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RESEARCH

Potent Intracellular Knock-Down of Influenza A Virus M2 Gene Transcript by DNAzymes Considerably Reduces Viral Replication in Host Cells

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Abstract Influenza A virus has been known to be an important respiratory pathogen and cause of several epidemics and devastating pandemics leading to loss of life and resources across the globe. The M2 ion channel protein is highly conserved and essentially required during the trafficking, assembly, and budding processes of virus, thus an attractive target for designing antiviral drugs. We designed several 10–23 DNAzymes (Dz) targeting different regions of the M2 gene and analyzed their ability to specifically cleave the target RNA in both cell-free system as well as in cell culture using transient transfections. Dz114, among several others, directed against the predicted single-stranded bulge regions showed 70 % inhibition of M2 gene expression validated by PCR and Western blot analysis. The activity was dependent on Mg^{2+} (10–50 mM) in a dose-dependent manner. The mutant-Dz against M2 gene showed no down-regulation thereby illustrating high level of specificity of designed Dz114 towards the target RNA. Our findings suggest that Dz may be used as potential inhibitor of viral RNA replication and can be explored further for development of an effective therapeutic agent against influenza infection. These catalytic nucleic acid molecules may further be investigated as an alternative to the traditional influenza vaccines that require annual formulation.

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Introduction

Influenza epidemics remain the most prevalent and unconquered acute threat to human health, causing damage and death far beyond expectations [1]. There are three types of influenza virus: type A, B, and C. The seventh segment of influenza A and B virus genome encodes an integral membrane protein, M2 which is a 92 amino acid, unique protein that is expressed abundantly at the surface of infected cells and is also present in small quantities on the surface of mature virions [2]. These proton channels are among the smallest ion channel proteins with the properties of ion selectivity and activation. When a virion enters the host cell by receptor-mediated endocytosis and the virus particle is in the acidic environment of the endosomal lumen, the M2 ion channel is activated and conducts protons across the viral membrane as a prerequisite for release of genetic material to the cytoplasm. The virus is regularly becoming resistant to the drugs developed against them because of abrupt mutations. The current nucleic acid-based antiviral approaches include ribozymes (Rz), DNAzymes (Dz), short-hairpin RNA, and small interfering RNA (siRNA), which have down-regulated several target RNAs [3–5]. The in vitro selection has made possible to generate catalytic DNA sequences with RNA-cleaving activity comparable to Rz while maintaining the chemistry of antisense oligodeoxynucleotides [6]. Dz are synthetic catalytic DNA molecules that can be designed to specifically bind to its target RNA sequence and cleave the phosphodiester linkages. The catalytic efficiency of these molecules is dependent on metal ions, and they target the

phosphodiester bond between purine and pyrimidine nucleotides [7]. Dz are easier to prepare as compared to its RNA counterparts. They are also less sensitive to chemical and enzymatic degradations. The DNA enzymes have been reported to have a catalytic efficiency (kcat/Km) of $\approx 10^9$ M^{-1} min^{-1} under multiple turnover conditions, which exceeds than any other known nucleic acid enzyme [8]. The Dz have been shown to be ≈100,000-fold more stable and ideal for storing genetic information [9]. Several studies have already been published showing successful down-regulation of vital genes of many viruses. These catalytic molecules have been modified to increase stability and have been shown to inhibit the replication of the Japanese encephalitis virus up to 99.99 % in mice brain [10]. In a study, authors have shown a down-regulation of the S gene of Hepatitis B virus up to 95 % after 48 h of transfection [11]. Similar experiments have also been done where expression of HIV integrase protein in the transiently transfected cell lines has been inhibited up to 80 % by a novel Dz [12]. Dash and Banerjea used 10–23 Dz to inhibit the expression of the HIV-1 envelope (env) gene in a gene fusion assay [13]. Previously, we reported the successful use of novel 10–23 Dz, hammer-head Rz, and antisense molecules to compare their efficacy in selectively achieving the post-transcriptional gene silencing of M1 gene of influenza A virus [14]. We have recently reported the successful down-regulation of BM2 transcript of influenza B viruses based on similar approaches [15]. In the present study, we have targeted the M2 gene transcript of influenza A virus by specific 10–23 Dz to inhibit the propagation of virus in the host cells. The strategy has already been validated on influenza B viruses, yet the study holds importance as influenza A viruses are known to acquire frequent mutations by genetic drift and shift leading to devastating pandemics. The designed nucleic acid molecules will help in inhibiting the virus replication by targeting a conserved gene, thus bypassing the fear of annual formulation as is the case with traditional influenza vaccines.

Material and Methods

Cells and Viruses

Madin Darby canine kidney (MDCK) cells (purchased from National Centre for Cell Sciences, Pune, India) were maintained in minimum essential medium (Sigma, St. Louis, MO) and supplemented with 10 % fetal calf serum and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). The influenza virus strain A/PR/8/34 (H1N1) used for this study was obtained from Centers for Disease Control and Prevention, Atlanta, and propagated in MDCK cell line in our laboratory as per standardized protocol. The culture supernatants containing the virus were collected and stored at −80 °C for further use. All experiments with virus were done in a BSL-2 cabinet.

Plasmid Constructs and In Vitro Transcription of M2 Gene

Viral RNA was isolated using QIAamp viral RNA isolation mini kit (Qiagen, GmBH, Hilden, Germany), total cDNA was prepared using IM-PROM reverse transcription kit (Promega, Madison, WI, USA), and the 290 nucleotides M2 gene was amplified from the cDNA using gene-specific primers: (Forward primer 5′ TCGggtaccTTATGATCTTCTTGAAAATTTGC 3′ and reverse primer 5′ TCGctcgagTTTTACTCCAGCTCTATG CTGAC 3′). Nucleotides in lowercase indicate restriction sites for *Kpn*I and *Xho*I in the forward and reverse primers, respectively. The M2 gene was cloned in mammalian expression vector pSecTag2B, and plasmids were sequenced to check the authenticity of the recombinant clone (M2-pSecTag2B). The clones were linearized with suitable restriction enzymes and subjected to in vitro transcription using Large-Scale RNA Production System (Promega, Madison, WI, USA). Non-structural gene 1 (NS1) of influenza A virus was also cloned in pcDNA3.1 (+) vector for assessing the efficacy and specificity of Dz and named NS1-pcDNA3.1 (+).

Selection of Cleavage Sites and Designing of DNAzymes

Secondary structures of full-length M2 gene (290 nt) were obtained and analyzed using the M-fold RNA folding program for nucleic acid folding and hybridization prediction [http://www.bioinfo.rpi/applications/mfold]. Based on the predicted secondary structure, four Dz (Fig. 1a) were designed against the single-stranded looped regions targeted to cleave the AU at various regions in M2 RNA. The Dz, Dz114, Dz128, Dz165, and Dz211, named based on their respective cleavage position (Table 1), were chemically synthesized and obtained from Sigma Genosys. The conserved 15 nt (5′-GGCTAGCTACAACGA-3′) 10–23 catalytic motif was flanked on both sides by substrate-binding arms (7 nt each) of the Dz that were made complementary to the target RNA. A mutant-Dz possessing a single-nucleotide substitution (G–C) in the conserved catalytic motif [16] and the controls having scrambled arms were synthesized to demonstrate the target sequence specificity.

In Vitro Cleavage of the Substrate RNA by DNAzyme

The DNAzyme and the in vitro synthesized M2 RNA were heated separately for 2 min at 95 °C and chilled on ice for

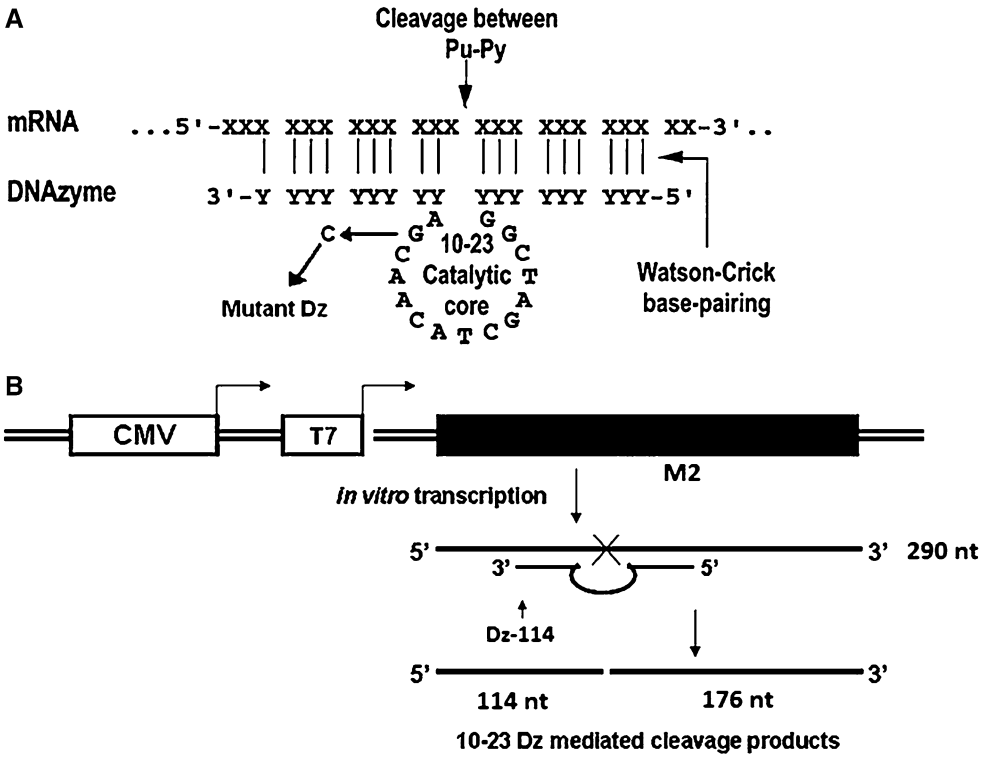

Fig. 1 The 10–23 DNAzymes were designed using the secondary structures of M2 RNA. **a** General structure of 10–23 DNAzyme. The G–C replacement of nucleotide in the catalytic motif leads to mutant-Dz. **b** Schematic representation of the cleavage of M2 RNA with Dz114

Table 1 Sequence of DNAzymes for M2 transcript of influenza A virus

DNAzyme	Sequence 5′–3′
Dz114	TCCACAAGGCTAGCTACAACGAATCAAGT
Dz128	AAGACGGGGCTAGCTACAACGACAAGAAT
Dz165	GTCCGTAGGCTAGCTACAACGATTAAAGC
Dz211	TCCCTCAGGCTAGCTACAACGAAGACTTT
Mt-Dz114	TCCACAAGGCTAGCTACAACCAATCAAGT

The mutated residue in the mutant Mt-Dz114 is highlighted in bold

5 min. The cleavage reaction was carried out by mixing equimolar amounts (100 pmol each) of substrate RNA and Dz in a final volume of 10 µl in a buffer containing 50 mM Tris–HCl, pH 7.5 in the presence of 10 mM $MgCl_2$ at standard conditions [17] for 2 h at 37 °C (Fig. 1b). The M2 transcripts were further subjected to cleavage reactions in the presence of 10–50 mM concentrations of $MgCl_2$.

RT-PCR and Real-Time RT-PCR

The cleavage reactions were analyzed by performing RT-PCR of full-length M2 gene followed by gel electrophoresis and documentation through AlphaImager-2200 (Alpha Innotech Corporation).

The specific cleavage was analyzed using TaqMan probes (Invitrogen) synthesized to hybridize to the Dz114 cleavage region in the substrate RNA. The probes were designed based on the genome sequence and structure of influenza A virus [A/PR/8/34 (H1N1)] mRNAs using RNAdraw, Primer Premier 5.0, and NCBI BLAST.

The real-time RT-PCR consisted of One step RT-PCR 2X-Super Mix (Ambion), 0.1 µM of each forward (Fd: AAACGAATGGGGGTGCAGATGCAA), reverse (Rv: CGGTAAATGCATTTGAAAAAAAGA) primers and probes (FAM': AAGAATCCACAATATCAAGTGCAA-BHQ1), 5 µL of template, and RNAse-free water up to a volume of 25 µl. The reaction was carried out with the following cycling conditions: 50 °C for 30 min for RT step, 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s, and 55 °C for 30 s. All the experiments were performed in triplicates, and the data were expressed as mean ± standard deviation (SD).

Co-transfection of DNAzymes and Plasmids

MDCK cells were seeded in a 6-well tissue culture plate at density of 0.5×10^6 cells/well. The cells, at 70 % confluency, were co-transfected with 1 µg of the sequence confirmed M2-pSecTag2B plasmid along with Dz (100 pmol) complexed with lipofectamine-2000 (Invitrogen) as described by us earlier [14, 18]. Briefly, for each co-transfection, 4 µl of Lipofectamine 2000 was diluted in 100 µl of DMEM (without antibiotics) and 1 µg of plasmid DNA (one clone at a time) was diluted in another 100 µl

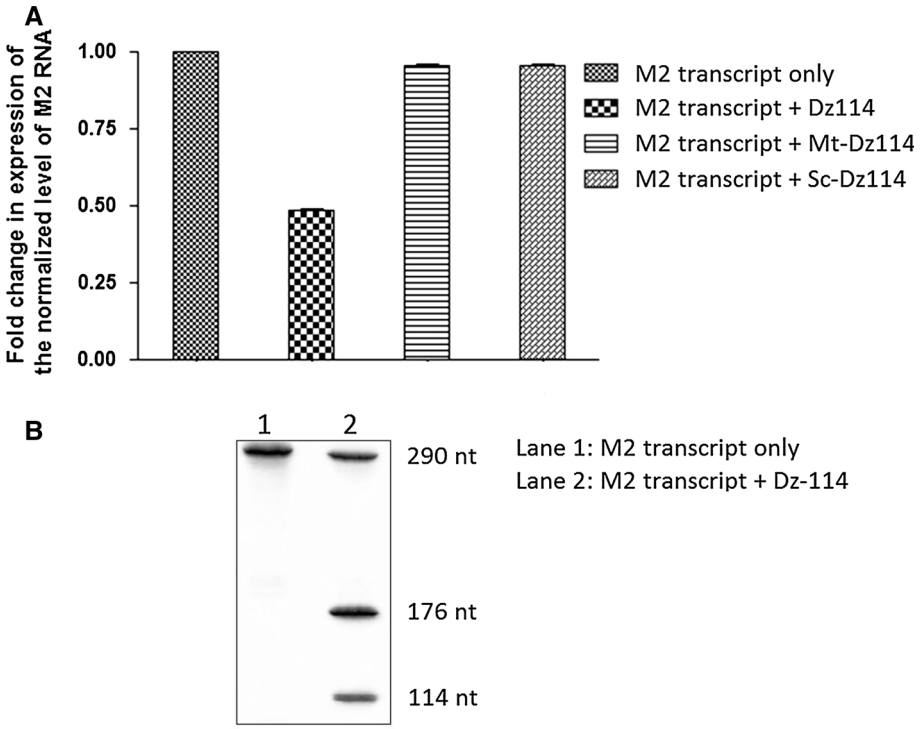

Fig. 2 The M2 gene was in vitro transcribed using the Large-Scale RNA Production System (Promega). The labeled full-length M2 transcripts were subjected to cleavage with Dz114. **a** The real-time RT-PCR based on the TaqMan chemistry was performed using primer and probes targeted to bind to the Dz114 cleavage site on M2 RNA and detect the fold change in expression of M2 gene in Dz114-treated samples. The experiments were performed in triplicate, and the error bars indicate standard deviation. *P* values for the graph plotted were ≤ 0.005. **b** The labeled M2 transcripts were subjected to cleavage by Dz114. The *lane 1* represents the full-length M2 transcript, while the *lane 2* represents the cleaved M2 transcript

DMEM. The two suspensions were mixed (total volume 200 µl) and incubated at RT for 10 min. The mixture was then added to 70 % confluent MDCK cells and incubated at 37 °C/5 % CO_2. Mock-transfected and only M2-pSec-Tag2B-transfected MDCK cells served as controls for the experiment.

We also assessed the efficacy and specificity of Dz114 by analyzing the effect of irrelevant Dz against NS1 gene (NS1-Dz) or mutant-Dz (Mt-Dz114) on M2 gene expression and effect of Dz114 on the gene expression of NS1 gene respectively. MDCK cells treated with 100 pmol Dz114 or Mt-Dz114 or irrelevant Dz against NS1 (NS1-Dz) were transfected with M2-pSecTag2B clone. For specificity analysis, NS1-pcDNA3.1 (+) clone with or without 100 pmol Dz114 was transfected in MDCK cells. Total cellular RNA was isolated, after 24 h of transfection, as previously described and subjected to conventional PCR and real-time RT-PCR analysis [14]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal gene control for each sample. The experiment was performed in triplicates, and the data were expressed as mean ± (SD).

DNAzyme Transfection and Virus Challenge

MDCK cells, at 70 % confluency, were transfected with 1, 3, and 5 µg of Dz using Lipofectamine 2000 as described earlier. The cells, 8-h post-transfection, were infected with A/PR/8/34 strain of influenza A virus (MOI of 0.1) in a total volume of 150 µl in 1X PBS containing 2 µg/ml TPCK-treated trypsin, for 1 h at 37 °C and 5 % CO_2. After 24 h of infection, total cellular RNA was isolated using Ribozol RNA extraction reagent (Amresco, Solon, USA). The concentration of the RNA was determined spectrophotometrically, and real-time RT-PCR using specific primers for the M2 gene was done as described earlier in materials and methods. All the experiments were performed in triplicates, and the data were expressed as mean ± SD. The cycle times (Ct value) were analyzed, and the Ct value that varied by >1 unit between the triplicates was discarded. The average of the triplicate Ct values was calculated and normalized to the Ct value of β-actin. The cytopathic effect (CPE) of the virus on the cells transfected with Dz114 was observed at 24-h post-infection, and effect of the Dz114 on virus multiplication was analyzed. Ten-fold dilutions of the culture supernatants were prepared and used for plaque assay as described earlier [19]. The results were expressed as mean p.f.u. per mL + SD.

SDS-PAGE and Immunoblotting

At 24-h post-infection, the cells were lysed using mammalian cell lysis buffer [0.1 M NaCl, 0.01 M Tris Cl (pH 7.6), 0.001 M EDTA (pH 8.0), 1 mM protease inhibitor cocktail (Amresco), and 100 µg/ml phenylmethylsulfonyl fluoride (PMSF)]. The protein concentration was determined by Bradford assay (Amresco), and whole cell lysate

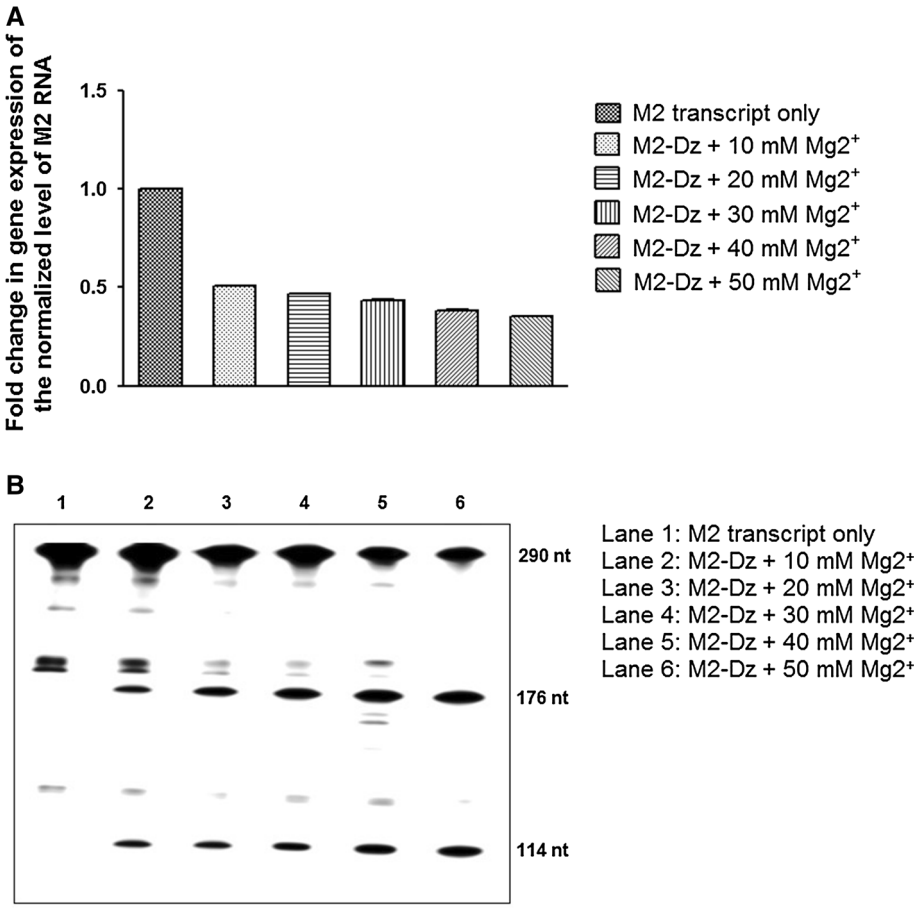

Fig. 3 The M2 gene was in vitro transcribed using the Large-Scale RNA Production System (Promega). The full-length labeled M2 transcripts were subjected to cleavage with Dz114 in the presence of increasing concentration of $MgCl_2$. **a** The real-time RT-PCR was performed to detect the fold change in expression of M2 gene in Dz114-treated samples. The experiments were performed in triplicate, and the *error bars* indicate standard deviation. *P* values for the graph plotted were ≤0.005. **b** The labeled M2 transcripts were subjected to cleavage by Dz114 in the presence of 10, 20, 30, 40, and 50 mM $MgCl_2$, respectively. The *lane 1* represents the full-length M2 transcript, while the *lanes 2–6* represent the cleaved M2 transcript

(40 μg/lane) was fractionated on 12 % polyacrylamide for Western blotting. The blot was developed using primary (1:100 dilutions) mouse monoclonal antibody (Santa Cruz Biotechnology, USA) against M2 protein of influenza A virus and horse-radish peroxidase-conjugated goat anti-mouse IgG (1:1000 dilutions) as secondary antibody (Santa Cruz Biotechnology, USA) using enhanced chemiluminescence reagent as per manufacturer's instructions.

Results

Construction of RNA-Cleaving DNA Enzymes and Selection of Target Sites in the M2 Gene

Predicted secondary structures of the entire RNA of M2 gene were obtained through the M-fold software. We designed 4 Dz (Table 1) with the lowest ΔG values against the nucleotides position at 114 (Dz114), 128 (Dz128), 165 (Dz165), and 211 (Dz211), and all designed to cleave an unpaired purine and paired pyrimidine residue (AU) in the presence of a divalent cations. Of the four Dz, Dz114 gave significant 70 % down-regulation of the M2 gene in in vitro (Fig. 2) conditions, while Dz128, Dz165, and Dz211 failed to show significant inhibition (data not shown). The mutant version of the Dz114 (Mt-Dz114), synthesized by substituting a single base (G–C) in the conserved catalytic motif, and controls having scrambled binding arms were evaluated to demonstrate the target sequence specificity of the designed DNAzyme.

Sequence-Specific Cleavage of M2 RNA by Dz114

The M2-pSecTag2B clones were linearized and subjected to in vitro transcription using the Large-Scale RNA Production System (Promega, Madison, WI, USA). The 290 nucleotide long labeled M2 RNA was specifically cleaved in the presence of Dz114 (100 pmol each) under standard conditions as described by us earlier. The cleaved products of the expected size (114 and 176 nt) were also obtained which was validated by both real-time RT-PCR assay designed for the Dz114 cleavage region and PAGE (Fig. 2). The disabled DNAzyme carrying a point mutation in its catalytic motif and the Dz with scrambled arms failed to cleave the target RNA. The catalytic activity of the Dz114 significantly correlated to the increasing concentration of

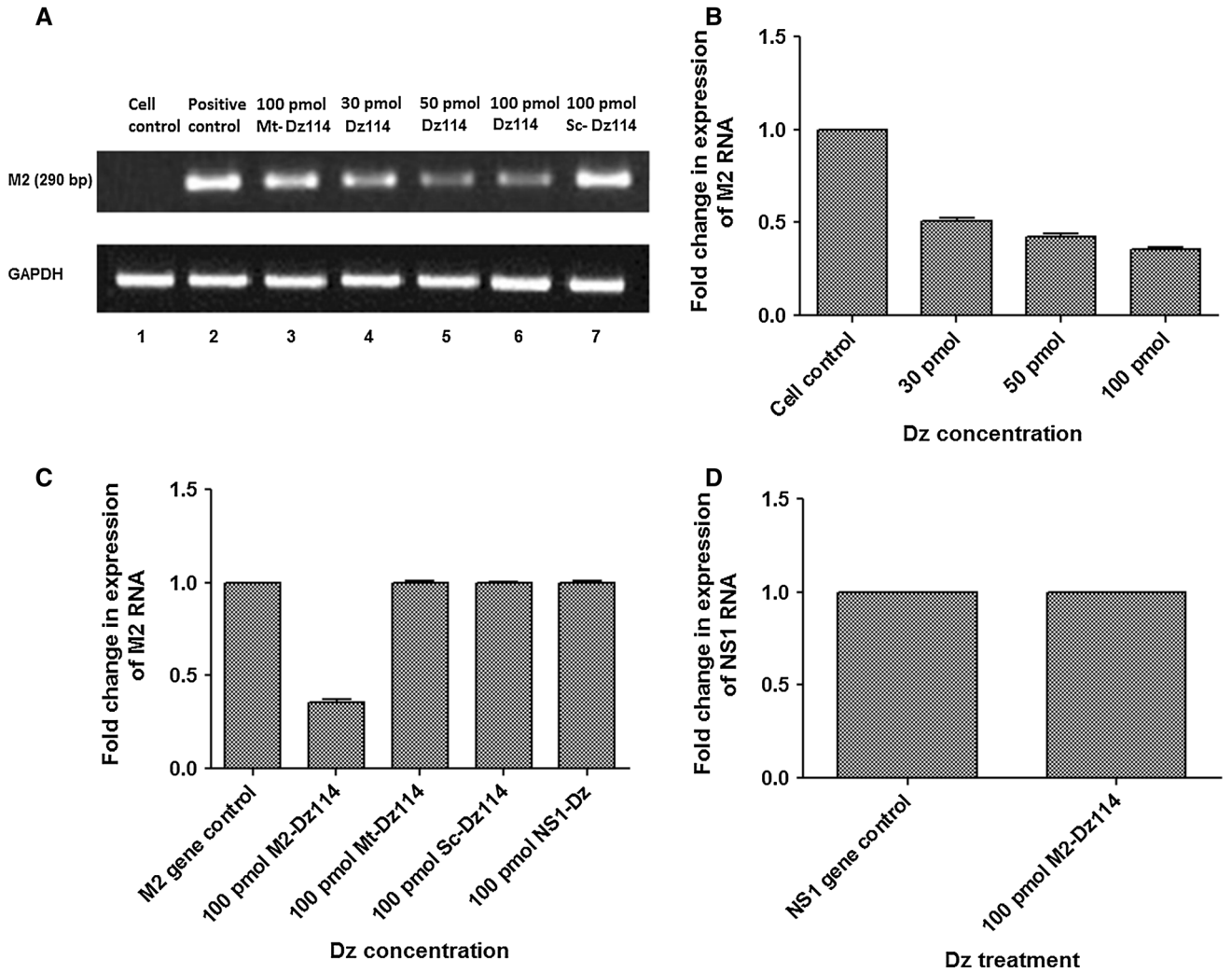

Fig. 4 RT-PCR analysis shows intracellular reduction of M2 RNA in MDCK cells treated with Dz114. **a** MDCK cells were transfected with 1 μg of M2-pSecTag2B with or without different concentrations of Dz114. Total cellular RNA was isolated 24-h post-transfection and subjected to separate PCR reactions for amplification of M2 and GAPDH genes. **b**, **c** TaqMan-based real-time RT-PCR analysis was performed for estimation of fold change in expression of the M2 gene. The M2-pSecTag2B-transfected cells were taken as positive control and reference for comparison of ΔΔCt values of Dz114 and NS1-Dz-transfected RNA samples. The experiments were performed in triplicate, and the *error bars* indicate standard deviation. **d** TaqMan-based real-time RT-PCR analysis was performed for estimation of fold change in expression of NS1 gene in the presence of 100 pmol Dz114. The NS1-pcDNA3.1 (+) clone-transfected cells were taken as positive control. The mRNA levels were normalized against GAPDH. The experiments were performed in triplicate, and the *error bars* indicate standard deviation (SD). *P* values were ≤0.005

$MgCl_2$ (10–50 mM) and showed an enhanced catalytic efficiency from 50 % at 10 mM $MgCl_2$ to 65 % at 50 mM $MgCl_2$ (Fig. 3).

Down-Regulation of M2 Gene Expression in MDCK cells

The sequence confirmed M2 clone (M2-pSecTag2B), and DNAzyme co-transfected MDCK cells were harvested 24-h post-transfection for analysis of intracellular levels of M2 gene. Out of the set of 4 Dz, Dz114 was found to be highly effective against the target gene. Approximately, 65 % decrease in RNA levels of M2 gene was observed post-Dz114 treatment by RT-PCR (Fig. 4a) and real-time RT-PCR analyses (Fig. 4b). The result was observed to be almost constant at 100 pmol and higher concentration of Dz114.

The inability of the mutated Dz (Mt-Dz114) or irrelevant Dz (NS1-Dz) against NS1 gene or Dz with scrambled binding arms to down-regulate the expression of M2 gene confirmed the efficacy of Dz114 (Fig. 4c). The specificity of Dz114 was further validated by analyzing the effect of Dz114 on the gene expression of NS1 gene, used as unrelated viral RNA control. The level of NS1 RNA in the 100 pm Dz114-treated and NS1-pcDNA3.1 (+)-transfected MDCK cells was almost similar to the RNA levels in the

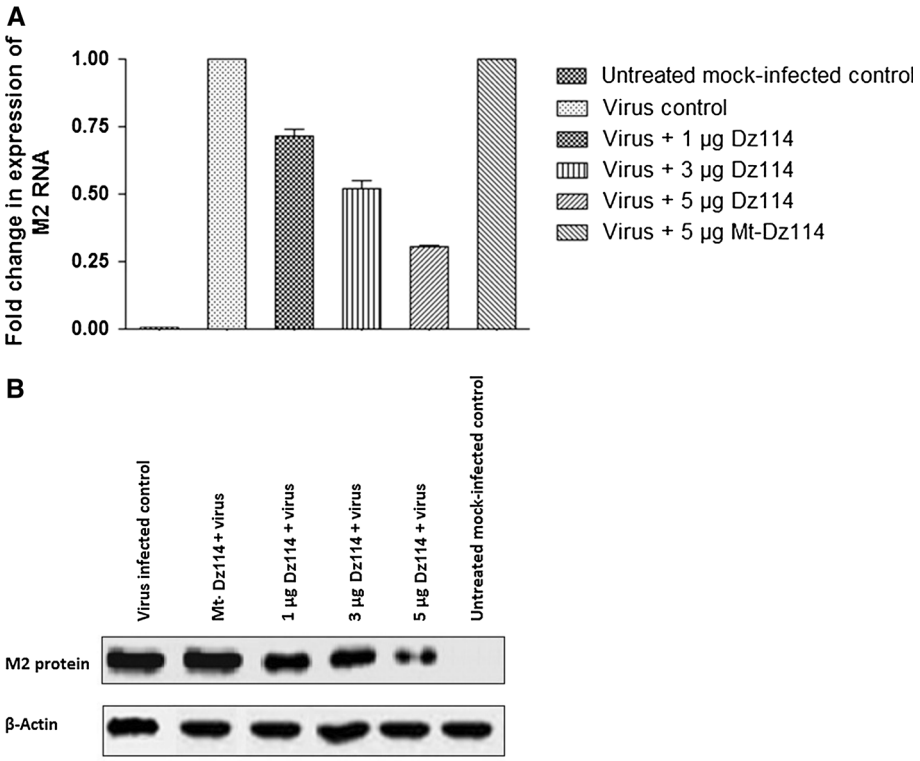

Fig. 5 Different concentrations of Dz114 were given to MDCK cells against the virus challenge. After 24-h post-virus challenge, the cells were harvested for RNA isolation and cell lysate preparation. **a** Real-time RT-PCR analysis of the RNA samples showed a dose-dependent decrease in M2 RNA level. Real-time PCR using the TaqMan chemistry was performed. The bars represent the ΔΔCt values. RNA samples isolated from Dz-treated cells were compared with RNA isolated from the untreated virus-infected cells, taking it as a reference and allotting it a Ct value of 1. The experiments were performed in triplicate, and the *error bars* indicate SD. *P* values were ≤0.005. **b** Similar dose-dependent reduction in the intracellular M2 protein level was observed, when the cell lysates from all the experimental groups were fractionated on 12 % SDS-PAGE and immunoblotted using antibody against M2 protein of influenza A virus. The data shown are from 1 of 2 experiments

MDCK cells transfected with the NS1-pcDNA3.1 (+) clone alone (Fig. 4d). This showed that the Dz were specific for the M2 gene and efficiently inhibited the target gene expression. The experiments were performed in triplicate, and the error bars in figures indicate standard deviation.

Variable Protection Against Virus Challenge with Dz114 in MDCK Cell Line

The cleavage potential of the Dz114 was further validated by transfection studies in MDCK cells. The cells, after 8 h of transfection followed by virus challenge (MOI of 0.1), were subjected to SDS-PAGE and Western blot analysis. The TaqMan-based real-time PCR assay was performed for assessing the extent of inhibition in virus replication. The ΔΔCt values were calculated and compared with the RNA level of M2 in untreated virus-infected cells. The PCR analysis showed a significant 70 % down-regulation in cells treated with 5 µg of Dz114 (Fig. 5a). The Mt-Dz114, however, failed to provide any protection against the virus challenge thereby proving the specificity of the designed DNAzyme against target RNA. The Dz114 with scrambled arms showed no down-regulation revealing specificity of the DNAzyme towards the target RNA. The intensity of bands corresponding to viral proteins also revealed that the DNAzyme considerably down-regulated the M2 protein expression (Fig. 5b) and thus inhibited the influenza virus replication in mammalian cell line. A significant reduction in viral protein was observed when subjected to 5 µg of Dz114. A considerable reduction in CPE was observed 24-h post-infection thus showing inhibition of whole virus replication in MDCK cells (Fig. 6). The plaque assay also revealed substantial inhibition of viral replication in cells transfected with Dz114 (Fig. 7). The experiments were performed in triplicate, and the error bars in Figs. 5(a) and 7 indicate SD.

Discussion

The RNA-cleaving activities of Dz have been the best characterized apart from the various other catalyzing functions like carbon–carbon bond formation [20], RNA ligation [21], and the hydrolytic cleavage of DNA. These

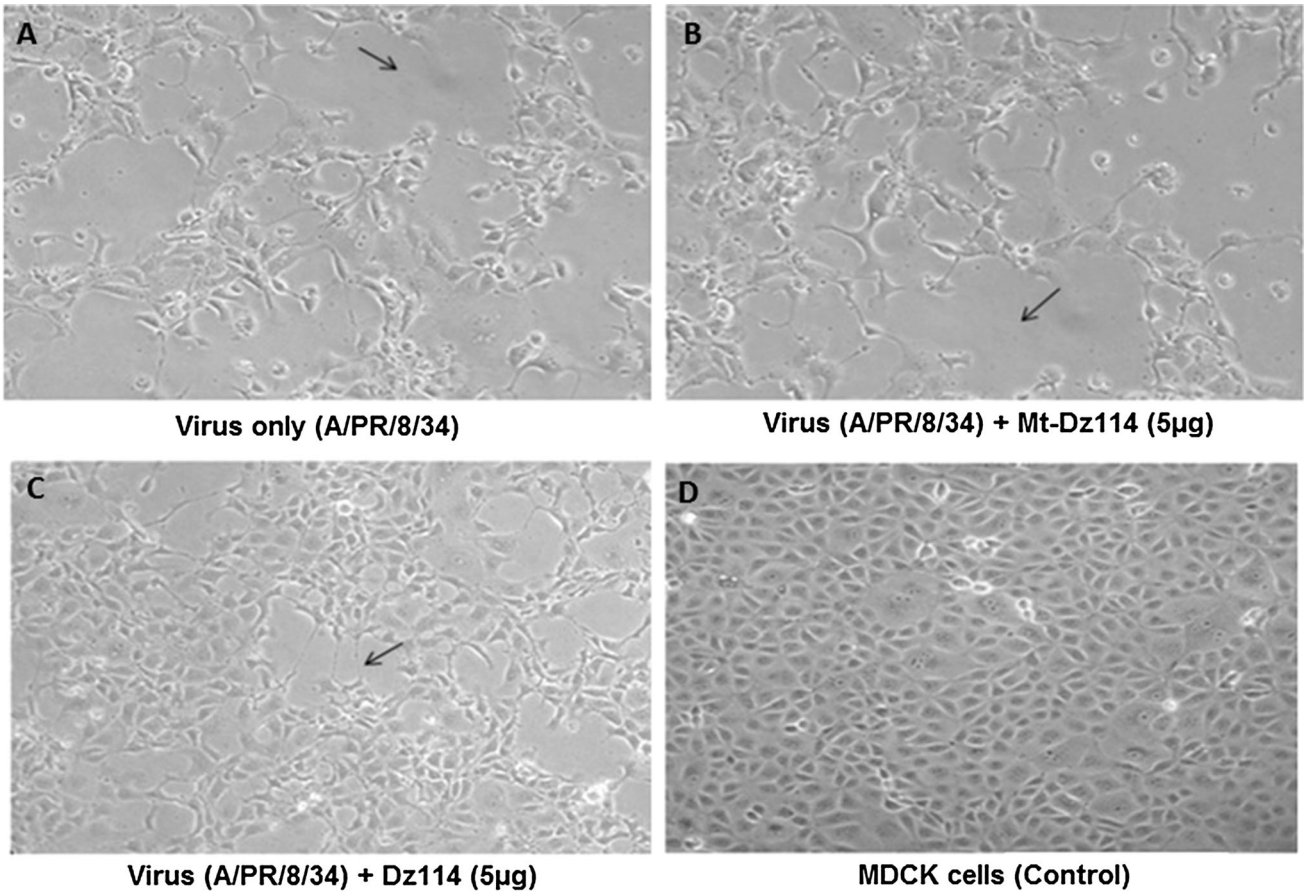

Fig. 6 Treatment of MDCK cell line with wild-type DNAzyme (Dz114) inhibited the multiplication of virus (infected at a MOI of 0.1) and reduced its cytopathic effect (CPE). Figures show the CPE at 24-h post-infection. **a**, **b** Maximum CPE was observed in the infected cells that were not transfected with any DNAzyme and with mutant DNAzyme (Mt-Dz114). **c** CPE was inhibited in the infected cells transfected with 5 µg of wild-type Dz114. **d** No CPE was observed in control MDCK cells. The CPE is marked by *arrows*

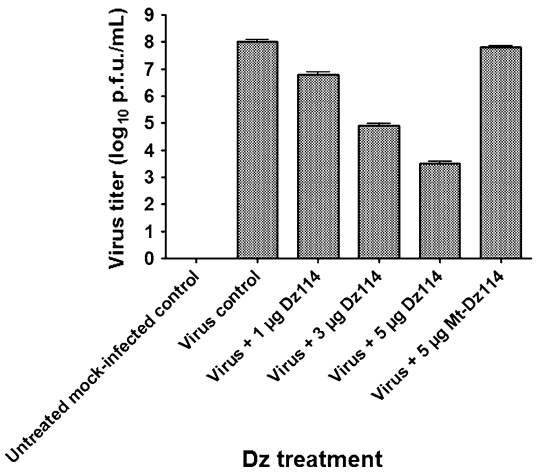

Fig. 7 The cells were collected 24-h post-virus challenge, and virus titration was performed by plaque assay. The virus titer in the cells treated with different concentrations of Dz114 was significantly reduced in comparison with virus-infected control or mutant-Dz groups. The experiment was performed in triplicate, and the *error bars* indicate SD. *P* values were ≤ 0.005

synthetic catalytic nucleic acids have gained importance in several research areas including cancer [22], allergy [23], cardiovascular diseases [24], and various other disorders [25]. The DNA molecules are predominantly double-stranded except for few viral genomes and replication intermediates, thus no naturally occurring Dz have been reported till date.

Several studies have also been conducted on 10–23 Dz to inhibit the replication of many RNA viruses such as the Japanese encephalitis virus [10], hepatitis [16, 26], HIV [12], and influenza [27]. When an influenza virion enters the host cell, the acidic environment of the endosomal lumen activates the M2 ion channel of the virus and conducts protons across the viral membrane as a prerequisite for release of genetic material to the cytoplasm. The M2 ion channel gets activated during transport of the M2 protein through the exocytic pathway; this activity raises the lumenal pH of the trans-golgi network, equilibrating pH with that of the cytoplasm [28]. The activity of M2 ion channel is essential for the viral replication in host cells, thus

making it an attractive target for post-transcriptional gene silencing strategies.

We previously demonstrated the use of 10–23 Dz targeted against the M1 gene of influenza A viruses, which plays crucial role in viral life cycle [14]. The 788 nucleotide M1 gene was successfully down-regulated by our novel Dz which gave a significant protection against the influenza A/PR/8/34-H1N1 strain. We achieved good results with both full-length (788 nt) and truncated (356 nt) M1 RNA. We have also shown the first report of down-regulation of the BM2 gene transcript of influenza B viruses using 10-13 Dz. The designed DNAzyme was able to inhibit the whole virus replication in MDCK cells [15].

In this report, we designed 4 Dz with the lowest ΔG values against the nucleotides position at 114 (Dz114), 128 (Dz128), 165 (Dz165), and 211 (Dz211) and designed to cleave the M2 transcript at AU site in the presence of $MgCl_2$. Only Dz114 showed significant 70 % down-regulation as compared to others (data not shown). This could be due to the fact that the nucleic acid enzymes are designed based on software predicted RNA structures, and during ex vivo analysis, all cleavage sites are not accessible for efficient cleavage by these enzymes. Dz with longer arms have been shown to be more efficient in cleavage activity as compared to shorter arms [26]; however, we have shown good results with shorter arms in our study. It has been reported that among the variable substrate-binding domain length, which ranged between 4/4 and 13/13, the maximum overall efficiency (kcat/KM) under physiological reaction conditions was found with an arm length of between 8 and 9 bp [7]. In our study, the gene silencing was found to be effective in a dose-dependent manner and was best at 50 mM $MgCl_2$ concentration which is comparable to other published reports on other viruses [29]. The inability of the mutant DNAzyme (Mt-Dz114) and DNAzyme with scrambled arms (Sc-Dz114) proved the highly specific nature of our designed Dz.

Our study revealed that the designed Dz114 could not only suppress the M2 transcript but also conferred significant protection against the virus challenge by the A/PR/8/34 (H1N1) strain. The inhibition of influenza A virus replication was revealed by the marked reduction in viral CPE and plaques. We further analyzed the effect of Dz114 against NS1 gene and found no significant difference in the levels of NS1 RNA levels, thereby suggesting the highly specific nature of our designed DNAzyme. The designed Dz114 provided protection against the virus challenge for up to 48 h in MDCK cells (data not shown).

The studies related to use of Dz for down-regulating influenza virus replication are still very limited and may be explored further for effective management of these highly mutating viruses. Our results, thus, provide a validation that the M2 gene transcript of influenza A viruses could be an effective target using 10–23 Dz to develop novel therapies against influenza infections.

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