Available online at www.sciencedirect.com
SCIENCE DIRECT
Antiviral Research 71 (2006) 31–41
Antiviral Research
www.elsevier.com/locate/antiviral

Inhibition of respiratory syncytial virus in cultured cells by nucleocapsid gene targeted deoxyribozyme (DNAzyme)

Yuan-Yuan Xie [a], Xiao-Dong Zhao [a], Li-Ping Jiang [a], Hong-Li Liu [d], Li-Jia Wang [a], Ping Fang [a], Kun-Ling Shen [b], Zheng-De Xie [b], Ya-Ping Wu [c], Xi-Qiang Yang [a,*]

[a] *Division of Immunology, Children's Hospital, Chongqing University of Medical Sciences, Chongqing 400014, China*
[b] *Division of Virology, Peking Children's Hospital, Peking 100021, China*
[c] *Division of Pathology, City of Hope National Medical Center, Duarte, CA 91010, USA*
[d] *Institute of Immunology, Third Military Medical University, Shapingba, Chongqing 400038, China*

Received 5 July 2005; accepted 27 February 2006

Abstract

Respiratory syncytial virus (RSV), which presents the primary cause of bronchiolitis and pneumonia among infants and causes significant morbidity and mortality in immunodeficient patients, remains a health problem worldwide. Unfortunately, an effective vaccine is currently unavailable and pharmacologic treatment needs further optimization for RSV disease. Because RSV is a non-segmented negative-strand RNA virus, it may be sensitive to the genome RNA cleaving by DNAzyme, an artificial nucleic acids molecule with high catalytic capability of cleaving complementary RNA molecules. Thus, RSV-targeted DNAzymes potentially present as a therapeutic candidate of RSV diseases. In this study, DNAzymes targeting the RSV genomic RNA or mRNA were designed and synthesized, one of which (DZn1133) did cleave RSV RNA in vitro, inhibit the transcription and expression of F viral gene, reduce the RSV yield by about 7 logs and protect more than 90% RSV-infected Hep-2 cells from a cytopathic effect at 8 μM. Moreover, 10 wild RSV strains isolated from clinic patients including both subgroups A and B were all suppressed by DZn1133 with greater anti-RSV activity than antisense DNA or ribavirin.

Keywords: Deoxyribozyme (DNAzyme); Respiratory syncytial virus; Antiviral; Nucleocapsid

1. Introduction

Respiratory syncytial virus (RSV) is the one of the most important pathogens of respiratory tract infections in the pediatric population worldwide (Collins et al., 2001; Hall, 2001; Klig and Shah, 2005), particularly problematic among infants that are born prematurely or with congenital lung diseases or heart diseases (Collins and Pollard, 2002; Walsh et al., 2004). Furthermore, RSV can be considered a prime candidate for the development of long-term pulmonary sequelae, because 70% of children with RSV lower respiratory tract infections, even whose initial illness did not require hospitalization (Long et al., 1995), experience impaired pulmonary function for up to 10 years afterwards (Schwarze et al., 1997).

Although the importance of RSV as a respiratory pathogen has been acknowledged for over 30 years and the highly prioritized research on RSV vaccines has spanned nearly 4 decades, unfortunately a successful vaccine remains elusive and the only prophylactic therapies available are RSV-IVIG and palivizumab antibodies (Groothuis et al., 1993; Johnson et al., 1997). While effectively reducing the hospitalization rate (The Impact-RSV Study Group, 1998), these prophylactic administrations are problematic and costly and, therefore, only recommended for use in high-risk infants (Meissner et al., 2004; Stevens and Hall, 2004). To date, the nucleoside analog ribavirin, licensed for the treatment of RSV infection, is the only clinically available anti-RSV drug. Despite evidence that ribavirin-treated patients have experienced fewer subsequent sequelae, the antiviral efficacy of ribavirin in infants with RSV needs to be further confirmed and the significant effect on long-term pulmonary morbidity is still under debate (Law et al., 1997; Ventre and Randolph, 2004). Thus, the development of novel pharmacologic treatment holds the promise of reducing morbidity, mortality and

* Corresponding author. Tel.: +86 23 6363 2756x3514/24479; fax: +86 23 6362 4479.
E-mail address: xi_qiang_yang@yahoo.com.cn (X.-Q. Yang).

0166-3542/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2006.02.011

chronic consequences caused by RSV (Chavez-Bueno et al., 2005; Domachowske and Rosenberg, 2005).

Currently, viral genome destruction represents an attractive target for therapeutic intervention, particularly for non-segmented negatively stranded RNA virus such as RSV, because arresting virus replication by cleavage of viral genome would be desirable (Zhang et al., 2005). Single-stranded DNA can also fold into well-defined, sequence-dependent tertiary structures, specifically bind to a variety of target sequences, and exhibit catalytic activities (Santoro and Joyce, 1997) similar to those of ribozymes or RNases (Fedor, 2000; Raj and Liu, 2003). The 10–23 type deoxyribozymes (DNAzymes), selected via in vitro evolution from a DNA combinatorial library, are artificial active nucleic acids with high catalytic capability of cleaving complementary RNA molecules (Santoro and Joyce, 1997). They consist of two antisense arms and a catalytic core of 15 nucleotides (nt), which bind the target RNA in a sequence-specific manner through Watson–Crick base pairing and cleave it between a paired pyrimidine base and a free purine base (Cairns et al., 1999; Cairns and Sun, 2004).

In our previous study, the first report of the application of DNAzymes as an anti-RSV agent, the inhibition of RSV A2 strain in cultured cells by the subgroup A RSV NS2 gene targeted DNAzyme, DZ604, was demonstrated, which achieved a reduction of virus yield by only 2 logs at 5 μM and could not effect the subgroup B RSV (Zhao et al., 2003a). In this study, with the aim to develop a universal efficacious DNAzyme covering both subgroups A and B RSV, four different DNAzymes targeting the RSV N, M2 and F genes were synthesized. One of these DNAzymes, DZn1133, effectively inhibited viral replication, completely eliminated virus yield and dramatically protected cells from the RSV-induced cytopathic effect (CPE) with higher efficacy than ribavirin.

2. Materials and methods

2.1. DNAzymes design and synthesis

According to Santoro and Joyce (1997), two universal target sequences in N and M2 genes (genomic RNA) and two individually target sequences in F genes (mRNA) of subgroups A and B were identified for DNAzymes. The corresponding DNAzymes (DZn1133, DZm7601, DZfa1612 and DZfb1630) are shown in Table 1. The structures of DZn1133, an inactive control (muDZn1133) of DZn1133 with single-base mutation in the catalytic core (muDZ T4 → A) (Zaborowska et al., 2002), a 19 nt antisense oligonucleotide (ASn1133) targeting the same sequences of DZn1133, and the target RNA sequence of RSV A2 stain genomic nucleotides 1133 ± 9 are shown in Fig. 1A–C. All the oligonucleotides were chemically synthesized using an ABI 392 DNA/RNA synthesizer. To increase the stability of these oligonucleotides, the three bases at the 3′ end and two bases at the 5′ end were substituted with phosphorothioate and the 3′ end was further cholesterol conjugated to increase cellular uptake (Fig. 1D). These modified oligonucleotides were employed both in the in vitro cleavage assays and cell culture assays.

2.2. Cell culture and virus production

Hep-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), 2 mM L-glutamine, 100 unit/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. The RSV standard strain CH18537 (kindly gift from Prof. Qian Yuan, Capital Pediatric Research Institute, Peking, China), strain A2 (stored in our lab) and 10 isolated wild strains (six from Chongqing Children's Hospital of China and four from Peking Children's Hospital of China provided by Prof. Kun-Ling Shen, Table 2) were grown in Hep-2 cells and purified by centrifugation on discontinuous sucrose gradients (Ueba, 1978) before the viral titer was determined by the end-point dilution method in Hep-2 cells. Viral identifications were confirmed by virus-specific monoclonal antibody (Chemicon International Inc. catalog no. 3105, USA), and the subgroups of the isolated wild strains were determined by semi-nest PCR as described previously (Zhao et al., 2003b). To confirm the target sequences of DZs in the wild strains of RSV, cDNAs were obtained from genomic RNA containing the target sites, DNA sequencing was performed subsequently.

2.3. In vitro DNAzyme cleavage assays

To assess the cleavage activity of DNAzymes to the target sequence of RSV, in vitro cleavage assays were performed. The cDNAs of N, M2 and F genes were obtained by RT-PCR from genomic RNA or mRNA of RSV (A2 strain and CH18537 strain) using specific primers, and were subcloned into the plasmid of pBluescript II KS+ (Stratagene, La Jolla, CA) before in vitro transcription with T7 polymerase using MAXIscript kit (Ambion, Austin, TX). Five picomole transcript RNA (final con-

Table 1
Targets and sequences of oligonucleotides

Target gene	Cleavage site	Oligonucleotide	Sequence
N gene of both subgroups	1133 ± 9 on A2 genomic RNA	DZn1133	5′−TGGGGCAAAGGCTAGCTACAACGAACAAAGATG −3′
M2 gene of both subgroups	7601 ± 8 on A2 genomic RNA	DZm7601	5′−AACTGGGGGGCTAGCTACAACGAAAATATGT −3′
F gene of subgroup A	1630 ± 10 on A2 mRNA	DZfa1630	5′−CAATTAATGAGGCTAGCTACAACGAAACAATATTA −3′
F gene of subgroup B	1612 ± 10 on CH18537 mRNA	DZfb1612	5′−CTATTAATGAGGCTAGCTACAACGAAACAATACTA −3′

Bases in the frames are the catalytic cores.

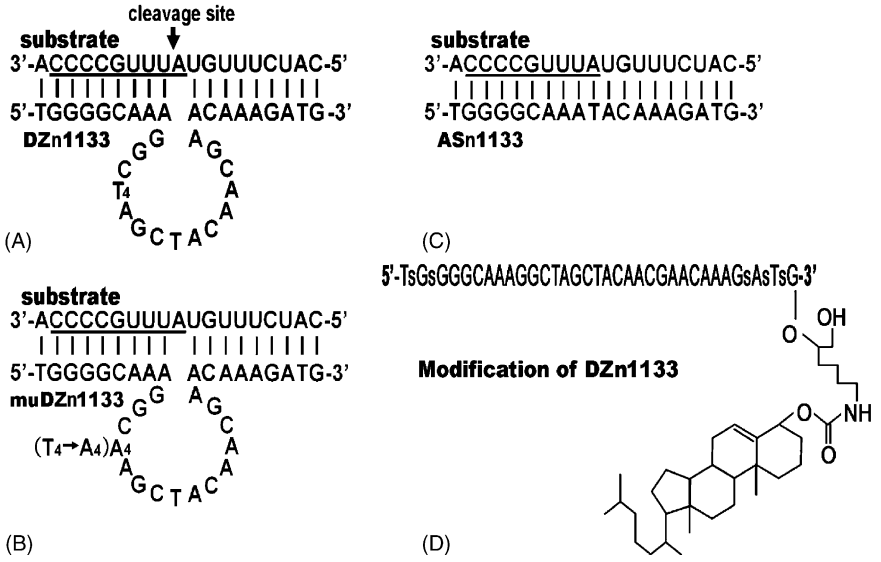

Fig. 1. Structures and modifications of oligonucleotides. (A) The sequence of DZn1133 in complex with its substrate RNA. The conserved 9 nt gene-start sequence of N gene is underlined and the cleavage should occur at the arrow-indicated position. (B) Inactivated DZn1133 (muDZn1133) with a mutation at the fourth position from T4 to A in the catalytic core. (C) A 19 nt antisense (ASn1133) targeting the same substrate of DZn1133. (D) The structure of cholesterol-conjugated and phosphorothioate-modified DNAzyme. Here only modified DZn1133 is shown, Structures of modification of muDZ and AS were same as in (D).

centration was 5×10^{-7} mol/l) was subjected to in vitro cleavage with 5 pmol DZ (final concentration was 5×10^{-7} mol/l), muDZ or AS (Fig. 1D) by incubation at 37 °C in 10 mM $MgCl_2$, 250 mM Tris–HCl (pH 7.4) solution for 30, 60, 90, 120 and 240 min. The cleavage products were electrophorized on 8% polyacrylamide–7 M urea gels, and quantified with a densitometer (Totallab, Nonlinear Dynamics Ltd., UK) after silver stain according to Palfner et al. (1995).

2.4. Virus infections and cell treatments

Monolayers of cultured Hep-2 cells were infected by incubating with RSV (multiplicity of infection, MOI = 0.1) at 37 °C for 2 h in 96-well plates, followed by adding DZ, muDZ and AS (Fig. 1D) or ribavirin (ICN Biochemicals, Aurora, OH) in a serial of 2-fold dilution ranging from 1/8 to 16 times of median effective concentration (EC_{50}, determined in MTT assays of cell viability, as described below). Media were refreshed at 12 h post-infection and the same compounds were added at the corresponding concentrations.

Table 2
Information of RSV strains

RSV stain	Subgroup	Resource
A2	A	Stored in our lab
CH18537	B	Kindly gift from Prof. Qian Yuan
CQ 381513	A	Isolated in Chongqing, China
CQ 381573	A	Isolated in Chongqing, China
CQ 381942	A	Isolated in Chongqing, China
CQ 381946	A	Isolated in Chongqing, China
CQ 381170	B	Isolated in Chongqing, China
CQ 033116	B	Isolated in Chongqing, China
Beijing 01	A	Isolated in Peking, China
Beijing 02	B	Isolated in Peking, China
Beijing 03	A	Isolated in Peking, China
Beijing 04	A	Isolated in Peking, China

2.5. Inhibition assays of viral cytopathologic effect

In order to demonstrate the antiviral effect of DNAzymes on RSV-induced CPE, we monitored viral syncytium formation by light microscopy and quantitated the cell viability by the MTT assay.

After infections and treatments as described above, all the cells were photographed microscopically when nearly 100% of untreated RSV-infected cells showed CPE. The assay of cell viability in this study was based on the quantitative colorimetric MTT assay as described previously (Watanabe et al., 1994). Briefly, at the time of complete CPE in untreated RSV-infected cells, 10 μl solution of MTT (Sigma, St. Louis, MO, 7.5 mg/ml) was added to each well and incubated for 4 h at 37 °C before adding 100 μl of DMSO to dissolve the crystallized formazan. Absorbance of supernatant at 450 nm was measured and the viability percent was calculated by the following formula: percent = $[(OD_T)_V - (OD_C)_V]/[(OD_C)_M - (OD_C)_V] \times 100\%$. Here, $(OD_T)_V$, $(OD_C)_V$ and $(OD_C)_M$ correspond to the absorbance of supernatant from the treated virus-infected cells, the untreated virus-infected cells, and the mock infected controls, respectively. The EC_{50} was then determined by regression analysis.

2.6. Inhibition assays of RSV replication

A series of experiments were undertaken to estimate the anti-RSV activity of the DNAzymes on the RSV gene transcription, protein expression and virus yield. On day 3 post-infection, RSV F mRNA was semi-quantified by RT-PCR of total RNA obtained from RSV-infected Hep-2 cells. The amplified prod-

ucts were separated by electrophoresis on 1.5% agarose gels and the corresponding quantities of F mRNA were normalized to the internal-control of human β-actin mRNA.

At 48 h post-infection, the expressions of RSV F protein in cells were assayed by the cell-ELISA technique as described (Kang and Pai, 1989), using anti-F monoclonal antibody (Cat. No. R1595-36, USBiological, MA, USA) as primary antibody. After the incubation with secondary antibody and colorization, the absorbance was measured at 460 nm and the inhibition percentage of virus F protein expression was determined as follows: percentage = $[(OD_C)_V - (OD_T)_V]/[(OD_C)_V - (OD_C)_M] \times 100\%$. Here, $(OD_T)_V$, $(OD_C)_V$, $(OD_C)_M$ represent the absorbance of the treated RSV-infected cell, the untreated RSV-infected cell, and the mock-infected control, respectively.

When nearly 100% of untreated RSV-infected cells showed CPE on average 5 day post-infection (the timing and severity of CPE were variable among different RSV strains), the supernatants were harvested and virus titer was determined by plaque forming assay on confluent monolayer of Hep-2 cells. The titer of virus produced by untreated RSV-infected cells was used as a control to calculate the percent reduction of virus yield by the test compounds.

2.7. Cytotoxicity

Cytotoxicity of the compounds was examined by the determination of cell viability by the similar procedure of MTT method described in Section 2.5. In brief, DZn1133, mutDZn1133 and ASn1133 were serially diluted in 2-fold from 4 to 128 μM, ribavirin was serially diluted in 2-fold from 64 to 4096 μM. Drug-infected CPE was observed microscopically every day. After 5 days, the MTT method was performed and the 50% cytotoxic concentration (CC_{50}) was determined by logistic regression analysis. The selectivity index (SI) was determined using the formula: $SI = CC_{50}/EC_{50}$.

2.8. Statistical analysis

A statistical program SPSS® (Version 10.0, SPSS Co. Ltd., USA) was used to calculate all means and standard deviations. Differences between two groups and among multiple groups were evaluated by the Student's *t*-test and the Kruskal–Wallis non-parametric analysis of variances, respectively. Differences with *P*-values of less than 0.001 were considered statistically significant.

3. Results

3.1. In vitro cleavage reaction of DNAzymes on RNA

To investigate the cleavage of RSV genomic RNA by DNAzymes that is prerequisite for virus inhibition, a series of cleaving experiments with in vitro transcribed RNA of RSV genome were performed. One of these DNAzymes, DZn1133, targeted against bases 1133 ± 9 of RSV genomic RNA, was found to be most efficient in the cleavage of 453 nt in vitro-transcribed N gene RNA substrate at the anticipated position after 2 h incubation, generating the size-matching products with 297 and 156 nt fragments (Fig. 2A). Moreover, RNA substrates decreased and the products increased with the incubation time prolonged in the cleavage reaction (Fig. 2B), resulting the cleavage percentages of 15.09, 26.45, 42.13, 53.20 and 67.90% at the incubation time of 30, 60, 90, 120 and 240 min, respectively (Fig. 2C). Whereas neither its inactive controls (muDZn1133)

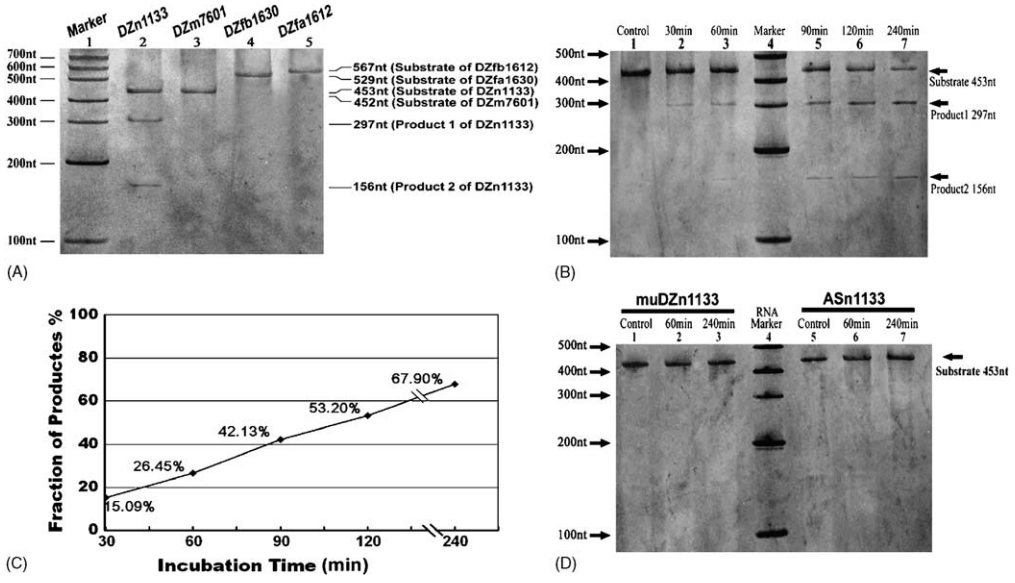

Fig. 2. In vitro cleavage activity of DNAzymes. (A) RNA substrates were incubated with DZn1133, DZm7601, Dzfa1612 and DZfb1630 at an equimolar concentration for 240 min. RNA substrates of DZn1133 and DZm7601 are negative-strands (genome RNA), while for Dzfa1612 and DZfb1630, the substrates are positive-strands (mRNA). DZn1133 cleaved the substrate at the anticipated site generating 297 and 156 nt products. There was not a detectable cleavage by DZm7601, Dzfa1612 or DZfb1630. (B) RNA substrates were incubated with equimolar DZn1133 and the cleavage increased with the incubation time. (C) Fragments cleaved by DZn1133 were quantified after silver stain and the fraction percentage plotting incubation time is illustrated (substrate without DZn1133 was taken as no cleavage). (D) muDnZ1133 or ASn1133 did not show cleavage activity after 60 and 240 min incubation.

nor 19 nt antisense oligonucleotide (ASn1133) targeting the same sequences gave any detectable cleaved products (Fig. 2D), suggesting the sequence-specific, time-dependent and catalytic cleavage capacity of DZn1133 on RSV N genome RNA. Therefore, we used DZn1133 for further investigations.

3.2. Protection effect of DNAzyme against RSV-induced CPE

The protection of DNAzyme on the RSV-infected Hep-2 cell was evaluated by the assays of viral syncytium formation and cell viability. On 5 day post-infection averagely, nearly 100% of untreated RSV-infected cells showed RSV-induced CPE. However, DZn1133 did block the syncytial pathologic change of Hep-2 cells caused by RSV, which became more potent along with the increased concentration of DNAzyme. In fact, the RSV-infected cells were morphologically indistinguishable from uninfected control on the day 5 post-infection when treated with 2 μM DZn1133. However, ribavirin and ASn1133 showed moderate ability to inhibit the RSV-induced CPE, and muDZn1133 did not showed a protective effect (data not shown).

The quantitative evaluation of cell viability was based on the colorimetric MTT assay (Watanabe et al., 1994). Excitingly, DZn1133 did suppress RSV-induced cell destruction, and the viability of infected cell was increased in a dose-depended manner with DZn1133, resulting in nearly complete protection of infected cells at 4 μM (representative data from the tests with 6 RSV strains covering both subgroups A and B are shown in Fig. 3A). According to the results from all 12 RSV strains illustrated in Fig. 3B, though the treatment of ribavirin provided significant effect with an average viability percent of 74.4% at 400 μM, an optimal inhibition of RSV-induced cell destruction was obtained with the DZn1133 resulting in average protection percent of 95.7% at a concentration of 8 μM ($P < 0.001$). The calculated EC_{50} of DZn1133, ASn1133 and ribavirin are 0.9955 ± 0.49, 11.1716 ± 2.3 and 47.39 ± 11.32 μM, respectively (Table 3). The muDZn1133 was ineffective, confirming that catalytic cleavage of RSV RNA by DNAzyme is crucial for cell protection from RSV-induced CPE.

3.3. Inhibition effect of DNAzyme on RSV gene transcription

The capacity of the DNAzyme to inhibit RSV mRNA transcription was assessed on 3 day post-infection by semi-quantified RT-PCR using F mRNA specific primers, and the representative data from A2-infected cells are shown in Fig. 4A. After the normalization of RT-PCR amplification by the internal-

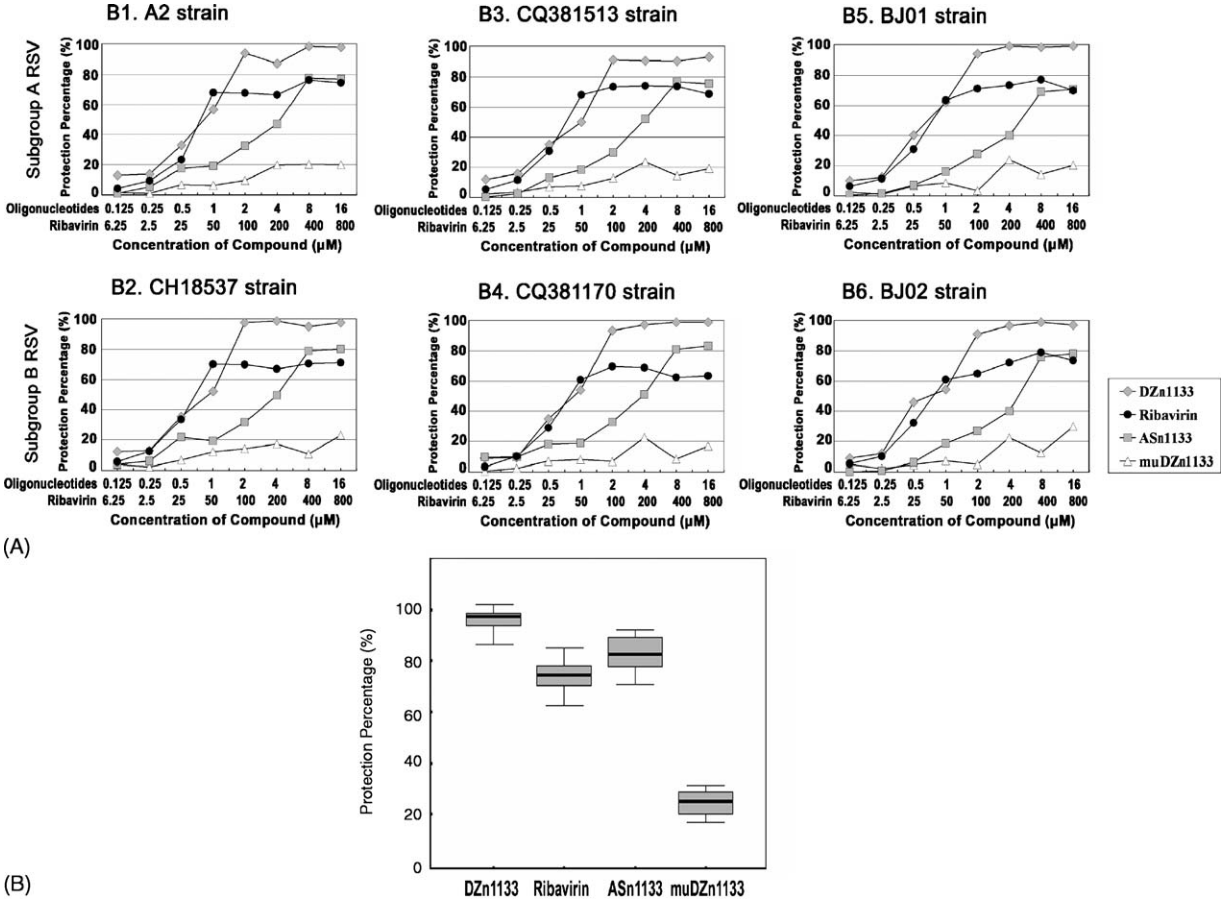

Fig. 3. Inhibition of viral CPE. (A) RSV-infected Hep-2 monolayers were grown in the absence or presence of compounds at various concentrations. Dose-dependent viabilities of RSV-infected cells were assessed by MTT assay and presented as protection percentage. Representative results from six RSV strains covering subgroups A and B are illustrated here. (B) Boxplots show the median, interquartile range and outliers of cell viability percentage protected by oligonucleotides at 8 μM and by ribavirin at 400 μM against 12 individual RSV strains.

Table 3
Anti-RSV activity and cytotoxicity in Hep-2 cells

RSV strain	EC_{50} (μM)			CC_{50} (μM)			SI[a]		
	DZn1133	ASn1133	Ribavirin	DZn1133	ASn1133	Ribavirin	DZn1133	ASn1133	Ribavirin
A2	1.2073	13.7045	52.79	45.90	50.88	606.48	38.02	3.71	11.49
CH18537	0.7404	9.7738	47.11	44.36	45.89	643.64	59.91	4.70	13.66
Beijing 01	0.9821	12.0935	35.9	45.05	43.78	536.67	45.88	3.62	14.95
Beijing 02	2.0304	14.9038	57.47	50.90	36.00	598.00	25.07	2.42	10.41
Beijing 03	1.7392	11.2111	70.05	57.02	67.00	633.68	32.79	5.98	9.05
Beijing 04	0.3387	10.3004	36.85	42.96	56.93	640.75	126.84	5.53	17.39
CQ 381573	0.9403	8.9038	40.73	36.79	52.46	600.48	39.12	5.89	14.74
CQ 381513	1.0043	9.9803	58.6	42.17	48.11	674.12	41.99	4.82	11.5
CQ 381942	0.7844	11.0606	53.46	36.77	35.67	554.68	46.88	3.22	10.38
CQ 381946	1.1097	12.5490	44.67	46.24	45.57	576.07	41.67	3.63	12.9
CQ 381170	0.5337	6.4903	39.08	43.07	43.09	569.79	80.7	6.64	14.58
CQ 033116	0.5355	13.0881	31.97	40.61	38.86	559.64	75.84	2.97	17.51
Average	0.9955 ± 0.49	11.1716 ± 2.30	47.39 ± 11.32	44.32 ± 5.61	47.02 ± 8.99	599.50 ± 42.01	54.56 ± 28.13	4.43 ± 1.36	13.21 ± 2.74

[a] The selective indices (SI) displayed were obtained by dividing the CC_{50} obtained for each compound for each RSV strain by its respective EC_{50}.

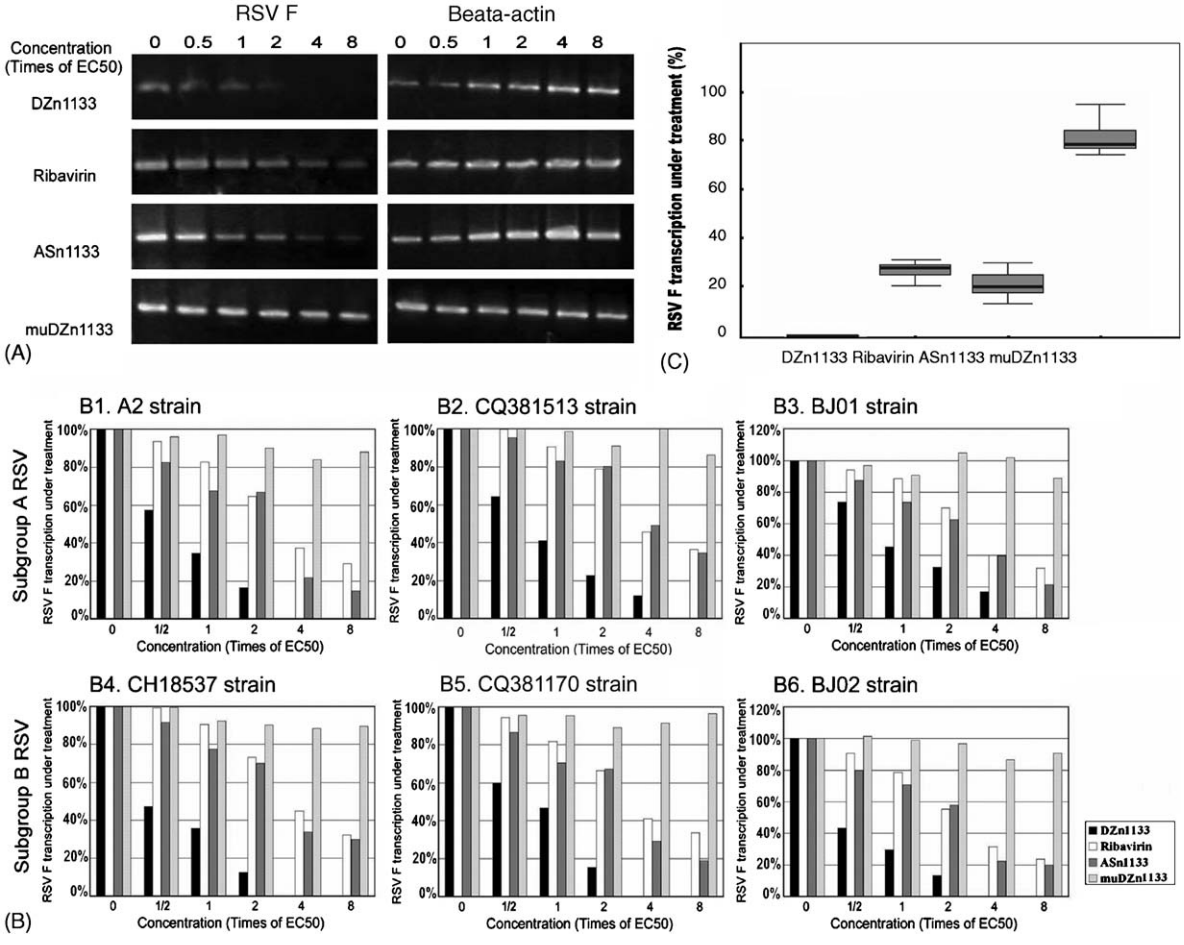

Fig. 4. Inhibition of RSV F gene transcription. RSV-infected cells were grown in the absence or presence of compounds. After 3 days, RT-PCR was performed using RSV F gene and human β-actin-specific primers. (A) PCR products of F mRNA from A2-infected cells under the treatment of compounds are shown as a representative example. (B) After the quantification of RSV F gene transcription by measuring the density of electrophoresis bands, which was further normalized to the internal-control β-actin mRNA, dose-dependent reductions of F mRNA in six RSV strains covering subgroups A and B are illustrated (taken the amounts of F mRNA in non-treated RSV-infected cells as 100%). (C) Boxplots show the median, interquartile range and outliers of F mRNA amounts in 12 individual RSV-infected cells under the treatment of compounds at eight times of EC_{50}.

control of β-actin mRNA, the analysis revealed a gradual decrease in the levels of the F mRNA with increasing concentrations of DZn1133 and ribavirin. However, DZn1133 displayed greater inhibition efficacy on the F gene transcription of RSV A2 than ribavarin, because the amounts of F mRNA under the treatments of DZn1133 and ribavirin were 100% versus 100%, 57.49% versus 93.56%, 34.44% versus 82.9%, 16.38% versus 64.47%, 0% versus 37.22% and 0% versus 29.19% at serial 2-fold dilution of agent concentration (Fig. 4B1). Similar results were obtained when either subgroups A or B RSV was used (Fig. 4B2–B6). Moreover, the F gene transcriptions of all 12 RSV strains were complete arrested when treated with DZn1133 while remained 30.0% under the treatment of ribavirin (at eight times of EC50 of DZn1133) with statistically significant difference ($P < 0.001$, Fig. 4C). Same suppression of viral transcription ($P = 0.003$, Fig. 4C) under the treatment of ASn1133 was achieved at 1/8 of the doze of ribavirin. The slight decrease of F mRNA induced by muDZn1133 demonstrated its low capacity on the inhibition of RSV gene transcription ($P = 0.013$, Fig. 4A–C).

3.4. Inhibition effect of DNAzyme on RSV protein expression

At 48 h post-infection, the inhibitory effect of DZn1133 on RSV replication was further evaluated at virus protein level by cell-ELISA technique. The F protein suppression of 6 representative strains was illustrated as percent inhibition (Fig. 5A). In all the cases, DZn1133 showed higher activity on the inhibition of F protein expression in comparison to ribavirin. In fact, the quantifications of F protein in the cells, infected with 12 strains of RSV, showed that DZn1133 vigorously inhibited viral F protein expression by 86.69% at eight times of EC_{50} (Fig. 5B). However, mutated DNAzyme could not decrease the expression of F protein effectively (Fig. 5A and B).

3.5. Inhibition effect of DNAzyme on RSV virus yield

After infection with RSV and treatment with DZn1133, muDZn1133, ASn1133 or ribavirin at serial 2-fold dilution of concentrations, the supernatants were harvested and the virus titers were determined by plaque forming assay when complete CPE was shown in untreated RSV-infected cells. DZn1133 drastically diminished the virus production in a dose-dependent manner and inhibited the virus yield of RSV by 5–7 logs when compared with untreated RSV-infected cells, resulting in complete elimination of both subgroups A and B RSV at eight times the EC_{50} (representative data shown in Fig. 6A and B). However, the titers of RSV remained at an average of 2.55 logs in the supernatants of RSV-infected cells, regardless of the treatment of ribavirin at eight times the EC_{50} (Fig. 6B). Treatment of ASn1133, an antisense DNA corresponding to the DZn1133

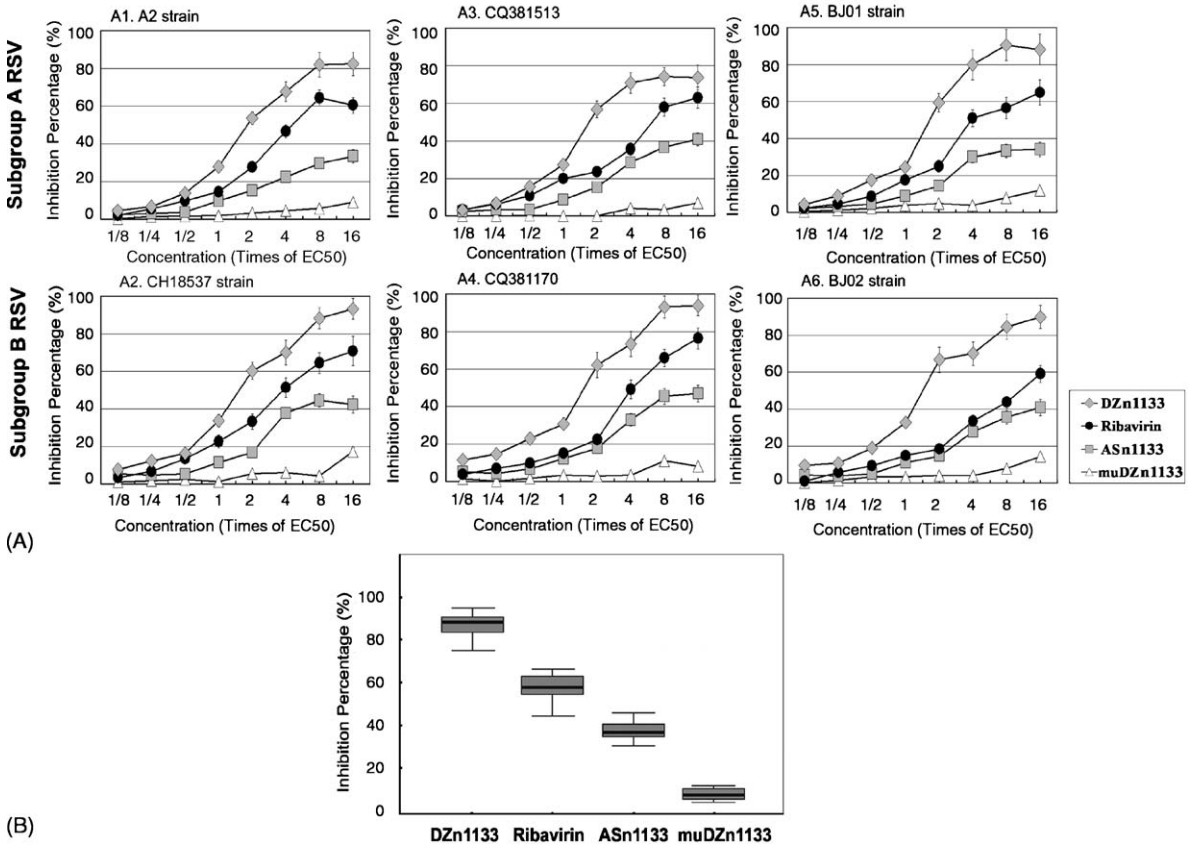

Fig. 5. Inhibition of RSV F protein expression. F protein expression in RSV-infected cells was measured by cell-ELISA at 48 h post-infection. (A) The dose-dependent inhibition of F protein expression from six RSV strains covering subgroups A and B are shown here (taken the amounts of F protein in non-treated RSV-infected cells as 100%). Error bars indicate the standard deviation of triplicate experiments. (B) Boxplots show the median, interquartile range and outliers of the inhibition percent on F protein expression in 12 individual RSV-infected cells by the treatment of compounds at eight times of EC_{50}.

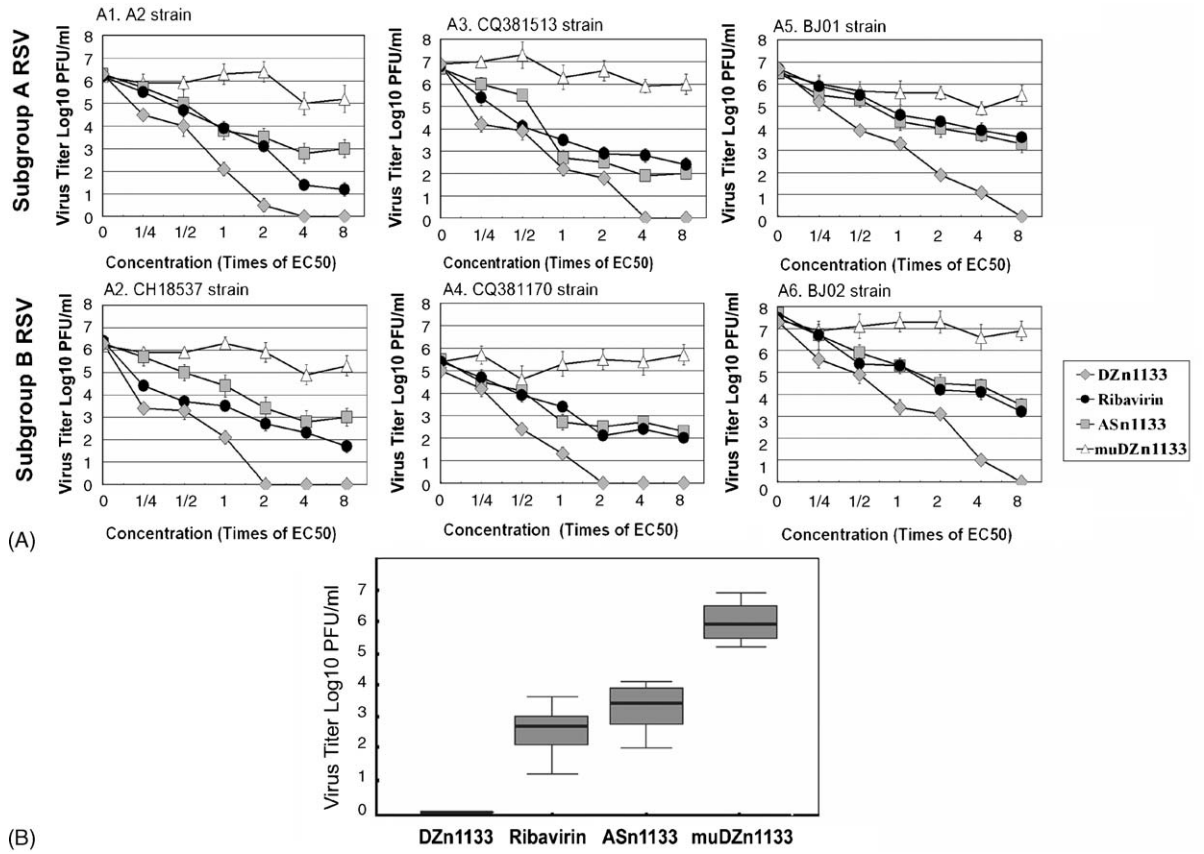

Fig. 6. Reduction of virus yield. On day 5 post-infection, supernatants from infected cells in the absence or presence of compounds were titrated by plaque forming assay. (A) Dose-dependent reductions of virus yield in the tests with six RSV strains are shown here. The error bars indicate the standard deviation of triplicate experiments. (B) Boxplots show the median, interquartile range and outliers of virus yield from the cells infected with 12 individual RSV strains under the treatment of compounds at eight times of EC_{50}.

binding sequences, provided similar results and blocked RSV production by an average of 3.26 logs. Conversely, the inactive mutant of DZn1133, muDZn1133, had a slight effect on virus yield (Fig. 6A and B).

3.6. Cytotoxicity

The CC_{50} and SI values obtained from DZn1133, ASn1133 and ribavirin in Hep-2 cells are shown in Table 3. Thus, DZn1133 and ASn1133 showed a similar CC_{50}. Because of the lower EC_{50} of DZn1133, it has an SI of 54.56 ± 28.13 against RSV in Hep-2 culture cells in vitro. The SI value obtained from ribavirin and ASn1133 against RSV was 13.21 ± 2.74 and 4.43 ± 1.36, respectively. In contrast, mutDZn1133 did not have a detectable selective antiviral activity against RSV because its SI was lower than 1.

4. Discussion

Two main factors should be considered for the selection of DNAzyme cleavage site against the target RNA. Firstly, the target site should be essential for the virus. Among the viral proteins encoded by RSV genomic RNA (3′-NS1-NS2-N-P-M-SH-G-F-M2-L-5′) (Collins et al., 2001), three viral proteins (nucleocapsid protein N, large protein L and phosphoprotein P) are minimally required to reconstitute the functional transcription complex of (−)RNA viruses (Teng and Collins, 1998; Oomens et al., 2003). For RSV, the optimal transcription, although not replication, further requires the transcription anti-termination protein M2-1 (Collins et al., 1995). In addition, the fusion protein F is essential for the characteristic cytopathology of RSV, marked by the formation of syncytia (Pastey et al., 2000; Cianci et al., 2005). Another consideration is that the target sequence should be highly conserved in different RSV strains. RSV isolates can be classified into two subgroups, designated A and B (Mufson et al., 1985; Sullender et al., 1993), which differ in all 11 known viral proteins and can be further classified into subtypes. Epidemics of RSV are accompanied by the occurrence of numerous strains of both A and B subgroups (Guney et al., 2004), the genotype varies from year to year (Kuroiwa et al., 2004), although subgroup A usually predominates and causes slightly more severe disease (Devincenzo, 2004). An anti-RSV agent should protect against the divergent subgroups A and B and try to cover all the subtypes. Fortunately, each gene in RSV genomic RNA begins with a conserved 9 nt gene-start signal, 3′-CCCCGUUUA, and transcription starts at the first nucleotide. This holds for all but the L gene, which has the signal sequence of 3′-CCCUGUUUUA (Kuo et al., 1997). However, the binding

site for DNAzymes should include 14–22 nucleic acids (7–11 nt for two binding arms on each side).

According to these strategies in target site choosing, we identified the gene-start signal sites of N, M2 genes as the universal targets and F gene signal sequences for individual targets of subgroups A and B, and four corresponding DNAzymes (DZn1133, DZm7601, Dzfa1612 and DZfb1630). One of these DNAzymes, DZn1133, targeted against N gene at the bases 1133 ± 9 of RSV genomic RNA (Fig. 1A), was found to be most efficient in the cleavage of in vitro-transcribed RNA substrate in a time-dependent manner (Fig. 2B). DZm7601, Dzfa1612 and DZfb1630 failed to exhibit cleavage activity in several repeated assays (Fig. 2A). These failures may be due to the fold of single-stranded substrate RNA into secondary structures through Watson–Crick base pairing, which probably makes the binding sites inaccessible to the DNAzymes and affect the cleavage results (Cairns et al., 1999). With the computer-assisted analysis of the secondary structure of RNA, the M2 gene target site and both F gene signal sequences of subgroups A and B were predicted at stably pairing locations, while the target of N gene signal should be unstably paired and accessible to DZn1133 (data not shown). As well as the results in the RNA cleavage assay, no effective results were observed in protecting the RSV-induced CPE by DZm7601, Dzfa1612 and DZfb1630 (data not shown).

The effects of DZn1133 and ribavirin on RSV were further assessed side by side in a cell culture system. DZn1133 displayed more inhibition efficacy on viral replication by testing with 12 strains of RSV covering subgroups A and B, resulting in complete ablation of F gene transcription (Fig. 4C) and the decrease of F protein expression by 86.69% (Fig. 5B) at eight times of EC_{50} (approximately 8 μM). Surprisingly, DZn1133 could dramatically reduce the virus yield with an elimination of RSV yield (Fig. 6B) and provide almost complete protection of infected cell from RSV-induced CPE with an EC_{50} of 0.9955 ± 0.49 μM (Fig. 3). Since the syncytium reduction and cell protection of RSV-infected cells perhaps reflect the functional protection of anti-RSV agents (Kahn et al., 1999; Techaarpornkul et al., 2001), DZn1133 may provide potent activity against RSV diseases. The ribavirin as well as ASn1133 moderately affected on RSV in our research, and muDZn1133 whose catalytic loop was inactivated while the target-binding flanks were intact, lost the anti-RSV activity that DNAzyme and antisense DNA experienced. The ineffective inhibition of muDZn1133 on RSV may be explained by its losing capacity to cleave the target sequence in comparison to DZn1133 and its bonding to the complementary sequence with less stability than ASn1133 due to the redundant inactive catalytic loop.

The majority of RSV inhibition by DZn1133 may be associated with the cleavage of RSV genomic RNA at the site of N gene start signal. By this cleaving, replication of vRNA is inhibited, consequently resulting in all the viral proteins are reduced. Thereinto, our data demonstrate that the destruction of viral genome promotes the decrease of F gene transcription and expression. Since the fusion protein F is by far the most important viral glycoprotein that is central to the pathologic cell fusion activity (Pastey et al., 2000; Cianci et al., 2005), one of the potential mechanisms for the functional protection of DZn1133 to RSV-infected cell is probably the effective inhibition of the F protein expression. Furthermore, cutting of N genome RNA may lead to ablation of N protein expression. The N protein binds tightly to genomic and antigenomic RNA of RSV to form an RNAse-resistant nucleocapsid, protecting the RNA from degradation by RNAses in cells (Bitko and Barik, 2001). Without the protection of N protein, RNA of RSV progeny template may change to be highly sensitive to nuclease and thus be unstable in the nuclease-rich cytoplasm. Previously, we have demonstrated that DZ604, the first reported DNAzyme against RSV by targeting the start signal of the NS2 gene, was found efficient to cleave corresponding substrate of in vitro-transcribed RNA. However, this DNAzyme only achieved a reduction of virus yield by 85.56% and could not protect RSV-infected cells from CPE at the concentration of 5 μM. An explanation for the lack of protection by NS2-targeted DNAzyme to infected cells may be that the protein NS2, as a nonstructural protein, was not crucial for RSV replication, because it can be deleted only with decreases in virus growth in vitro and in vivo (Teng and Collins, 1999; Jin et al., 2000).

Antiviral DZ against virus may have potential side effects because of the presence of multiple target nucleotides within the human genome. Because the available target sites of RSV for DZ choosing are limited, such potential side effect is inevitable in DZ design. It is noted that parts of target sequence for DZn1133 (17 nucleosides at most) of RSV is also found in a number of human EST as well as in some genes with known functions (Dias et al., 2000; Matsuda et al., 2003). However, theoretically DZ strictly targets to RNA rather than affecting genomic DNA and mRNA is continuously express in most cases, it is hardly possible to completely silent a certain human mRNA that is identical with sequence. And clinically, anti-RSV DZ will probably have minimal side effect as they are designed to be administered as an aerosol directly into the respiratory tract, which may limit acute systemic toxicity. In our cell culture study, the CC50 of DZn1133 in Hep-2 cell, a human larynx epidermoid carcinoma cell line, is about 44μM (Table 3), thus demonstrated a more acceptable SI value than ribavirin. This result should be careful assayed and cautious evaluated in vivo.

In conclusion, DZn1133 is the first DNAzyme can efficiently inhibited wild strains of RSV covering both subgroups A and B in cultured cells and provide protection against RSV-induced CPE, presumably by directly cleaving N gene of RSV genomic RNA, sequentially reducing the expression of viral proteins. This compound might be a potential anti-RSV therapeutic agent with improved efficacy for clinical applications.

Acknowledgments

The authors acknowledge the technical expertise of Ding Yu in synthesizing the oligonucleotides and Prof. En-Mei Liu for clinical specimen collection. We thank Dr. Helen Law for helpful comments on the manuscript. We also gratefully thank Prof. Qian Yuan, Ru-Nan Zhu and Den-Jie for generous gifts of RSV and technical help. This study was supported by the National Science Foundation of China (No. 30340045).

References

Bitko, V., Barik, S., 2001. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. BMC Microbiol. 1, 34.

Cairns, M.J., Hopkins, T.M., Witherington, C., Wang, L., Sun, L.Q., 1999. Target site selection for an RNA-cleaving catalytic DNA. Nat. Biotechnol. 17, 480–486.

Cairns, M.J., Sun, L.Q., 2004. Target-site selection for the 10–23 DNAzyme. Methods Mol. Biol. 252, 267–277.

Chavez-Bueno, S., Mejias, A., Jafri, H.S., Ramilo, O., 2005. Respiratory syncytial virus: old challenges and new approaches. Pediatr. Ann. 34, 62–68.

Cianci, C., Meanwell, N., Krystal, M., 2005. Antiviral activity and molecular mechanism of an orally active respiratory syncytial virus fusion inhibitor. J. Antimicrob. Chemother. 55, 289–292.

Collins, C.L., Pollard, A.J., 2002. Respiratory syncytial virus infections in children and adults. J. Infect. 45, 10–17.

Collins, P.L., Hill, M.G., Camargo, E., Grosfeld, H., Chanock, R.M., Murphy, B.R., 1995. Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proc. Natl. Acad. Sci. U.S.A. 92, 11563–11567.

Collins, P.L., Chanock, R.M., Murphy, B.R., 2001. Respiratory syncytial virus. In: Knipe, D.M., Howley, M. (Eds.), Fields Virology, fourth ed. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 1443–1485.

Devincenzo, J.P., 2004. Natural infection of infants with respiratory syncytial virus subgroups A and B: a study of frequency, disease severity, and viral load. Pediatr. Res. 56, 914–917.

Dias Neto, E., Correa, R.G., Verjovski-Almeida, S., Briones, M.R., Nagai, M.A., da Silva Jr., W., Zago, M.A., Bordin, S., Costa, F.F., Goldman, G.H., Carvalho, A.F., Matsukuma, A., Baia, G.S., Simpson, D.H., Brunstein, A., de Oliveira, P.S., Bucher, P., Jongeneel, C.V., O'Hare, M.J., Soares, F., Brentani, R.R., Reis, L.F., de Souza, S.J., Simpson, A.J., 2000. Shotgun sequencing of the human transcriptome with ORF expressed sequence tags. Proc. Natl. Acad. Sci. U.S.A. 97, 3491–3496.

Domachowske, J.B., Rosenberg, H.F., 2005. Advances in the treatment and prevention of severe viral bronchiolitis. Pediatr. Ann. 34, 35–41.

Fedor, M.J., 2000. Structure and function of the hairpin ribozyme. J. Mol. Biol. 297, 269–291.

Groothuis, J.R., Simoes, E.A., Levin, M.J., Hall, C.B., Long, C.E., Rodriguez, W.J., Arrobio, J., Meissner, H.C., Fulton, D.R., Welliver, R.C., Tristram, D.A., Siber, G.R., Prince, G.A., Van Raden, M., Hemming, V.G., The Respiratory Syncytial Virus Immune Globulin Study Group, 1993. Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. N. Engl. J. Med. 329, 1524–1530.

Guney, C., Kubar, A., Yapar, M., Besirbellioglu, A.B., Doganci, L., 2004. An outbreak of respiratory infection due to respiratory syncytial virus subgroup B in Ankara, Turkey. Jpn. J. Infect. Dis. 57, 178–180.

Hall, C.B., 2001. Respiratory syncytial virus and parainfluenza virus. N. Engl. J. Med. 344, 1917–1928.

Jin, H., Zhou, H., Cheng, X., Tang, R., Munoz, M., Nguyen, N., 2000. Recombinant respiratory syncytial viruses with deletions in the NS1, NS2, SH, and M2-2 genes are attenuated in vitro and in vivo. Virology 273, 210–218.

Johnson, S., Oliver, C., Prince, G.A., Hemming, V.G., Pfarr, D.S., Wang, S.C., Dormitzer, M., O'Grady, J., Koenig, S., Tamura, J.K., Woods, R., Bansal, G., Couchenour, D., Tsao, E., Hall, W.C., Young, J.F., 1997. Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. J. Infect. Dis. 176, 1215–1224.

Kahn, J.S., Schnell, M.J., Buonocore, L., Rose, J.K., 1999. Recombinant vesicular stomatitis virus expressing respiratory syncytial virus (RSV) glycoproteins: RSV fusion protein can mediate infection and cell fusion. Virology 254, 81–91.

Kang, J.O., Pai, C.H., 1989. In situ enzyme immunoassay for antiviral susceptibility testing of respiratory syncytial virus. Am. J. Clin. Pathol. 91, 323–326.

Klig, J.E., Shah, N.B., 2005. Office pediatrics: current issues in lower respiratory infections in children. Curr. Opin. Pediatr. 17, 111–118.

Kuo, L., Fearns, R., Collins, P.L., 1997. Analysis of the gene start and gene end signals of human respiratory syncytial virus: quasi-templated initiation at position 1 of the encoded mRNA. J. Virol. 71, 4944–4953.

Kuroiwa, Y., Nagai, K., Okita, L., Tsutsumi, H., 2004. Genetic variability and molecular epidemiology of respiratory syncytial virus subgroup a strains in Japan determined by heteroduplex mobility assay. J. Clin. Microbiol. 42, 2048–2053.

Law, B.J., Wang, E.E., MacDonald, N., McDonald, J., Dobson, S., Boucher, F., Langley, J., Robinson, J., Mitchell, I., Stephens, D., 1997. Does ribavirin impact on the hospital course of children with respiratory syncytial virus (RSV) infection? An analysis using the pediatric investigators collaborative network on infections in Canada (PICNIC) RSV database. Pediatrics 99, E7.

Long, C.E., McBride, J.T., Hall, C.B., 1995. Sequelae of respiratory syncytial virus infections. A role for intervention studies. Am. J. Respir. Crit. Care Med. 151, 1678–1681.

Matsuda, A., Suzuki, Y., Honda, G., Muramatsu, S., Matsuzaki, O., Nagano, Y., Doi, T., Shimotohno, K., Harada, T., Nishida, E., Hayashi, H., Sugano, S., 2003. Large-scale identification and characterization of human genes that activate NF-kappaB and MAPK signaling pathways. Oncogene 22, 3307–3318.

Meissner, H.C., Rennels, M.B., Long, S.S., Pickering, L.K., 2004. Immunoprophylaxis with RespiGam. Pediatrics 113, 629.

Mufson, M.A., Orvell, C., Rafnar, B., Norrby, E., 1985. Two distinct subtypes of human respiratory syncytial virus. J. Gen. Virol. 66, 2111–2124.

Oomens, A.G., Megaw, A.G., Wertz, G.W., 2003. Infectivity of a human respiratory syncytial virus lacking the SH, G, and F proteins is efficiently mediated by the vesicular stomatitis virus G protein. J. Virol. 77, 3785–3798.

Palfner, K., Kneba, M., Hiddemann, W., Bertram, J., 1995. Short technical reports. Quantification of ribozyme-mediated RNA cleavage using silver-stained polyacrylamide gels. Biotechniques 19, 926–929.

Pastey, M.K., Gower, T.L., Spearman, P.W., Crowe Jr., J.E., Graham, B.S., 2000. A RhoA-derived peptide inhibits syncytium formation induced by respiratory syncytial virus and parainfluenza virus type 3. Nat. Med. 6, 35–40.

Raj, S.M., Liu, F., 2003. Engineering of RNase P ribozyme for gene-targeting applications. Gene 313, 59–69.

Santoro, S.W., Joyce, G.F., 1997. A general purpose RNA-cleaving DNA enzyme. Proc. Natl. Acad. Sci. U.S.A. 94, 4262–4266.

Schwarze, J., Hamelmann, E., Bradley, K.L., Takeda, K., Gelfand, E.W., 1997. Respiratory syncytial virus infection results in airway hyperresponsiveness and enhanced airway sensitization to allergen. J. Clin. Invest. 100, 226–233.

Stevens, T.P., Hall, C.B., 2004. Controversies in palivizumab use. Pediatr. Infect. Dis. J. 23, 1051–1052.

Sullender, W.M., Sun, L., Anderson, L.J., 1993. Analysis of respiratory syncytial virus genetic variability with amplified cDNAs. J. Clin. Microbiol. 31, 1224–1231.

Techaarpornkul, S., Barretto, N., Peeples, M.E., 2001. Functional analysis of recombinant respiratory syncytial virus deletion mutants lacking the small hydrophobic and/or attachment glycoprotein gene. J. Virol. 75, 6825–6834.

Teng, M.N., Collins, P.L., 1998. Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles. J. Virol. 72, 5707–5716.

Teng, M.N., Collins, P.L., 1999. Altered growth characteristics of recombinant respiratory syncytial viruses which do not produce NS2 protein. J. Virol. 73, 466–473.

The Impact-RSV Study Group, 1998. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respi-

ratory syncytial virus infection in high-risk infants. Pediatrics 102, 531–537.

Ueba, O., 1978. Respiratory syncytial virus. I. Concentration and purification of the infectious virus. Acta Med. Okayama 32, 265–272.

Ventre, K., Randolph, A., 2004. Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. Cochrane Database Syst. Rev. 18, CD000181.

Walsh, E.E., Peterson, D.R., Falsey, A.R., 2004. Risk factors for severe respiratory syncytial virus infection in elderly persons. J. Infect. Dis. 189, 233–238.

Watanabe, W., Konno, K., Ijichi, K., Inoue, H., Yokota, T., Shigeta, S., 1994. MTT colorimetric assay system for the screening of anti-orthomyxo- and anti-paramyxoviral agents. J. Virol. Methods 48, 257–265.

Zaborowska, Z., Furste, J.P., Erdmann, V.A., Kurreck, J., 2002. Sequence requirements in the catalytic core of the "10–23" DNA enzyme. J. Biol. Chem. 277, 40617–40622.

Zhang, W., Yang, H., Kong, X., Mohapatra, S., San, Juan-Vergara, H., Hellermann, G., Behera, S., Singam, R., Lockey, R.F., Mohapatra, S.S., 2005. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. Nat. Med. 11, 56–62.

Zhao, C.A., Zhao, X.D., Yu, H.G., Wu, Y.P., Yang, X.Q., 2003a. Inhibition of respiratory syncytial virus replication in cultured cells by RNA-cleaving DNAzyme. Zhonghua Er Ke Za Zhi 41, 594–597.

Zhao, L.Q., Qian, Y., Wang, Z.L., Zhu, R.N., Deng, J., 2003b. Comparison of four methods used in detection of the respiratory syncytial virus in nasopharyngeal aspirates from children with the acute lower respiratory tract infection. Chin. J. Pediatr. 36, 20–24.