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Broad-spectrum and virus-specific nucleic acid-based antivirals against influenza

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1. ABSTRACT

Rapid increase in drug-resistant influenza virus isolates, and pandemic threat posed by highly pathogenic avian influenza A and swine flu viruses provide clear and compelling reasons for fast tracking development of novel antiviral drugs. Nucleic acid-based drugs represent a promising class of novel antiviral agents that can be designed to target various seasonal, pandemic and avian influenza viruses. Nucleic acids can be designed to elicit broad-spectrum antiviral responses in the host, by suppressing viral gene expression, or by inducing cleavage or degradation of viral RNA. Immunomodulating nucleic acids, such as double stranded RNA and CpG oligonucleotides, can be potent anti-influenza agents that work by eliciting protective innate and adaptive immunity in the host. By activating the toll-like receptor signaling pathways, these drugs can activate the host's antiviral and inflammatory defenses to combat influenza viruses. Antisense oligonucleotides, small interfering RNAs (siRNA), and nanoRNAs represent sequence specific gene-silencing approaches that could be deployed to suppress or inhibit viral protein gene expression. Lastly, catalytic nucleic acids such as DNAzymes and/or ribozymes can suppress viral replication by repeatedly cleaving viral mRNAs and template RNAs. In summary, nucleic acid-based antiviral agents are versatile, diverse and could complement existing antiviral drugs in combating influenza.

2. INTRODUCTION

Influenza and influenza-related complications are among the leading causes of death from infectious diseases in the elderly and people with underlying medical conditions (1). The present global crisis with the highly pathogenic avian H5N1 influenza and swine flu (H1N1) virus exemplifies the devastating effects of influenza on public health, agriculture and the economy. Should HPIA, which has a human case fatality rate exceeding 80% in some regions (2), develop efficient human to human transmission, it has the potential to cause a pandemic that could be more deadly than the Spanish flu pandemic. The major challenges to stockpiling anti-influenza drugs to combat future influenza pandemics caused by HPIA are the steady increase in resistance to oseltavimir (3) and the low efficacy for post-exposure drug treatment of symptomatic patients (4). Furthermore, the number of seasonal influenza A virus isolates resistant to oseltamivir has sharply increased. The Center for Disease Control and Prevention (CDC) reported that more than 98.5% of the H1N1 influenza viruses isolated in the 2008-09 influenza season in the USA were oseltamivir-resistant (5). The divergence and antigenic shift/drift in influenza viruses represent significant challenges for the development of effective antiviral drugs against influenza viruses. These challenges present opportunities for the development of novel anti-influenza drugs which are more robust, less likely to give rise to drug-resistance and are broadly effective.

Table 1. Prophylactic activity of Poly ICLC, LE Poly ICLC and IFNs against various strains of influenza A viruses in mice

Group	INFLUENZA STRAIN	LD_{50}	% SURVIVAL VS CONTROL
Poly ICLC	A/PR/8/34 (H1N1)	10	100% vs 0%
	A/Aichi /2 (H3N2)	10	100% vs 0%
	Henan/2005 (H5N1)	10	ND
LE Poly ICLC	A/PR/8/34 (H1N1)	10	100% vs 0%
	A/Aichi /2 (H3N2)	10	100% vs 0%
	Henan/2005 (H5N1)	1	100% vs 50%
	Henan/2005 (H5N1)	5	67% vs/ 0%
IFN-α	A/PR/8/34	10	50% vs 0%
IFN-γ	A/PR/8/34	10	50% vs 0%

ND = not determined

Rapid advances in the fields of molecular biology, genomics, nucleic acid chemistry and drug design have accelerated the development of nucleic acid-based drugs for the prevention and treatment of various cancer and infectious diseases. Since nucleic acids are naturally occurring macromolecules present in all cells, and highly diverse in their regulation of cellular functions, they represent important and comprehensive strategies for antiviral prevention and treatment.

Preclinical studies in experimental animals have demonstrated the safety and efficacy of various classes of nucleic acid-based antiviral drugs against seasonal and avian influenza viruses (6-8). These antiviral agents are versatile in their modes of action, diverse in their molecular structures and designs, and are currently in various stages of preclinical and clinical development. Although none of these drug candidates have been approved for the influenza market, it is expected that some of these leading compounds will be commercially available when clinical studies are successfully completed. The purpose of this review is to highlight the experimental applications of nucleic acid-based antiviral agents, particularly against influenza virus infections. Nucleic acid-based drugs are classified into 3 main categories based on their modes of antiviral activity: a) immunomodulating nucleic acids, b) gene-silencing nucleic acids, and c) catalytic nucleic acids. The following sections will provide examples for each class of nucleic acid-based antiviral agents, and will describe their mode of action, stability and antiviral efficacy assessed by *in vitro* tissue culture and/or *in vivo* animal infection model systems.

Furthermore, since nucleic acids are intrinsically susceptible to nuclease degradation in the body, protection against nucleases by optimizing nucleic acid backbone chemistry will be briefly discussed. The use of liposomes to protect against nucleases, to provide specific targeting of nucleic acids to intracellular sites of infection and to cells of the innate immune system has been extensively reviewed elsewhere (9). For a comprehensive review of the recent developments in the delivery of nucleic acids in liposomes, nanocapsules, viral vectors, please refer to recent article by Christopher and Wong (9).

3. IMMUNOMODULATING NUCLEIC ACIDS

Nucleic acid-based immunomodulators are designed to stimulate the host's innate immune system to combat invading viruses. The ability of these drugs to elicit broad-spectrum antiviral immunity is of particular importance in the prevention and treatment of influenza infection. The enhancement of innate, cellular immunity and antiviral activity has the potential to protect against seasonal and avian/swine strains of influenza viruses, regardless of genetic mutation, reassortment, recombination, zoonotic origin or drug-resistance. Nucleic acid-based drugs that can stimulate the host's immune responses against viral infections include CpG containing ODNs (10) and dsRNA such as Poly ICLC (11). These drugs are TLR agonists (7, 12) and are in various stages of clinical development.

Activation of TLR signaling pathways plays a pivotal role in the host's innate and adaptive immune system and in their ability to recognize pathogen derived nucleic acids. Of particular relevance are the TLRs recognizing nucleic acids, which include TLR-3 (dsRNA), TLR-7 (single stranded RNA; ssRNA), TLR-8 (ssRNA) and TLR-9 (unmethylated CpG motif-containing sequences) (13). In recent years, TLRs and TLR agonists have become a hot area in anti-infective drug design. In addition to binding TLR-3, dsRNAs activate key enzymes that play a central role in the host antiviral state, including interferon-inducible protein kinase R (PKR), 2′-5′ oligoadenylate synthetase (2′- 5′ OAS) (14), RIG-I Helicase, and MDA5 (15). It follows that dsRNA may play a critical role in arming the innate immune and inflammatory pathways to fight viral infections.

3.1. Double stranded RNA (Poly ICLC)

Wong *et al.* first reported the use of Poly ICLC to elicit a prolonged antiviral effect in mice against a lethal respiratory infection by influenza viruses (16). Poly ICLC, a synthetic, double-stranded polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine and carboxymethyl cellulose, is a potent immunomodulating agent (6, 7, 11). Elicitation of broad-spectrum antiviral immune responses by Poly ICLC is mediated by recognition and interaction with TLR-3 (7). This recognition of dsRNA by TLR-3 induces the production of interferons (IFN) -alpha, -beta and -gamma *in vivo* (7, 11, 13). This signalling pathway also accounts for stimulation of both innate and adaptive immune responses, including the activation of natural killer cells (14). When encapsulated in liposomes, Poly ICLC (LE Poly ICLC) was found to be broadly effective against a number of seasonal and avian influenza viruses (6, 7). Pre-treatment with two intranasal doses of 1 mg/kg body weight given 48 hrs apart was found to fully protect mice (100% survival rate) against various lethal challenge doses of mouse-adapted influenza A/PR/8/34 (H1N1) or influenza A/Aichi/2 (H3N2) virus, while all non pre-treated control mice succumbed to the infection ($p < 0.01$ vs. control) (Table 1; 7). LE Poly ICLC was also

found to provide a high level of protection to mice against highly pathogenic H5N1 avian influenza A virus (6, 7). Results from this study suggested that two intranasal doses of LE Poly ICLC (1 mg/kg body weight) provided high to complete protection against a lethal virus challenge with 1-4 LD_{50} of influenza H5N1 avian influenza virus, and 67% protection against 5 LD_{50}. This suggests that LE Poly ICLC has the potential to protect against a variety of influenza strains, possibly including H1N1 swine flu influenza. The windows of protection provided by Poly ICLC and LE Poly ICLC were compared by pre-treating mice with 2 intranasal doses (1 mg/kg/dose) of Poly ICLC at day 1 to day 21 prior to infection with 10 LD_{50} of influenza A/PR/8/34 virus. Mice pretreated with Poly ICLC were fully protected from the lethal virus challenge, provided the pre-treatment was administered within 12 days prior to virus challenge (6, 17). The window of protection provided by LE Poly ICLC was extended, with mice fully protected when the LE Poly ICLC pre-treatment was given at day 21 prior to virus challenge (6, 17). This suggests that LE Poly ICLC treatment could be effective in outbreak situations even if the causative strain has not been conclusively identified.

3.2. CpG oligonucleotides

In 1995, Kreig *et al* reported that ODNs containing the unmethylated (bacteria DNA-like) CpG motif were able to induce murine B cells to proliferate and secrete immunoglobulin *in vitro* and *in vivo* (18). CpG ODNs primarily stimulate B-cells and dendritic cells (DCs) through the constitutively expressed TRL-9 receptor (19). Human memory B-cells (high TRL-9 expressing), but not naïve B cells (low TRL-9 expressing), are stimulated to proliferate and differentiate to immunoglobulin-secreting cells in response to CpG. DCs form a link between the innate and acquired immune systems with stimulation of DCs shifting the balance from a Th-2 humoral response to a Th-1 cellular response. CpG motifs stimulate peripheral blood DCs (but not monocyte-derived DCs) and, in doing so, induce maturation, transiently increase antigen processing, and increase the half-life of peptide-MHC-II complexes, thus sustaining subsequent presentation (20, 21). Immune cells from both immature and aging animals reveal that the Th-2 humoral response forms a disproportionately large component of the immune response. After treatment with CpG, maturation of DCs from immature mice was hastened and CpG treatment of aging mice resulted in recovery and enhancement of Th-1 type immunity (22, 23).

In a lethal murine influenza model, a K-type CpG ODN (0.25 mg/kg) was observed to protect against 10 LD_{50} influenza A/PR/8/34 viral challenge when given 4 d prior to infection (24). K-type CpG ODNs possess a phosphorothioate backbone and multiple TCGT/A motifs. CpG ODNs have been shown to be effective in protecting against a variety of bacterial and viral infections and have also been shown to be effective adjuvants when administered with inactivated influenza virus, influenza protein or plasmid-based vaccines (reviewed in 25), thus possibly expanding the number of individuals that could be vaccinated in times of vaccine shortfall. Efficacy testing of CpG oligonucleotides against HPIA has yet to be reported.

4. GENE SILENCING NUCLEIC ACIDS

Gene-silencing is a highly specific approach for inhibiting the expression of selected key proteins involved in disease initiation or progression. This innovative technology allows for the specific suppression of viral replication in the host by targeting key viral proteins required for the virus life cycle. Initially, it was thought that the most effective mechanism for inhibiting viral RNA processing or translation was achieved through the use of a ssRNA such as antisense ODN hybridizing to viral mRNA (26, 27). It is now known that post-transcriptional gene silencing using dsRNA can be a very potent alternate means to silence expression of viral proteins. This process, first identified in *C. elegans* and designated RNA interference (RNAi), was initially performed with long dsRNA molecules which also induced unwanted immunological activity. For therapeutic application these problems can be avoided by using synthetic small interfering RNA (siRNA) that mimic the cytoplasmic product of the RNaseIII Dicer.

4.1. Antisense oligonucleotides

Antisense ODNs are ssDNA or ssRNA sequences that are complementary to specific target sites in mRNA. The experimental usage of antisense ODNs against influenza viral infections has been reported in both *in vitro* tissue culture as well as *in vivo* animal infection model systems (28-34). The rationale drug design for antisense ODNs against influenza has focused on viral gene targets that are less prone to antigenic shift and/or drift. Therefore, the highly variable regions of the mRNAs encoding haemagglutinin (HA) and neuraminidase (NA) are usually not considered to be suitable gene targets. However, the polymerase (PA, PB1, PB2) and nucleoprotein (NP) genes of influenza contain highly conserved regions which provide a number of potential broad range antisense target sites (30-32). One of the early key studies involved the development of a transient influenza gene expression assay by sandwiching a chloramphenicol acetyltransferase (CAT) gene between the 5' and 3' terminal sequences of influenza A/PR/8/34 RNA segment 8 (30). RNA from this expression vector was transfected into clone 76 cells into which ODN was delivered using a cationic lipid complex (31). Using either CAT expression or cell viability assays as measures of antiviral activity, antisense ODNs directed against PB2 were consistently found to be the most potent inhibitor of influenza replication and gene expression (30-32). Antisense ODNs directed against PB1, PB2 and PA were administered intravenously as cationic lipid complexes with Tfx-10 or DMRIE at twice daily intervals 1 day prior to, and 4 days post-infection in a lethal murine influenza A/PR/8/34 infection model (32, 33). An antisense ODN targeted to the AUG region of the PB2 gene, consistently increased both the mean survival time and survival at day 14 post-infection (45%). Concurrent with increased survival was a decrease in PB2 mRNA expression and viral titer measured in infected lungs 4 days after infection (32).

Antisense ODNs (15-mer) directed against a conserved region of the influenza A virus HA gene have also been shown to be effective in the post–exposure

treatment of influenza A virus infection in mice (6). In this study, both un-encapsulated and liposome-encapsulated antisense ODNs, administered intranasally, were completely effective in the treatment of mice against an otherwise lethal respiratory challenge. There was a significant therapeutic advantage achieved with liposome encapsulation of antisense ODNs: the total dosage of antisense ODNs required to achieve complete protection was reduced by up to 80% when liposome encapsulated ODNs were used (35). The level of dose reduction was presumably due to the protection of the antisense ODNs against nuclease degradation in the body, as well as enhanced delivery of the antisense ODN to the intracellular sites of viral replication.

A number of chemical modifications have been introduced to antisense ODNs to increase stability and decrease the non-specific effects of the phosphorothioate backbone. In phosphorodiamidate morpholino ODNs (PMOs), the ribose moiety is converted to a morpholino group and the charged phosphodiester internucleoside linkage is replaced by an uncharged phosphorodiamidate linkage. The resulting uncharged molecule is nuclease resistant, unable to recruit RNaseH, has reduced non-specific side effects and poor cellular uptake, the latter problem being alleviated by conjugation to arginine-rich peptides (36). Unlike antisense ODN, siRNA and nRNA that prevent translation by degradation of the mRNA, PMOs prevent translation by sterically blocking the translation initiation complex (37). In 2006 Ge *et al.* evaluated PMOs conjugated to arginine-rich peptides for their ability to inhibit influenza A/PR/8/34 virus (H1N1) replication in cell culture. These PMOs targeted sequences critical to the influenza A life cycle. Several PMOs were highly efficacious and two PMOs, targeted to the PB1 AUG translation start site and to the 3'-terminal region of viral NP RNA, proved to be potent against several other strains, including A/WSN/33 (H1N1), A/Memphis/8/88 (H3N2), A/Eq/Miami/63 (H3N8), A/Eq/Prague/56 (H7N7), and the highly pathogenic A/Thailand/1(KAN-1)/04 (H5N1), suggesting that PMOs may represent a broad-spectrum approach against influenza (38). *In vivo* studies in mice demonstrated that PMO administration improved survival from influenza infection from 0% to 30-95%, with the survival rate dependent on the dose, timing of PMO administration, mRNA/vRNA sequence targeted by the PMO and the viral strain (39, 40).

4.2. Small interfering RNA (siRNA)

RNAi is an endogenous sequence specific gene-silencing mechanism that evolved to control the expression of pathogenic nucleic acids such as transposons, retroviruses and other viruses (41). RNAi also provides the framework for processing microRNA, the cells own gene silencing molecules. This natural form of RNAi is used by eukaryotic cells to regulate cellular gene expression. The application of RNAi for silencing viral gene expression in mammalian hosts has been a subject of intense research in recent years. As a result, a number of siRNAs with promising antiviral function in tissue culture and animal infection model systems have been identified and a number of these have found their way into clinical trials. RNAi-based therapies targeting various respiratory viruses, including seasonal and avian influenza viruses, were recently reviewed extensively (42, 43), and are not revisited here.

4.3. NanoRNAs (nRNAs)

NanoRNAs are ssODNs containing a 2′-O-methyl modification and a 3′-butanol tag that increases *in vivo* stability of the ODN (44). Comprehensive studies have demonstrated a range of features of nRNAs including being stable to heat, acid and base, resistance to nucleases, rapid and strong binding to target RNAs and ability to disrupt the secondary structures of target RNAs (44, 45). The most significant advantage of nRNAs is that these RNA ODNs can be used for therapeutic purposes in combinations containing more than two nRNAs, which can target either different sites within a single gene or different genes involved in a defined disease pathology (46, 47)

In anti-influenza applications, nRNAs were shown to be able to suppress viral replication both *in vitro* and *in vivo* (48). At the cellular level, three nRNAs targeting the NS1 gene of H5N1 influenza virus, termed NS2, NS27, NS526, and a mixture of the three NS nRNAs (NS mixture), together with a random control were transfected into chicken embryo fibroblast cell monolayers (48). After 4 h the cells were infected with 5 $TCID_{50}$ of influenza H5N1 virus (m.o.i. of 0.2). Twenty hours post infection, culture supernatants were harvested for HA assays. Compared with the ODN and virus controls whose HA assay titers were 52.4 and 55.9 respectively, the cells treated with nRNAs all showed decreased titers, with the HA assay titers for NS2, NS27, NS526, and NS mixture being 7.3, 2.4, 5.1 and 3.2 respectively ($P<0.01$) (48). In order to investigate the *in vivo* activities of these nRNAs, an animal challenge was performed in 20 day old special pathogen free chickens that were randomly divided into groups of eight. Ten nanomoles of nRNA (NS2, NS27, NS526, NS mixture and control) or saline were sequentially mixed with Lipofectamine™ 2000 and influenza H5N1 virus (2 LD_{50}) and the nRNA/Lipofectamine™/virus mixtures were immediately administrated intranasally into chickens. Animals were given two more doses of nRNAs 24h and 48h post-infection. The clinical symptoms and mortality rate were recorded over the experimental period. The study demonstrated that while all the nRNAs exhibited some degree of delay in clinical symptoms in chickens, the NS mixture and NS526 nRNA protected the animals from H5N1 virus-induced death at a rate of 87.5% and 50%, respectively, compared with the virus control group ($P<0.05$) (48). This suggests that multiple nRNAs may exert conformational interference to the NS mRNA transcript, potentially exposing target sites that may be buried in the RNA tertiary structure. To confirm that the inhibitory effect of the nRNAs on H5N1 virus replication was due to a direct action on the viral RNA, real-time PCR analysis on total lung RNA isolated 24 h post-infection was performed. This analysis detected viral RNA in the nRNA mix-treated group only at very high cycle numbers (close to the assay limit), while a high level of the viral RNA was observed in the control group. Plaque assays performed on lung tissue collected at the same time corroborated the

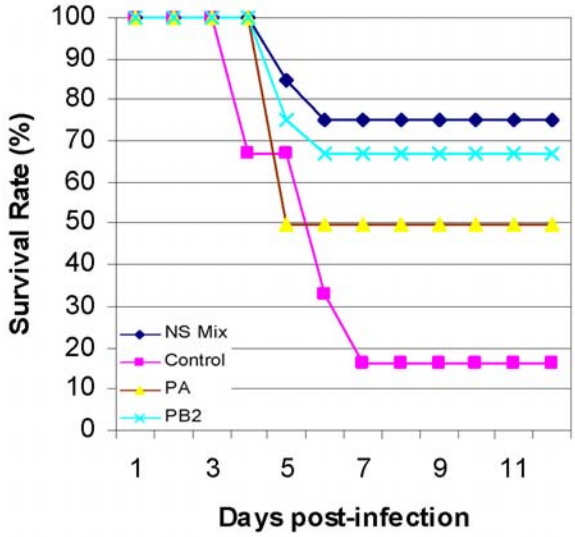

Figure 1. NanoRNAs and protection of H5N1 virus infection in mice. NS mixture, PA or BP2 nRNAs were mixed with Lipofectamine^{TM}2000. The nRNA:liposome mixture, together with H5N1 virus ($2LD_{50}$), were intranasally administered to the mice at time 0. The nRNA:liposome mixture was administered again at 24 h post-infection. The control was an irrelevant molecule with the same chemical modifications (2'-O-methyl and 3'-butanol tag). Survival rate is calculated by surviving animals/total animals x100 (n=8).

significant difference between nRNA mix and control-treated chickens with pfu's of control-treated chickens being significantly higher than that of nRNA mix-treated chickens (48). This clearly demonstrated that it was possible for nRNAs to suppress H5N1 virus replication by directly targeting the viral RNA in a sequence-specific manner.

Although influenza H5N1 virus replicates favourably in chickens, their natural host, without need for viral adaptation, such ovine models differ substantially from mammalian systems in many aspects. Thus the efficacy of nRNAs against H5N1 was also tested in a mammalian model. The efficacy of nRNAs was assessed in mice using NS-targeted nRNAs. Using a similar protocol to that used in chickens (Lipofectamine^{TM}/nRNA/virus administration at time 0 followed by a single treatment 24 h post-infection) it was demonstrated that mice could be protected from H5N1 infection by treatment with NS gene-targeted nRNAs (Figure 1). Seventy five percent of mice receiving NS mix survived the viral challenge compared to 12.5% for those receiving control ODN.

5. CATALYTIC NUCLEIC ACIDS

Catalytic nucleic acids which can be designed and expressed to cleave viral genetic materials are novel tools which can be employed to combat viruses. They can be classified either as RNA (ribozymes) or DNA (DNAzymes).

5.1. Ribozymes

Ribozymes are catalytic RNA molecules possessing enzymatic cleavage and ligation activities (49, 50). Although there are several types of ribozymes with unique catalytic mechanisms, the two primary ribozymes used for antiviral applications are hairpin and hammerhead ribozymes. These have been extensively investigated because of their relative simplicity, small size and the ability to alter the RNA-targeting sequences (flanking the active site) without loss of catalytic activity. The hammerhead ribozyme is derived from the satellite RNA strand (+) of the tobacco ringspot virus (TobSV) and its sequence consists of: (i) a conserved 22-base catalytic region, (ii) base-pairing flanking arm sequences and (iii) a recognition sequence on the target RNA (e.g. CUC). Cleavage occurs 3′ to the recognition sequence and results in a 2′, 3′ -cyclic phosphate and a 5′-hydroxyl terminus on the 3′-fragment. A two-dimensional hairpin structure is formed by the (-) strand of the sTobSV with the catalytic site located within 4 helical segments. The hairpin ribozyme catalytic moiety consists of a complex of 4 helices and 5 loop regions formed between the 50–base ribozyme and 14 base substrate sequence region. Cleavage occurs 5′ to a bNGUC recognition sequence (where b is C,G or U and N is any base) (51).

In nature, ribozymes are involved in intra-molecular RNA processing (cleavage) during rolling circle replication or splicing and are therefore *cis*-acting. However, by removing the enzyme-substrate stem loop and altering the substrate recognition sequences, ribozymes have been altered to cleave in *trans* a wide range of viral (and other medically relevant) target RNA molecules (52). While theoretically a ribozyme containing any sequence recognition site could be designed, empirical *in vitro* studies have revealed basic rules of ribozyme design. Hammerhead ribozymes for example can cleave any 5′-NUH-3′ where N is any nucleotide, U is conserved and H can be A, U, C, but not G. As reviewed by Sun *et al* (51), the work of several groups has revealed that k_{cat} decreases in the order AUC, GIC>GUA, AUA, CUC>AUU,UUC,UUA>GUU, CUA>UUU, CUU and this order of decreasing efficiency can, in general, be applied to levels of gene expression affected by ribozyme cleavage as well.

Ribozymes (hammerhead, hairpin and RNAse P) have been designed to cleave a number of viral targets in cell culture assays against a range of viruses including: HBV (53), HCV (54), HIV (55), HPV (56) and Influenza A (57, 58). Ribozymes (both hammerhead and ribonulease P) directed against various sites in influenza A PB1, PB2 and NP genes have been shown to inhibit viral particle production and or viral protein expression (57-59). In virtually all cases cells were transfected with plasmid or transduced with retrovirus or adenovirus vectors. Delivery in this manner resulted in endogenous expression of the ribozyme and potential co-localization with the viral RNA target.

In another therapeutic approach, ribozyme ODNs have also been synthesized and delivered exogenously.

However, the biological lability of RNA is a major limitation to therapeutic application *in vivo*. To overcome this, a range of chemical modifications have been employed to resist nuclease degradation. Typical ribonucleotide modifications include phosphorothioate linkages, 2′-O – substitutions such as; 2′-O-methyl, 2′-O-allyl-, 2′-fluoro-, 2′-amino- modifications and the 3′-3′ inversion, or combinations thereof, have been employed (60). Such modifications allow direct delivery (intravenous or direct tissue injection) of intact ribozyme and extend the biological half-life as much as 14,000-fold or more *in vivo* (61, 62). Recently a modified hammerhead ribozyme directed against influenza A has been developed and patented, although no data describing its anti-influenza activity has yet been reported (63).

5.2. DNAzymes

RNA-cleaving catalytic DNA enzymes (DNAzymes) are entirely synthetic and derived from selection protocols developed to evolve DNA sequences capable of cleaving RNA (64). The most efficient and well characterized molecules in this class are the "10-23" DNAzymes which resulted from this *in vitro* selection protocol. They are general-purpose enzymes capable of cleaving RNA at almost any purine-pyrimidine junction (65). The structure of the DNAzyme is reminiscent of the hammerhead ribozyme with a 16-mer 3′-AGCAACCATCGATCGG-5′ catalytic core flanked by binding arms.

The antiviral activity of the 10-23 DNAzyme has been examined against a range of viruses in cell culture experiments. Suppression of viral replication has been observed using DNAzymes directed against the HIV-1 tat, rev (66) gag (67) and env genes (68), the HBV X gene (69) and the HCV 5′-NCR (70). In a study describing the antiviral activity of 10-23 DNAzyme against the influenza A PB2 gene, >90% viral inhibition was observed (71). Surprisingly, few descriptions of potential gene targets for anti-influenza activity have been reported using either ribozyme or DNAzyme, and to date no studies have described selection of regions of the influenza genome that represent the most conserved sequences across all of the known virus subtypes. Such a strategy could produce a therapeutic enzyme less likely to be rendered ineffective by mutation of the viral genome. Furthermore existing studies have not considered the impact of target RNA secondary structure, which has been shown to have a significant impact on the activity of these molecules (72). The identification of both highly conserved and highly susceptible target site in the influenza genome will no doubt be an important step forward in the development of broadly effective therapeutic candidate.

6. PERSPECTIVES

Influenza viruses are extremely adaptable and potent infectious agents which are ever changing and unpredictable. Constant mutation, genetic reassortment and recombination ensures that new influenza viral variants will emerge and continue to pose public health threats. The global crisis involving HPIA and swine flu influenza virus are examples of this challenge. While immunization with the appropriate vaccines remains the most practical first line of defense against seasonal and potential pandemic influenza viruses, there are a number of unresolved issues and challenges with the production and effectiveness of these vaccines which necessitates the development of novel anti-influenza drugs. Vaccines, due to development of immunological memory, can be given up to years prior to exposure to the agent they are designed to protect against. However, influenza vaccines are administered on a yearly basis due to the changes in strain types circulating from one year to the next. Vaccine-mediated protection against influenza is designed to involve a humoral response. Towards this end, novel anti-influenza drugs which are versatile, potent and can respond to constant genetic changes in the viruses will need to be added to the arsenal of existing drugs to confront new influenza variants.

Nucleic acid-based therapeutic drugs represent a promising new class of antiviral agents. They offer significant therapeutic advantages over conventional antiviral drugs because of their versatility and specificity. This review has highlighted a wide range of applications for which ODNs are seeking employment in antiviral therapy. Immunomodulating nucleic acids, DNAzymes & ribozymes, and antisense ODNs are three main classes of nucleic acid based antiviral agents being evaluated in clinical studies. Double stranded RNA (such as poly ICLC) and CpG ODNs may provide broad-spectrum protection against new and drug-resistant influenza virus variants by virtue of the enhancement of the host's innate and adaptive immune responses. DNAzymes and ribozymes are catalytic nucleic acids that cleave viral mRNAs while antisense ODNs cause highly sequence specific and exquisite inhibition of viral gene expressions. These drugs, due to stimulation of the innate immune system or direct action on the virus, are designed to be administered close to the time of infection, unlike vaccines. Thus these drugs could be given at the start of an outbreak to help protect those not immunized against the outbreak strain, or could be given shortly after infection. However, in order for these agents to meet expectations, a number of significant issues will need to be addressed. The biological stability of the first generation phosphorothioate ODNs, the safety of second and third generations of chemically modified ODNs, and their general delivery and transport into intracellular sites of infection are the focus of intense research and development efforts. These efforts have been greatly aided by advances in synthetic medicinal chemistry resulting in better design of ODNs with improved stability and fewer side effects. These are currently being evaluated in clinical trials. Formulation improvements have also been made to enhance intracellular delivery of ODNs to sites of viral replication. Liposomes, nanoparticles and adenovirus delivery have all been successfully formulated to optimize antiviral efficacy of nucleic acid-based drugs and vaccines. As more of these novel formulations enter into clinical studies, it is anticipated that there will be an exponential growth in the number of nucleic acid-based therapeutics approved to combat seasonal, avian and pandemic influenza viruses.

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Abbreviations: CAT: chloramphenicol acetyltransferase; DC: dendritic cell; ds: double-stranded; CpG: unmethylated CpG motif; HA: haemagglutinin; HPIA: highly pathogenic influenza A; IFN: interferon; LD_{50}: lethal dose 50%; LE Poly ICLC: liposome-encapsulated Poly ICLC; m.o.i.: multiplicity of infection; NA: neuraminidase; NP: nucleoprotein; PA, PB1 and PB2: proteins comprising influenza virus polymerase; PMO: phosphorodiamidate morpholino ODNs; Poly ICLC: polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose; RNAi: RNA interference; siRNA: small interfering RNA; ss: single-stranded; TCID: tissue culture infectious dose; Th: T helper cell; TLR: toll-like receptor; ODN: oligonucleotides; .

Key words: Influenza, Nucleic Acids, Antiviral, Innate Immunity, Antisense, Gene Silencing, Catalytic, Immunomodulation, Protection, Pandemic, Review

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