a segment of the human population, a naturally occurring 32-bp deletion in the CCR5 gene results in the loss of this coreceptor thus conferring significant resistance to HIV infection. Homozygous or heterozygous individuals for this mutation remain physiologically normal. With regard to the CXCR4 coreceptor, it was found to be dispensable for T cell development and maturation in murine studies. These findings suggest that CCR5 and CXCR4 are promising targets for HIV therapies.

Cancer

Infection with viruses has been associated with several human malignancies for example Epstein-Bar Viruses (EBV) in nasopharyngeal carcinoma, Kaposi's sarcoma-associated herpes virus (KSHV) in primary effusion lymphoma and human T-cell leukemia virus type-1 (HTLV-1) in adult T cell leukemia. In models of each of these diseases RNAi targeting of virally encoded genes resulted in changes in invasive behaviour, tumor cell apoptosis or reduced tumourogenecity after transplantation into immunodeficient rats[25]

The Bcr-Abl fusion gene is characteristic of chronic mylogenous leukemia (CML) and some cases of acute lymphoblastic leukemia (ALL), and encodes a protein tyrosine kinase with aberrant activity and intracellular localization when compared with unrelated cellular Abl enzyme. Bcr-Abl breakpoint sequences have been targeted using chemically synthesized siRNAs and shRNA-encoding lentiviral vector[26].

Human epidermal growth factor receptor-2 (HER-2 which is found to be over expressed in approximately one in three breast and ovarian carcinomas and against which a therapeutically active monoclonal antibody has been developed. Targeting the HER-2 mRNA with synthetic or reterovirally encoded siRNAs resulted in compromised proliferation, survival and tumourogenecity in immunocompromised mice[27].

Since administration of aqueous siRNA, even chemically stabilized, is limited by a lack of tumor activity and non-specific responses, the use of gene therapy vector systems is one approach. Several efforts have evaluated cationic lipids and polymers originally developed for DNA plasmids where internalization is by non-specific electrostatic interactions. For DNA, means to improve on control of cellular interactions have focused on either addition of peptide ligands for cellular receptors or addition of steric polymer coatings to inhibit non-specific interactions, and recently their combination. The resulting systems generally are perceived of having yielded good cytoplasmic delivery, but the gene expression from the plasmid remains limited by poor trafficking into the nucleus. Recent efforts achieved some success by coupling dual antibodies to the distal end of polyethylene glycol (PEG) on a sterically stabilized liposome by a complex procedure using post modification and incurring substantial loss of antibody and siRNA. Nonetheless, a need exists for relatively small peptide ligands, such as a disulfide-stabilized Arg-Gly-Asp (RGD) peptide targeting integrin expression upregulated at sites of neovasculature and self-assembling nanoparticles without the problems of liposomes[28].

Neurological diseases

Recent studies have shown that the potential of RNAi extends to applications in the mammalian nervous system. Effective gene silencing using RNAi has been demonstrated in mouse neuroblastoma cells[29]. In P19 cells[30] and neural stem cells (unpublished observations), and in primary cortical and hippocampal neurons, where silencing of the endogenous neuronal genes, microtubule associated protein 2 and RNA binding protein YB-1 genes was shown[31]. Moreover, gene silencing by adenovirus vector shRNA expression has also been demonstrated in mouse brain *in vivo*[32]. Many of the major neurological diseases that are significant causes of mortality in developed nations are likely to be suitable targets for therapeutic gene silencing; these include central nervous system (CNS) tumors and neurodegenerative disorders. In addition, retinal disorders, on account of their accessibility, nervous system infections, given the intense focus of the RNAi field in this area[33], prion diseases[34] and pain[35] are other potential areas for therapeutic development.

Ribozymes

Traditionally therapies for infectious diseases are pharmaceutical. Recent advances in molecular biology have made gene-based therapy feasible. Ribozymes are RNA molecules having catalytic activity i.e. they cleave their target RNA molecule. The specificity of cleavage, potential for turn over and lack of immunogenicity make ribozyme attractive as a potential therapeutic agent.

The discovery of ribozyme by Cech and Altman (1981) has changed the view of the function of RNA in the chemistry, biology and medicine[36]. Earlier it was thought that RNA is only carrier of genetic information or it provides structure to RNP. After the discovery that RNA molecule has property of cleavage (self or trans cleavage), this view has been changed. Cleavage property permits the development of new therapeutics, which can suppress the expression of deleterious protein by catalyzing the cleavage of corresponding mRNA[37]. Target of ribozyme include oncogene growth factor, growth factor receptor, proinflamatory cytokines and their corresponding cell surface receptor and signal molecules. The field of ribozyme is developing at an impressive pace so it is impossible to cover the whole repertoire of ribozyme. We will focus mainly on hammerhead ribozyme.

Structure and function

Ribozyme has three helical structures H1, H2 and H3. Sequence in H1 is responsible for correct conformer of ribozyme. H1 and H3 forms complementary base pairing with target RNA. 3D structure of hammerhead ribozyme was elucidated by X-RAY crystallography[38]. FRET studies shows that Mg induces a conformational change required for complementary base pairing of H1 and H3 with target RNA while help in formation of active catalytic domain (H2). Hammerhead ribozyme recognizes any RNA which contains any cleavage triplets 5'NUX3' where N is any nucleotide and X is any nucleotide except G. It has been presumed that sequences upstream and downstream of triplet exert little or no effect on cleavage. Surprisingly when 2 U-A base pairs are present at 3'of cleavage site, the cleavage rate is enhanced 10 fold[38].
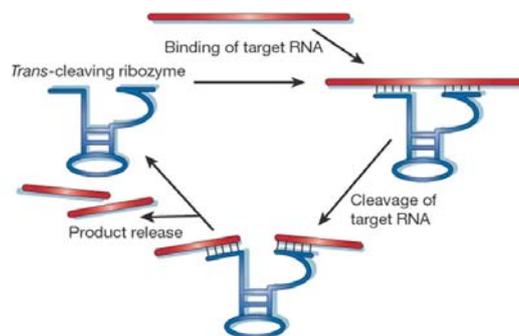

Fig. 3: Mechanism of ribozyme

Figure-3

Ribozyme gain their target specificity from Watson and crick base pairing between ribozyme 1st and 3rd arm and sequence flanking NUX. The attack at 2' OH and destabilizes RNA backbone. Upon cleavage resultant product dissociate and ribozyme is again ready to cleave another RNA molecule. Ribozyme need to be designed so that they can survive in nuclease rich environment of biological tissue and body fluid. They are methylated at 2' OH in each nucleotide[36]. Additionally an inverted deoxyribose sugar residue is placed at 3' end of ribozyme to provide 3'-3' linkage and 3-4 phosphorothioate linkage are inserted at 5' end five purine in H2 should remain unchanged otherwise catalytic activity could be lost.

Applications

Much of the excitement in the ribozyme field stem from potential application of ribozyme in sequence specific inhibition of gene expression. Ribozyme can be administered exogenously or endogenously.

Exogenous delivery: In exogenous delivery presynthesised ribozyme are introduced in cell. There are examples of exogenous delivery of carrier free nuclease resistance ribozyme by injection in animal model. e.g.

1. Ribozyme against metalloproteinase: stromlysin a key mediator in arthritis disease. Ribozyme is taken by cells in synovial lining and reduced IL-1α induced stromylsin mRNA observed[39].

2.   Inhibition of myelogenin in newborn mice by injecting carrier free ribozyme close to developing mandibular molar teeth[40].

3.   A ribozyme against protein kinase Cα is made resistant to degradation by exchange of all pyrimidine with corresponding 2' amino derivatives. Cationic liposome delivery of this ribozyme inhibited glioma cell growth in vitro and inhibited tumor growth in rats[41].

Endogenous delivery: In endogenous delivery gene of ribozyme is introduced in cell with the help of vector (plasmid). Retroviruses are choice of vector because they get integrated in the genome and give stable expression of target gene. Adeno associated virus are another substitute due to the following reasons-

➢   Non pathogenic

➢   Integration into genome

➢   Has specific integration site at chromosome no. 19

Ribozymes against viral infection

HIV-1

Ribozyme have the potential to act at a variety of point in HIV infection cycle-

➢   The entry of genomic viral RNA into target cell prior to reverse transcription.

➢   During transcription of structural and coding RNA molecule.

➢   Prior to and during translation of mRNA to viral protein.

➢   Prior to encapsidation of genomic RNA during virion formation.

The cleavage of HIV RNA at these stages can result in decrease in intracellular viral replication, extending cell survival[42]. HIV mutate very rapidly so ribozyme are synthesized for most conserved sequence of its genome. One of them is Ψ region and other is 5' region of the first coding exon of tat. Ribozyme targeted to these site results into inhibition of wide range HIV isolates[42]. Sarver and colleagues[43] showed inhibition of HIV replication by gag targeting ribozyme. Another group introduced ribozyme targeting HIV 5' leader sequence into a stable MT4 cell line by MoMLV based vector transfer and demonstrated an acquired resistance to HIV-1 replication. Ribozyme construct termed RZ2 was one directed to a site in first coding exon of tat[42]. When cloned in MoMLV based vector LNL6, this construct was designated as RRZ2.

For gene therapy using ribozyme CD34^{+} stem cells appear to be cell of choice. A simple approach to treat HIV infected patient might be the infusion of transduced CD4^{+} peripheral blood lymphocytes (PBLs). Studies on these cell types should provide the foundation for future gene therapy approaches that may be used to treat HIV infection.

HCV

Chronic infection with HCV can lead to cirrhosis, liver failure and hepato cellular carcinoma over a period of 20-30 years. A ribozyme against HCV, named HEPTAZYME, is in phase two clinical trials. HEPTAZYME and type 1 interferon in combination have a more potent antiviral activity than either compound individually.

Cancer

Ribozyme might be used to block tumorogenesis itself or might act at later stage to inhibit growth and metastasis. Angiogenesis is also required for sustained tumor growth[36], making VEGF pathway an attractive target. Of particular importance interest, are receptor protein tyrosine kinase Flt-1 (VEGFR-1) and KDR (VEGFR-2) which are expressed primarily on endothelial cells and activate cell division in response to VEGF. There are two ribozyme ANGIOZYME against Flt-1 and anti KDF against KDFR (44).

Lucien Bergeron and Jean Perreault (2005) reported molecular engineering of a ribozyme possessing new biosensor molecule. This biosensor in the presence of its target molecule renders the ribozyme cleavage activity from off to on status[45].

Allosteric ribozyme

Allosteric ribozyme are those ribozyme, which assume different conformation in response to various effectors. These effectors may be small molecule e.g. ATP or protein (tat). But these allosteric ribozyme can not be used in vivo because they relies on a third molecule for their function as well as they do not increase the specificity of ribozymes.

Lucien et al (2005) rationally designed a ribozyme, which has a biosensor module, which can recognize presence or absence of its target molecule. This biosensor module is a specific on/off adapter (SOFA) molecule. Original δ ribozyme (δRz) derived from hepatitis delta virus was used as suitable model[46,47]. SOFA includes three sequence segment- a blocker, a biosensor and a stabilizer. In absence of target molecule blocker forms intramolecular stem with p1 strand resulting in no cleavage. In presence of target biosensor anneal with substrate. Now P1 is free and subsequently hybridize with substrate, which result in to active conformer. Thus target has two roles

➢   Activator

➢   Substrate

This is the first report of a ribozyme, which harbors a safety lock. This new approach provides an improved tool with significant potential in gene therapy.

Numerous example which demonstrate that ribozyme can interfere with gene expression in a sequence specific manner, in vitro as well as in vivo, have been reported. Thus ribozyme have the potential to be developed as drugs or tools for elucidation for gene function. As therapeutic, ribozyme could be administered either by successive external application akin to chemotherapy or by endogenous transcription in a gene therapy fashion.

RNA aptamer

They are single stranded RNA molecules that assume specific 3D folded conformation to achieve high affinity binding interaction with the defined molecular target. The affinity and specificity of aptamer for their target often surpass that can be achieved with monoclonal antibodies[48]. Considerable efforts have been devoted for developing aptamers as therapeutic agents.

An anti VEGF aptamer, known as MACUGEN (pegabtenib sodium injection) was approved by FDA in Dec 2004 for treatment of age related macular degeneration. Similarly an anti-thrombin aptamer entered phase 1 clinical trial in 2004 and numerous other aptamer are currently in preclinical development[48]. Antiviral aptamer have been described for HIV-1, HCV, influenza virus and cytomegalovirus[48]. It is anticipated that therapeutic aptamer could be delivered as intercellular and intracellular agents. Intracellular expressed aptamers have proven their efficacy for protein antagonism in both prokaryote and bacterial system[49,50].

The possibility of forming alternative inactive secondary structure in aptamer transcript can be minimized by including self cleavage ribozyme with in expressed RNA adjacent to core target binding structural element. In this strategy self-cleavage of ribozyme releases aptamer from nascent transcript[51]. Intracellular expression of aptamer obliges the use of unmodified biological RNA. In contrast aptamer derived as extra cellular agent may contain non biological covalent modification e.g. 2'flouro and other substitute on ribose sugar, PO_4 backbone modification etc. They all stabilize RNA against degradation by serum nuclease while tethering a PEG or cholesterol moiety to aptamer reduces uptake by kidney and liver[48].

Inhibition of HIV-1 enzymatic function

Most of the anti HIV-1 drugs currently approved for use by FDA inhibit the function of either RT or PR, making these viral enzymes primary target of current therapeutic approaches[52,53].

Reverse Transcriptase: Retrovirus carries an RNA genome that is copied to double stranded cDNA by viral reverse transcriptase before integration into host genome. HIV-1 reverse transcriptase is released from pol polyprotien as a 66 KDa protein product that assembles into a P^{66}/P^{66} homodimer. Protease removes the C terminal 120 amino acid from one of the P^{66} subunit to generate mature P^{66}/P^{51} heterodimer[54].

The highly positively charged template-binding cleft of reverse transcriptase is well suited for aptamer binding. Atleast 300 different RNA and ssDNA aptamers have been isolated [48,54]. Nearly all RNA aptamers are predicted to fold into pseudoknot. Many of RNA aptamers from different structural classes binds reverse transcriptase (RT) with dissociation constant in low nanomolar range when assaying using gel shift and nitrocellulose binding[48]. RNA aptamer retain their RT-inhibitory effect in vivo, severely attenuating viral replication in cultured human T lymphoid cells. When Joshi and Prasad [51] expressed six different pseudoknot RNA aptamer in human 293-t kidney cells co-transfected with pro-viral HIV DNA, virion harvested from these cells demonstrated a 90-99% reduced capacity for subsequent infection. Dot blot analysis of RNA show that they were present in mature virion where they may already be bound to RT in position to inhibit next round of replication. Researchers have speculated that viable aptamer resistant mutant will be rare [48, 50].

Protease: HIV-1 protease (PR) plays a critical role in viral replication by processing the gag and pol polyprotein into their mature structural protein and enzymes. Anti PR drugs developed during last decade are associated with toxic side effects including lipodystrophy and hyperlipidimea due to cross reactivity with cellular protease [55,56]. Nucleic acid aptamer targeted to HIV-1 PR have not yet been reported. Nevertheless there is precedence for their potential utility based on the studies of aptamers selected to bind the NS3 protease of HCV. Nishikawa et al showed inhibition of NS-3 protease in HELA.

Integrase: HIV-1 integrase (IN), the 3rd of pol-encoded enzymes, catalyzes the insertion of a dsDNA copy of viral genome into preferred location within actively transcribed genes in the host chromosome. Aptamer to IN include several RNA species[48]. Allen et al obtained high affinity IN binding RNA aptamers, the best of which displayed Kd for IN of 2mM when assayed in presence of 50nM NaCl.

Inhibition of HIV gene expression [transcriptional regulation and RNA transport]

1. Rev: Rev facilitates the movement of partially or unspliced mRNA from nucleus to cytoplasm. Several aptamers have been generated with affinities of Rev that are 10 times greater than that of wild type RRE (Rev response element) [40,41]. Symensma et al found that several different Rev aptamer could direct the export of unspliced mRNA in a Rev dependent in vivo reporter system.

2. Tat: The Tat protein enhances the expression of viral genome by host RNA pol II. Tat binds directly to a stem loop structure near the 5'end of viral RNA known as Trans activator response element. Although its role as a transcriptional upregulator make it an ideal target for anti HIV therapeutics, the number of Tat inhibitory compound to reach clinical trial has been limited and not has yet been approved for use in patients[48]. The need for continued development of novel Tat inhibitor remains urgent, suggesting a utility for Tat aptamer in that role.

Inhibition of viral entry

gp140 and gp41: targeting the HIV-1 envelope glycoprotein (gp) or host cell receptor provides an opportunity for aptamer therapies without the need for intracellular delivery or expression from gene therapy vectors. As such it is conceivable that aptamer to viral gp could proceed more rapidly through testing in animal models (and reach clinical trial sooner) than expressed aptamers.

Aptamer to gp120 have been generated in several labs in recent years [57, 58]. James lab RNA aptamer libraries were stabilized against serum nucleases by substituting fluorine in place of OH at 2'position of pyrimidine nucleotides[57].

Aptamer in cancer

Constitutive high level of nuclear NF-kB activity has been described in many type of cancer cells and abrogation of constitutive NF-kB activity results in apoptosis of treated tumor cells [59,60]. Jing mi et al (2006) successfully produced an adenoviral vector to express the pure RNA aptamer of NFkB p50 protein termed A-P50. The expressed A-P50 effectively inhibits NFKB transactivation and induces apoptosis in both human lung adenocarcinoma cells and murine carcinoma cells and also delays tumor growth in human tumor xenograft model [59].

Aptamer inactivation of HIV-1

- IN
- gp120

- RT
- Rev
- Tat
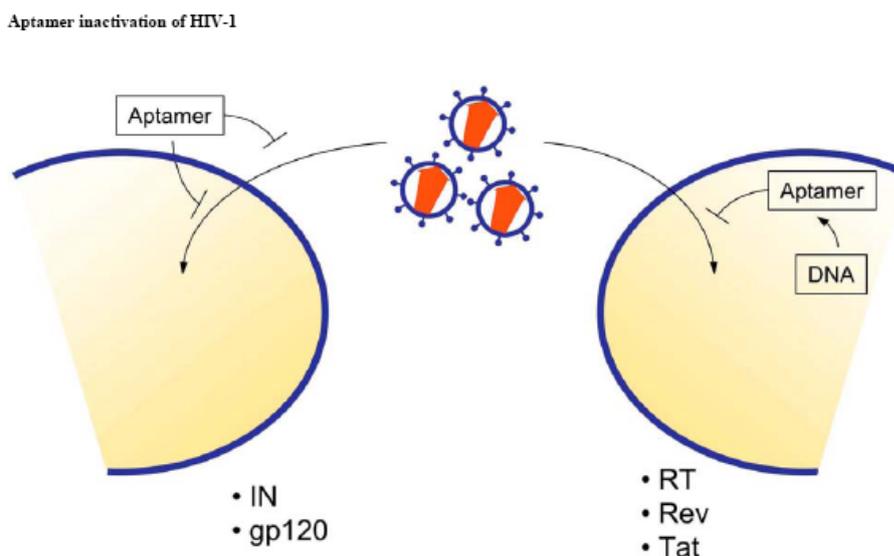
Fig. 4: Neutralization of HIV-1 replication by nucleic acid aptamer can be accomplished either through exogenous delivery (left) or through intracellular expression (right). Targets for which viral neutralization has been best demonstrated are noted below the corresponding pathway. (Kathi et al, 2003.)

Table 2: Anti-HIV-1 aptamers

Target	Aptamer	Kd (nM)	Viral inhibition
RT	RNA	0.025-20	10 to 100 fold[a,d]
PR[1]	RNA	10	NA
IN	RNA	2	NA
Rev	RNA	1.3	10 fold[a,c,d]
Tat	RNA	0.12-120	2 to 10 fold[a,c] 100 fold[c,d]
NC	RNA	0.5 to 50	2 fold[c,d]
gp120	2'F-RNA	5-100	10,000 fold[b,d]

(Daniel et al. 2006. Frontiers in Bioscience 11:89-112)

RNA decoy

RNA decoys are single stranded RNA molecule that assume specific folded structure and bind with high affinity to the active site of enzymes essential for replication and transcription. They contain essential cis acting element that bind trans acting proteins. They function by luring away trans acting proteins from their true target sequence. When expressed at high levels, they can successfully compete against viral cis acting sequences that are indispensable for viral replication. Decoys can also be devised to upregulate genes that are transcriptionally suppressed by the binding of a factor.

There are two RNA decoys, which target HIV-1 gene expression. These are tar (transactivator response element) RNA decoy and RRE (rev response element) RNA decoy. Sullenger and colleagues[48] achieved a 99% decrease (2 logs) in HIV-1 replication in cultured human T-lymphoid cell line expressing a tRNAmet-TAR fusion transcript. At least four additional studies demonstrate the efficacy of TAR RNA decoy for inhibition of viral replication in cultured cells. Two of these used constitutive promoters to express HIV-2 TAR or a nucleolus targeted U16 snoRNA-TAR chimera[48]. The other two systems used Tat regulated promoters to express the decoys. The transcript contained polymeric TAR motifs of up to 50 tandem repeats in one case, and an HIV-2 TAR/HIV-1 RRE hybrid transcript in the other[48].

Both the TAR and RRE RNA elements interact with several essential cellular proteins, including nuclear protein p140, cellular factor TRP-185, interferon-induced double-stranded RNA-activated protein kinase, the single stranded DNA binding protein and others[48]. While this circumstance raises the possibility of cytotoxicity resulting from expressed decoys, no detrimental effects on cell viability were observed during continuous expression of wild type and mutant TAR sequences in cultured T-lymphoid cells[61]. Because TAR and RRE decoy systems utilize viral RNA sequences, the probability of HIV-1 evolving escape mutations that evade this mode of inhibition would appear to be extremely low, as such an event would require simultaneous mutations in both the protein (Tat or Rev) and RNA (TAR or RRE) components.

Riboswitch

Riboswitches are specialized stretches of non-coding mRNA that can specifically bind to their cognate metabolites, inducing conformational changes and/or ribozyme activity that lead to physical occlusion of the ribosome, transcription attenuation, or self-cleavage of the mRNA. In this way, mRNAs can directly regulate the biosynthesis of protein products associated with the metabolites that bind them[62]. Like many other discoveries riboswitches were discovered serendipitously by Ron Breaker of Yale University in 1999. The first natural riboswitch was found in genes involved in vitamin biosynthesis. Investigations by Winkler et al and Nahvi et al found riboswitch regulation in B_1[63] and B_{12}[64] biosynthesis, respectively. Although at this point, most known riboswitches have been identified in bacteria, there is emerging evidence of these systems in archaea and eukaryotes as well, particularly in the case of the THI-box riboswitch [65,66].

Until very recently, metabolic regulation was thought to be carried out exclusively by proteins. Recently, however, the unchallenged supremacy of proteins in metabolic regulation has been called into question through the discovery of riboswitches.

In all cases, specific, high affinity binding of a metabolite effector to a highly conserved non-coding region of its cognate mRNA initializes a conformational change in the mRNA that leads to the formation of a stem-loop structure. Where this stem-loop forms, the characteristics of the region involved determine the overall effect of the binding. Figure 1 illustrates the known possibilities, which will be discussed below.
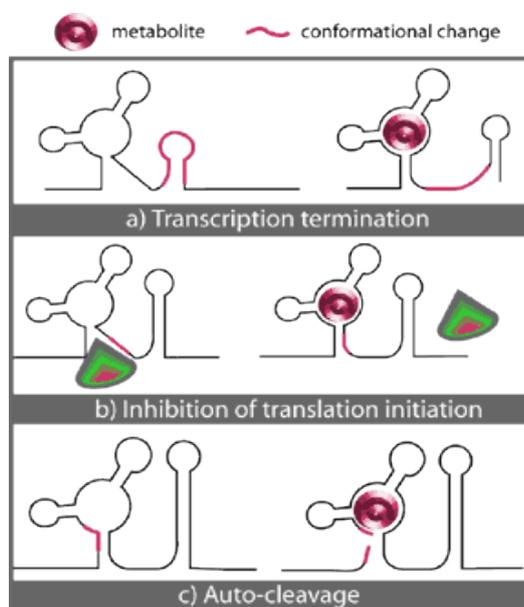

Fig. 5: Three known mechanisms of riboswitch action upon binding of metabolite (M): a) Transcription termination. b) Inhibition of translation initiation. c) Auto-cleavage. *Science creative quaterly, Issue6, year2011(74).*

Transcription-termination

In conditions of low effector concentrations, these transcripts have an anti-terminator in their secondary structure that prevents the formation of a terminator loop. Binding of a metabolite to a nearby riboswitch element in the mRNA induces a change in secondary structure that destabilizes the hairpin anti-terminator in the mRNA. As the anti-terminator dissociates, the sequence formerly part of the stem is revealed and allowed to pair with a terminator sequence. The resultant terminator loop is usually located in the 5' UTR and is often followed by a downstream polyuridine tract[67]. The terminator loop thus reduces the stability of either the mRNA:RNA polymerase interaction and/or of the DNA:RNA hybrid in a rho-independent manner[68]. This causes the RNA polymerase to dissociate, terminating transcription prematurely.

Inhibition of Translation Initiation

Alternatively, riboswitches may act at the level of translational control through the sequestration of the ribosome binding sequence in the mRNA. When no metabolite is bound, the Shine-Dalgarno (SD) site is exposed and the ribosome can bind and initiate translation. Binding of the metabolite to the 5' leader region of the mRNA induces the formation of an SD: anti-SD stem-loop structure that masks the ribosome binding site such that initial step of translation, binding of the ribosome to the mRNA, is not achieved.

Auto-cleavage

Auto-cleavage is the most recently discovered and possibly the most remarkable riboswitch mechanism known thus far. Whereas the previous two mechanisms have each been observed in multiple riboswitches and in a variety of bacterial systems, the only riboswitch with ribozyme action known to date is the *glmS*-box discovered by Winkler and his group as recently as 2004 [18].

Located in the 5' untranslated region of the *glmS* gene, this riboswitch is astonishingly specific to its ligand, glucosamine-6-phosphte, whose binding increases the rate of cleavage 1,000 fold[62]. The precise mechanism of cleavage remains unknown, but Winkler has proposed that it is accomplished through internal phosphoester transfer, which he noted has been studied in other known riboswitches[62]. In short, it seems that the conformational change induced by the binding of the ligand to the riboswitch brings adjacent nucleotides in line with each other in an orientation that favours cleavage.

Other Methods of Action

All of the riboswitch effects described above have related to repression of gene function upon binding of a specific metabolite involved somehow in that gene's function. It has, however, also been suggested that the same mechanisms could be involved in positive gene regulation as well [67, 39]. In these cases, binding of a metabolite would release the terminator hairpin or liberate the SD-site, permitting full transcription or translation, respectively. Although this type of control has not yet been observed in natural systems, there is no reason to rule it out.

There is also speculation that eukaryotic riboswitches have the potential for more complex methods of regulation. For example, it has been suggested the riboswitches could also potentially play a role in the processing and transport of mRNA in eukaryotes[39]. Again, this has not yet been observed, but bearing in mind how recent is the discovery of riboswitches, it may just be a matter of time. Our understanding of these elegant control systems is only just beginning to develop.

Yen et al 2004[73] have shown the upregulation of gene expression with the help of riboswitch. They embedded a riboswitch in a reporter gene. This interspersed RNA motif affects the cleavage of mRNA but in presence of its cognate regulator molecule prevents destruction of mRNA to produce protein. The injection of a reporter gene that contained a riboswitch into the eyes of mice resulted in moderate expression of the reporter gene product; subsequent injection of a small-molecule regulator increased the expression of the gene product by a factor of up to 190 in the eyes of the treated animals. Yen et al. went on to show that riboswitches might work when inserted into various other types of mRNA.

Thus, riboswitches can, in principle, be used to control the expression of a variety of gene products.

Riboswitch	Ligand	Precursor	Mechanism	Related Studies	Found in
B12-box	Ado-Cbl (Adenosylcobalamin)	B12	Transcription termination, inhibition of translation initiation	25, 26, 28, 33, 42	*E. coli, S. typhimurium, Bacillus/Clostridium, Thermotogales, Chloroflexus.*
thi-box	TPP (thiamine pyrophosphate)	B1 (thiamine)	Transcription termination, inhibition of translation initiation, dual action, inhibition of RNA processing in archea/fungi	17, 23, 24, 32, 37, 46	*R. etli, B. subtilis, E. coli, Deinococcus, Chlorobium, Thermoplasma.*
rfn-box	FMN (flavin mononucleotide)	B2 (riboflavin)	Transcription termination, inhibition of translation initiation	24, 41, 45	*B. subtilis, Deinococcus, cyanobacteria, Chloroflexus.*
S-box	Methionine	SAM (S-adenosyl-methionine)	Transcription termination, inhibition of translation initiation	8, 22, 47, 48	*B. subtilis, Petrotoga, Chloroflexus, Deinococcus, Geobacter.*
G-box	Purines (guanine)	n/a	Transcription termination	20	*B. subtilis, Clostridium, Fusobacterium, Vibrio vulnificus.*
L-box	Lysine	n/a	Transcription termination, inhibition of translation initiation	10, 20, 13, 30, 38	*Bacillus, Clostridium, Fusobacterium, Thermoanaerobacter.*
glmS box	GlcN6P (glucosamine-6-phosphate)	n/a	RNA cleavage	49	*B. subtilis, B. cereus, Thermoanaerobacter tengcongensis.*

Fig. 6: Overview of the riboswitches and its mechanism of Action [from 75,76,77,78,79,80]

So how, might the riboswitches be used in the clinic? In the setting of gene therapy, the expression of therapeutic proteins must be tightly and properly controlled for most gene therapies to be efficacious and for many of them to be safe in complex organisms such as humans. For example, for the genetic treatment of type-1-diabetes, the ability of transferred genes to modulate insulin production by means of riboswitches that sense and respond to glucose levels should not only improve the efficacy of insulin therapy but also curb the side effects that would be expected to result from the inappropriate expression of insulin.

However, as with the development of any new therapy, a number of major issues must be addressed before riboswitches can be evaluated in the clinical setting. First, riboswitches that respond to clinically relevant drugs or metabolites must be developed and shown to function when they are incorporated into clinically relevant genes. Next, riboswitches must be tested in a variety of animal tissues. And finally, as with all gene-therapy strategies, methods for the efficient and safe delivery of riboswitches into endogenous genes or as part of exogenously administered therapeutic genes must be developed and optimized. Future studies that address these issues will ultimately reveal whether riboswitches can fulfill their clinical potential.

RNA-based approaches used in the clinics

A clinical trial involving RNA decoys was conducted in a pediatric population using an RRE decoy to modify CD34+ bone marrow cells. Kohn et al. (1999) showed that retroviral mediated CD34+ cell transduction had no significant adverse effects and that leukocytes containing an RRE decoy could be isolated from peripheral blood, even one year post-infusion; however, the numbers of transduced cells were extremely low[69]. In 2004, Amado et al. demonstrated long-term maintenance of a therapeutic transgene in a phase I clinical trial involving ribozymes[69]. They used MoMLV vector virus transduction of CD34+ heamopoetic stem cells for introduction of a ribozyme targeted to highly conserved regions in the HIV- 1 *tat* and *vpr* genes. Ten patients participated in the study and researchers could detect the vector in naïve T cells for up to three years, the last time-point evaluated. There was an average increase of 10 CD4+ T cells per µl from the beginning of the trial until the third year. In six patients, viral loads decreased by an average of 2.25 logs. Three patients had undetectable viral loads. One patient showed an increase of one log.

More recently in 2005, Macpherson *et al.* published the results of a phase I clinical trial on ribozymes involving identical twins with discordant HIV status[69]. Again, one twin acted as the donor (HIV-negative) and the other was the recipient (HIV-positive) of genetically engineered CD4+ T cells expressing a ribozyme. Specifically, the cells were transduced with an anti-HIV-1 *tat* ribozyme (RRz2) and a control LNL6 retroviral vector (for cell marking). Patients were followed initially for 24 weeks and then at regular intervals over a 4-year period. Peripheral blood cells (PMBs) containing both RRz2 and LNL6 were detected consistently. However, the effect of this therapy on HIV-1 viral load or the CD4 count was not specifically addressed.

Lastly, Morgan et al. (2005) published data from a clinical trial involving anti-sense RNA in conjunction with a transdominant negative protein[69]. This study employed 10 pairs of twins. Like the earlier study involving twins with discordant HIV status, one twin served as the donor (negative) and the second twin as the recipient (HIV-positive). Lymphocytes from the donors were transduced to express a control gene (*neo* gene) or anti-HIV gene(*s*); a transdominant mutant Rev protein (TdRev) was used alone or with an anti-sense element directed against the HIV-1 TAR sequence on the same construct. Polymerase chain reaction demonstrated increased survival of modified lymphocytes in the initial weeks post-infusion in 9 of 10 recipients. In six of six recipients followed for approximately two years, T cells containing anti-HIV genes could be consistently detected and there was preferential survival of modified cells in one patient during a period of high HIV load[69] beside this can also be one of the potent therapy against HIV along with the other therapies mentioned by researchers[70].

Potential opportunities of RNA based therapies

There is an extraordinary opportunity for development of RNA based therapeutics with the completed human genome providing ample target sequences. It is already possible to suppress dominant disease genes in vitro by using RNA based therapies, and in some cases, this suppression has been allele-specific, silencing the disease-causing allele while maintaining expression of the normal allele. The benefits of RNA based therapies are:

➤ RNA based therapeutics can be rationally designed to block the expression of any target gene, including genes for which traditional small molecule inhibitors cannot be found.

➤ High specificity, as RNA based therapies specifically inhibit production of single proteins.

➤ RNA based therapies lend themselves to customization more readily than many other drugs.

➤ RNA based therapeutics can persist longer than traditional drugs in cells, leading to bi-weekly or weekly dosing regimens.

➤ Due to their sequence specificity, RNA based therapeutics have dramatically improved toxicity profiles compared to the chemotherapy.

➤ Relative ease of synthesis and low cost of production (compared to proteins such as antibodies or recombinant growth factors at the concentration needed for therapeutic effects), makes RNA based therapeutics an attractive new class of drugs.

In principle, RNA based therapies might be used to treat any disease that is linked to elevated expression of an identified gene. This might make these approaches suitable for combating viral diseases, cancer, and inflammatory diseases. However, there is a disparity between achieving such results in vitro and in a whole animal or patient. Although use of RNA based therapies have been explored in a large number of disease models, clinical trials are in progress only in the following conditions:

➤ Age-related macular dystrophy

➤ Respiratory syncytial virus

➤ Parkinson's disease

➤ Cancer

➤ HIV/AIDS

➤ Hepatitis C virus infection

➤ Amyotrophic lateral sclerosis

Given sufficient improvement in delivery methods, some of these diseases will probably eventually be treated effectively by RNAi-based therapeutics.

CONCLUSION

Within the last few years, RNA based therapeutics have made considerable progress and some products have entered clinical trials. Currently the major use of RNA based therapeutics is in reagents for research and drug discovery. The future potential is in clinical applications. The specific complementary base pairing, specific catalytic properties, the ability to sense micro-environmental signals and property of assuming different secondary structure to regulate gene expression make RNA based therapeutics specific, potent and conditional. The major obstacle is the targeted delivery of these molecules inside cells and stability in serum but with the emergence of application of nanoparticle in drug targeting, these problems can be solved. RNA based therapeutics interacts with technologies such as drug delivery and molecular diagnostics and will play a role in the development of personalized medicine.

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