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RNA Interference (RNAi) - An antiviral Strategy for Enteroviruses

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Abstract

Human enteroviruses are a genus of RNA viruses which affects millions of people worldwide each year. They are associated with wide spectrum of diseases ranging from mild respiratory illness like the common cold, hand, foot and mouth disease (HFMD) to more severe neurological and cardiac complications which are fatal. Currently, there is no an effective vaccine or specific antiviral treatment to prevent or treat non-polio enteroviral infections. Since its discovery in 1998, RNA interference has emerged as a potential therapeutic strategy against infectious diseases. In this review, we focus on the developments of using RNAi to prevent or treat enteroviral infections, highlighting the potential of RNAi as a potential antiviral strategy against enteroviruses.

Keywords: Enteroviruses; Wide spectrum human diseases; RNA interference; Potential antivirals

Introduction

Human enteroviruses are a genus of positive-sense, single-stranded RNA viruses classified under the Picornaviridae family and are associated with several human and mammalian diseases. On the basis of their pathogenesis in human, enteroviruses were originally classified into four subgroups, Polioviruses, Coxsackie A viruses (CA), Coxsackie B viruses (CB), and Echoviruses (Echo). This original classification is then later revised within the Enterovirus genus into these four major phylogenetic clusters: cluster A (CA16-like viruses, including Enterovirus 71 (EV71), cluster B (CB-like group containing all CB, including CA9 and EV69, as well as echoviruses), cluster C (poliovirus-like viruses) and cluster D (EV68 and EV70) [1,2]. Serologic studies have distinguished 66 human enterovirus serotypes on the basis of antibody neutralization tests. Currently, there are 62 non-polio enteroviruses that can cause disease in humans: 23 Coxsackie A viruses, 6 Coxsackie B viruses, 28 echoviruses, and 5 other enteroviruses [3].

Human enteroviruses affect millions of people worldwide each year, and are often found in the respiratory secretions and stool of an infected person. Poliovirus, as well as Coxsackieviruses and Echoviruses, are mainly spread through the fecal-oral route. The stability of enteroviruses in the acidic environment allows them to be ingested and inhabit the alimentary tracts of humans and animals [4]. Upon infection of the cell, the single positive-strand genomic RNA is translated in a cap-independent manner into a single polyprotein, which is subsequently processed by virus-or host encoded proteases into the structural capsid proteins and the non-structural proteins, which are mainly involved in the replication of the virus [5]. Infection can result in a wide variety of symptoms ranging from mild respiratory illness like the common cold, hand, foot and mouth disease (HFMD), acute hemorrhagic conjunctivitis, acute flaccid paralysis, aseptic meningitis, myocarditis and severe neonatal sepsis-like disease which could be fatal [3].

Poliovirus is probably the most well known enterovirus which still poses a threat in developing countries. Poliovirus is highly contagious via the oral-oral (oropharyngeal source) and fecal-oral (intestinal source) routes and causes poliomyelitis. Two different vaccines, the inactivated polio (IPV) developed by Jonas Salk (licensed in 1955) and the oral polio (OPV) developed by Albert Sabin (licensed in 1963) have been available for several decades and are effective in providing individual protection against poliomyelitis. However, it was noted that the attenuated poliovirus used for OPV is genetically unstable and reverts to neurovirulence and causes vaccine-associated paralytic poliomyelitis (VAPP) in vaccinees or results in transmissible vaccine-derived poliovirus (VDPV) strains. In addition, OPV recipients with an inborn immunodeficiency can become asymptomatic long-term excretors of immunodeficient vaccine-derived poliovirus (iVDPV) [6]. As such, we are still far from the endgame of the poliovirus and more effective antiviral strategies may be needed for the eradication of poliovirus.

While efforts to eradicate poliovirus through vaccination programs have limited the number of polio-endemic countries to just a few (Afghanistan, India, Nigeria and Pakistan) [3], EV71 has recently emerged as a medically important non-polio, neurotropic enterovirus with worldwide distribution. EV71 was first isolated from patients with central nervous system diseases in California between 1969-1974 [7]. Since then, EV71 outbreaks have been reported in several countries beyond North America, including Bulgaria [8], Hungary [9], and more recently Taiwan [10], Australia [11], Malaysia [12] and Singapore [13]. EV71 outbreaks have also been associated with a variety of severe neurological complications that can deteriorate rapidly to involve cardiopulmonary failure with high mortality rates [14,15]. The spectrum of neurological diseases caused by EV71 includes aseptic meningitis, brain stem encephalitis and poliomyelitis-like acute flaccid paralysis [16]. Similar to poliomyelitis, severe EV71 infections may also result in permanent neurological damage. During the largest EV71 outbreak to date, more than 100,000 children were affected in Taiwan, 1998, with 405 severe cases involving neurological or cardiopulmonary

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complications, of which 78 were fatal [10]. It has been estimated that more than 3.0 million cases of EV71 infection occur worldwide every year, and the death rate associated with the more severe form of EV71 encephalitis is approximately 0.15%. Furthermore, 2.8 billion of people are at risk for EV71 infection around the world in the absence of effective intervention.

Despite the significant morbidity and mortality rates, particularly in immunocompromised patients and children, there is no an effective vaccine or specific antiviral treatment to prevent or treat non-polio enteroviral infections to date.

RNA interference (RNAi)

Since the first report on inhibition of respiratory syncytial virus (RSV) in 2001 [17], RNAi was shown to hold promise as potential antiviral therapeutic agents. By targeting viral transcripts and/or host genes which produce co-factors critical for viral replication, many successful studies were reported using RNAi against a wide range of viruses both in vitro and in vivo, including Human Immunodeficiency virus [18-20], Hepatitis C virus [21-23] and the influenza virus [24].

RNAi is a defense strategy which was first described in Caenorhabditis elegans by Fire et al. [25]. He first stumbled upon RNAi when they found that dsRNA introduced into C. elegans silenced expression of a homologous target gene approximately 10–100 folds more efficiently than the corresponding antisense RNA. RNAi is a sequence-specific, post-transcriptional gene silencing process of initiated by double-stranded RNA (dsRNA) molecules [25], and the machinery has been best characterized in Drosophila melanogaster. The first step involves Dicer which has an N-terminal helicase domain, a Piwi/Argonaute/Zwille (PAZ) motif, a dsRNA-specific binding domain and two RNase III motifs at the C-terminus. The dsRNA-specific endonuclease activity of the Dicer will cleave the dsRNAs to produce functional siRNAs. These siRNAs are subsequently loaded into the RNA-induced silencing complex (RISC) [26]. The loading of the siRNAs into the RISC requires the RISC loading complex (RLC) that contains the DCR2 (one of two Dicer molecules) and a dsRNA-binding domain-containing protein, R2D2 [27]. R2D2 is involved in binding to the more thermodynamically stable strand of the siRNAs (passenger strand), orienting the DCR2 to bind to the less thermodynamically stable strand of the siRNAs (guide strand) [28]. Argonaute 2 (Ago2), which is a core component of the RISC and is also the endonuclease that cleaves the target mRNA, will then bind to the siRNA and displaces it from the RLC component [29,31]. The unwinding of the siRNA is facilitated by Armitage which is a DEAD-box helicase in an ATP-dependent manner [28]. The Ago2 then cleaves the passenger strand, preparing the way for the guide strand to pair with the complementary target mRNA sequence [32,33]. The nucleotide positions 2 to 8 of the siRNA guide strand are exposed on the surface of the RISC and form the seed sequence that directs the target recognition [34,35]. The paired siRNA-mRNA is thought to form an A-type helix that aligns the cleavage site (10 nucleotides from the 5'-end of the guide siRNA) on the target mRNA with the Ago2 PIWI endonuclease domain [36,37]. The Ago2 cleaves the phosphodiester bonds in the target mRNA in the central region of the paired siRNA-mRNA complex in a Mg^{2+}-dependent manner [38,39]. The mutations of the key nucleotides that disrupt siRNA-mRNA pairing within this central region will interfere with the cleavage but have no effect on the binding of the siRNA guide strand [40]. After the target mRNA is cleaved, the activated RISC containing the siRNA guide strand will be released to direct subsequent rounds of target mRNA cleavage [41] (Figure 1).

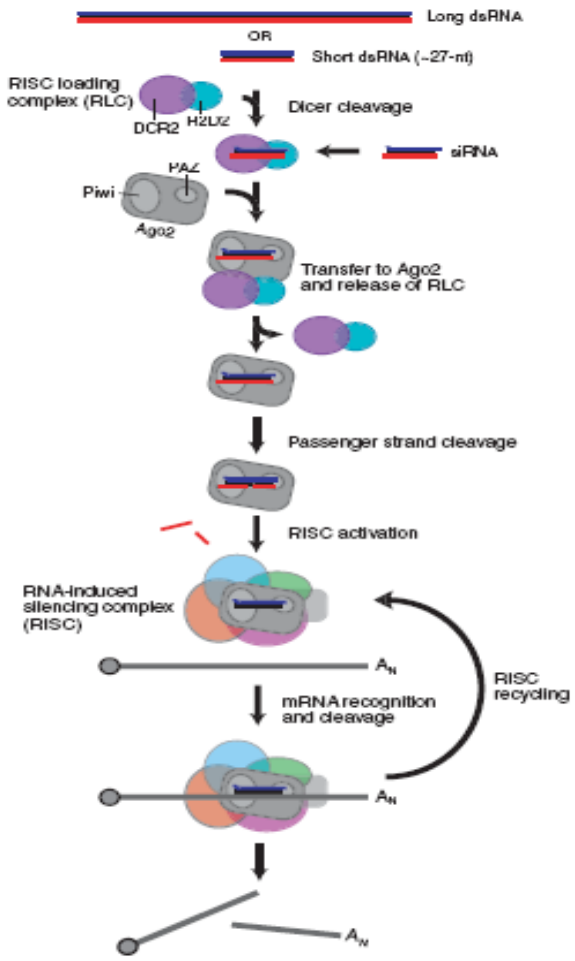

Figure 1: The RNA interference pathway. RNAi is triggered when a cell encounters a long double-stranded RNA (dsRNA). The dsRNA will be processed into 21 nucleotide small interfering RNAs (siRNAs) by an RNase III-like enzyme known as Dicer (known as Dicing). The siRNAs will then unwind and the sense strand will be degraded. The antisense strand will be assembled into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). The antisense strand will subsequently guide the RISCs to complementary mRNA molecules, where they will then cleave the mRNAs (known as Splicing) [87].

RNAi against enteroviruses

Polioviruses: One of the earliest reports on the potential of siRNAs on enteroviral infections was by Gitlin et al. [42] on poliovirus. Two 19-mer siRNAs were evaluated, targeted at the viral protein 3 (VP3) and the non-structural RNA dependent RNA polymerase (3D) regions. The results showed almost complete inhibition of poliovirus replication with both siRNAs [42]. In another study by Gitlin et al. [43], it was reported a single nucleotide change within the siRNA was reported to be capable of completely abrogating the functioning of the siRNA [43]. This is phenomenal especially in RNA viruses like enteroviruses, which mutates easily as a result of selective pressure. Gitlin et al. [43] overcame this problem by using a mixture of siRNAs targeted at different sites on the poliovirus genome, thus preventing escape mutants.

Echoviruses: Echoviruses are responsible for a broad variety of diseases including fever, mild rash, and mild upper respiratory tract (URT) illness, aseptic meningitis, neonatal carditis, encephalitis,

hepatitis, and diseases of the upper respiratory tract [44,45]. Previous studies have reported that Echovirus 30 (Echo 30) was the most frequent serotype isolated from samples of patients from aseptic meningitis in France and Spain [46,47]. A previous report by Rothe et al. [78] evaluated thirty 19-mer siRNAs and 5 of them which were targeted at the 3D region showed high efficiency in inhibiting Echo 30 replication. In a more recent study, the same group reported the use of viral-vector encoded multiple short hairpin RNAs (shRNAs) targeted at both 3D region and an important cellular cofactor of Echo 30 replication and achieved more than 90% silencing effects on Echo 30 replication [49].

Coxsackie viruses: Coxsackie viruses are generally classified into Coxsackie A (CA) and B (CB) viruses, causing a relatively wide range of diseases ranging from the milder cold, HFMD to the more serious complications such as myocarditis, pericarditis, meningitis and pancreatitis. Coxsackievirus A16 (CA16) is one of the main causative agents of Hand, Foot and Mouth Disease, a disease which commonly affects children below 6 years old and prevalent mainly in the Asia Pacific Region [50]. In a study by Wu et al. [51], a total of 13 plasmid-encoded siRNAs targeted against different conserved regions of the CA16 were identified to block CA16 replication in in vitro system. It was also shown that a combination transfection of these 13 siRNAs produced a higher inhibitory effect.

CB4 has been known to elicit a wide variety of diseases, from asymptomatic infection, undifferentiated febrile illness, or mild upper respiratory symptoms, to more severe disease symptoms recognised by the presence of fever, chest pain, pleural inflammation, headache and sore throat [52]. Infections by CB4 have also been known to cause aseptic meningitis, encephalitis, pleurodynia, myocarditis, and pericarditis [53]. However, the most significant chronic disease associated with CB4 infection is the juvenile onset of insulin dependent diabetes mellitus (IDDM) [52]. In 2010, our group employed RNAi to treat CB4 infections in in vitro system [54]. Three 19-mer siRNAs were designed to target at 2C, 3C and 3D regions of CB4. All the 3 siRNAs showed high efficacy in inhibiting CB4 replication, and the siRNA targeted at 3C region was shown to be the most effective [54].

Coxsackie virus B3 (CB3) is probably the most widely studied enterovirus using RNAi strategy. CB3 infections have been associated with different forms of subacute, acute, and chronic myocarditis, causing cardiac arrhythmias and acute heart failure [55]. In some cases, the myocardial inflammation may persist chronically and progress to dilated cardiomyopathy, requiring heart transplantation [56]. Since 2005, there were a total of 13 studies, employing different strategies of RNAi targeting different regions of the CB3 genome or their receptors being reported (Table 1). Majority of the studies were based on siRNAs which targeted the 3D region and showed high efficiency in CB3 inhibition both in in vitro and in vivo systems. Two of the studies used Locked Nucleic Acid (LNA)-locked siRNAs to target the highly structured but conserved 5UTR region, complementing the previous study by Schubert et al. [57], who suggested that the efficiency of siRNAs depends greatly on their accessibility to the target sequences [57,66,67].

Enteroviruses

Enterovirus 70 (EV70) is widely recognized as the main causative agent of acute haemorrhagic conjunctivitis (AHC), a highly contagious viral disease [70], and some cases eventually develop non-ophthalmic symptoms such as neurological dysfunction resembling paralytic poliomyelitis as well as respiratory and gastrointestinal disturbances [71]. Two studies were reported previously on the use of 19-mer siRNAs targeted at 3D region and have shown good efficacy in inhibiting EV70 replication [72,73]. In the latter report, the siRNAs was also shown to be able to inhibit CA24, another common causative agent of AHC [73].

EV71 is the main causative agent of HFMD in young children, together with CA16. It is often associated with neurological complications and has caused high mortalities in recent outbreaks in the Asia Pacific region. Previous studies using 19-mer siRNAs targeted at various regions of EV71 genome have shown high inhibitory effects on the virus [75,76]. In 2007, an enhanced anti-EV71 effect was reported by Tan et al. [77], who used 29-mer shRNAs to target 2C, 3C and 3D regions of EV71 in in vitro system. In all the studies, 3D region was the most effective region for the RNAi effect [77]. In 2008, the same group reported that both 19-mer siRNAs and plasmid-encoded siRNAs were able to treat EV71 infections in suckling murine model [78]. However, despite showing enhanced potency in inhibiting EV71 infections in the in vitro system, the 29-mer shRNAs failed to protect the mice from EV71 infections in the in vivo model [78].

Advantages of RNAi as antiviral drugs

As a therapeutic tool, RNAi has been shown to be more efficient than "traditional" anti-viral drugs because of its ease of use, high efficiency and specific modes of action. In most of the studies reported so far, the 3D region of the enteroviral genome was the main target gene. The 3D region encodes the viral RNA-dependent RNA polymerase which oligomerizes into a complex and subsequently binds to the viral RNA. Since the 3D gene and the other cellular factors form an important component in facilitating viral replication, its down-regulation could produce the most potent inhibitory effect on enteroviral replication [77].

No.	RNAi strategy used	System tested	Target region	Reference
1	Plasmid-encoded dual siRNAs	In vitro	3D	57
2	19-mer siRNAs	In vitro	2A	58
3	19-mer siRNAs	In vitro and in vivo	2A	59
4	19-mer siRNAs	In vitro	3D	60
5	19-mer siRNAs	In vitro	3D	61
6	Plasmid-encoded shRNAs	In vitro and in vivo	3D and VP1	62
7	Plasmid-encoded shRNAs	In vitro	CAR gene coding for CVB3 receptor	63
8	Viral-vector encoded shRNAs	In vivo	2C	64
9	Viral-vector encoded shRNAs	In vitro and in vivo	3D	65
10	19-mer siRNAs and siRNAs containing locked nucleic acids (LNAs)	In vitro	3D and 5′UTR	66
11	siRNAs containing locked nucleic acids (LNAs)	In vitro	5′UTR	67
12	Multiple 19-mer siRNAs	In vitro	3D	68
13	19-mer siRNAs and plasmid-encoded siRNAs	In vitro	3D and CAR gene coding for CVB3 receptor	69

Table 1: Previous studies reported on use of RNAi technology on Coxsackievirus B3 (CB3).

All results so far showed that technology exploits a well-characterized endogenous pathway which allows silencing of virtually any infectious targets, including those which were regarded as "undruggable" [79]. Pharmaceutical development of RNAi-based drugs has also be shown to be greatly reduced as compared to that of traditional drugs, which are mainly proteins, small molecules and antibodies (2 to 3 years compared to 4 to 6 years) [79].

A variety of approaches have been developed for silencing gene expression. Most notable are antisense oligonucleotides (ASOs), ribozymes, and RNAi [80,81]. All the approaches involve the recognition of a specific mRNA target site by complementary oligonucleotides, but the mechanisms in silencing the genes differ from one another. Similar to RNAi, ASOs silence gene expression by either inhibiting translation or directing mRNA cleavage [80]. However, unlike RNAi, where the degree of target site homology determines the mode of action, the charged characteristics of the ASO backbone largely determine the silencing mechanism [82,83]. Although studies that compare the different silencing approaches are limited, they generally have found that siRNAs silence gene expression more effectively than ASOs or ribozymes. Direct comparison of an optimized phosphorothioate-modified ASO with a siRNA directed against the same target mRNA site found that the siRNA was approximately 100 to 1000 folds more efficient. The siRNAs have also been found to have longer sustained silencing [84]. This could be due to the protection of the siRNAs from intracellular degradation by its incorporation into the RISC. Virtually, any gene can be specifically and efficiently silenced by RNAi. In comparison, ASO approaches have only been found to work effectively in a limited number of cases. In fact, some ASOs that showed early promise as effective therapeutic agents were found to accomplish their antiviral effects by stimulating an innate immune response owing to their high guanine-cytosine (GC) content, rather than by specifically silencing target gene expression [81].

Conclusion

Up til today, infectious diseases remain a major challenge for modern medicine. This is especially true in the case of viruses, due to their high mutation rates which allow them to escape immune systems and become resistant to the traditional antiviral drugs. This is where the potential of RNAi technology to revolutionize the treatment of viral infections lies. Indeed, within a relative short time since the discovery, RNAi has progressed so rapidly that there are several RNAi-based drugs which have been filed with Food and Drug Administration (FDA) and currently undergoing different phases of clinical trials. The first being Cand5, a siRNA used to treat age-related macular degeneration (AMD) in 2004. This was followed by few other RNAi-based antiviral drugs against viruses such as HIV-1, RSV and hepatitis C virus [85,86]. With the wider acceptance in clinical applications, RNAi technology proves to be an extremely invaluable tool as modern drugs to treat human diseases.

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