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RESEARCH

Nucleic Acid-Mediated Cleavage of M1 Gene of Influenza A Virus Is Significantly Augmented by Antisense Molecules Targeted to Hybridize Close to the Cleavage Site

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Abstract Influenza A virus genome segment 7 encodes protein M1, which is the matrix protein playing crucial role in the virus life cycle. Any antiviral strategy that aims at reducing, in particular, the expression of this genome segment should, in principle, reduce the infectivity of the virus. We developed a specific antiviral approach at the molecular level and designed several novel 10–23 DNAzymes (Dz) and hammerhead ribozymes (Rz), specifically targeted to cleave at the conserved domains of the influenza virus M1 RNA. We sought to use antisense molecules with the hope that it will facilitate the ribozyme-mediated cleavage. We observed that the Mg^{2+}-dependent sequence-specific cleavage of M1 RNA was achieved by both the Dz and Rz in a dose-dependent manner. This combination of catalytic Dz and Rz with antisense molecules, in principle, resulted in more effective gene suppression, inhibited the whole virus replication in host cell, and thus could be exploited for therapeutic purposes.

Keywords Influenza virus · DNAzyme · Ribozyme · Antisense · Gene silencing

Abbreviations

Dz	DNAzymes
Rz	Ribozymes
AS	Antisense

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Introduction

Influenza virus, since time immemorial, has been a major concern for public health and is responsible for significant morbidity and mortality [1]. They have been circulating worldwide with established lineages in avian and mammalian species. A rapid evolution in the field of gene silencing strategies has taken place in the past decade following the enhanced understanding of gene functions in the pathogenesis of a disease. Among the several range of agents that encompasses gene silencing therapeutics, the DNAzymes (Dz), ribozymes (Rz) and antisense molecules have shown proven results in downregulating the replication of many pathogens in mammalian hosts [2–5]. The function of nucleic acids, other than storing genetic information, has been associated recently with the catalysis of sequence-specific cleavage reactions. The Dz are short DNA molecules capable of cleaving target RNA in a sequence-specific and catalytic manner. Out of several types, the 10–23 Dz are exploited extensively by several researchers [6]. The Dz having 10–23 catalytic motifs cleave the target RNA between a purine and pyrimidine both under the in vivo and in vitro conditions. This type of Dz has previously been shown to work effectively against the HIV-I genes and other viral genes [7]. mRNAs from oncogenes and viral genomes and transcripts are the ideal targets for Dz therapeutic agents, although the 8–17 Dz are also studied for target RNA cleavage but the intracellular efficacy has not been established well. Also, the cleavage occurs between the A and G nucleotides and has a special requirement for a rG–dT wobble pair in the enzyme–substrate complex, located immediately after the cleavage site, which also makes it less popular as compared to the 10–23 Dz [8]. There are several other nucleic acid-based approaches also that have been used to specifically target a

gene. The Rz are one such class of catalytic nucleic acids that have been studied equally as the Dz. These Rz specifically target RNAs and their efficiency is comparable with those of the protein enzymes. Rz, like the Dz, have been designed to specifically target the HIV-I genes and have shown proven results [9]. Further, the viral genes have also been successfully targeted by several mono as well as multi-target Rz. Of all the Rz, the hammerhead motif-containing Rz have been exploited by several investigators because of its smaller size and impressive catalytic properties making it a promising therapeutic molecule. Any target RNA with NUX (where N is any nucleotide and X is any nucleotide except guanosine) sequence can be targeted by the hammerhead Rz. This consists of 22-nt-long conserved hammerhead Rz catalytic motif flanked by 8-nt-long hybridizing arms that are made complementary to the target RNA sequences [5]**.** Earlier, some attempts were made to interfere with the replication of influenza virus using hammerhead Rz [10, 11].

Several investigators have used both Dz and Rz individually to target-specific genes, but in this study, we have aimed to target the M1 gene of the influenza virus by both Dz and Rz in combination and also in the presence of antisense molecules. We targeted M1 gene of influenza virus as it plays an important role in several stages of virus replication [11]. In this study, we have shown the individual and synergistic post-transcriptional gene silencing potentials of Dz and Rz and identified antisense molecules that significantly enhanced the cleavage potential of Rz against the influenza virus M1 gene that is conserved among all the subtypes of influenza A viruses, thus suggesting their wide application.

Materials and Methods

Cloning and Transcription of the M1 Gene

Viral RNA was extracted from the influenza A virus strain (A/Puerto Rico/8/34) using viral RNA extraction kit (Qiagen, Hamburg, Germany). Total cDNA was prepared using IM-PROM reverse transcription kit (Promega, Madison, WI, USA), and the 788-bp M1 gene was amplified from the cDNA using gene-specific primers: (a) Forward 5′ GGC gaa ttc CC ACC ATG AGT CTT CTA ACC GAG GTC GAA 3′ (*Eco*R1 site is written in lowercase) and (b) Reverse 5′ GGC gag ctc TCA CTT GAA CCG TTG CAT CTG C 3′ (*Xho*I site is written in lowercase). The PCR-amplified M1 gene was subjected to digestion using *Eco*R1 and *Xho*1 restriction enzymes (RE) [12] and cloned in mammalian expression vector pcDNA3 (Promega, Madison, WI, USA). A truncated version of the M1 gene (356 bp) was also selectively cloned in the same vector. The plasmids then were sequenced to check the authenticity of the recombinant clone (M1-pcDNA3). The clones were linearized with the suitable RE and subjected to in vitro transcription in the presence of labeled αP^{32} UTP using T7 RNA polymerase (Promega, Madison, WI, USA).

Selection of Cleavage Sites, Designing of Dz, and Construction and Cloning of Rz

Secondary structures of M1 gene were analyzed using the M-fold RNA folding program [13]. Based on the predicted secondary structure, four Dz having the 10–23 catalytic motifs (Fig. 1a) were designed that were targeted to cleave the AU at 89, 132, 245 and 272 base positions (Table 1) [3] and four hammerhead Rz (Fig. 1b) were designed against the single-stranded looped regions that contained the cleavage sites for these catalytic enzymes at 60, 126, 163 and 351 base positions (Table 2) [14]. The Rz were constructed as described in reference 15. Briefly, a sequence of eight bases complementary to the M1 genome segment was synthesized on either side of the target site (shown by arrows in Fig. 1b) along with the central conserved catalytic domain. The Rz sequences were PCR amplified using specific terminal primers at standardized conditions (initial denaturation at 95°C for 10 min, denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s (40 cycles), and final extension temperature was 72°C for 7 min.), cloned in pcDNA3 vector and subjected to restriction digestion by *Hin*dIII and *Bam*H1 enzymes already engineered in the primers. The Dz and Rz were named based on their respective cleavage position.

In vitro Cleavage of the Substrate RNA by Dz and Rz

The cleavage reaction was carried out by using equimolar amounts (100 pmol each) of labeled substrate RNA and unlabeled Dz and Rz 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 [3, 14] for 2 h at 37°C (Fig. 1c–d). The cleavage products were resolved by 7 M urea-6% PAGE using the mini-protean gel system of Biorad [15]. The M1 transcripts were further subjected to cleavage reactions in the presence of 10–50 mM concentrations of $MgCl_2$ for the most active Dz and 1–100 mM concentration of $MgCl_2$ for the most active Rz.

Efficiency of Rz in the Presence of Antisense Molecules and Synergistic Effect of Dz and Rz

Antisense molecules (AS) against M1 gene were designed and the cleavage efficiency of Rz in the presence and absence of AS molecules (AS1: 5′-GTGACAGGATTGGTCTTGT CT-3′ designed to hybridize upstream of Rz cleavage site and

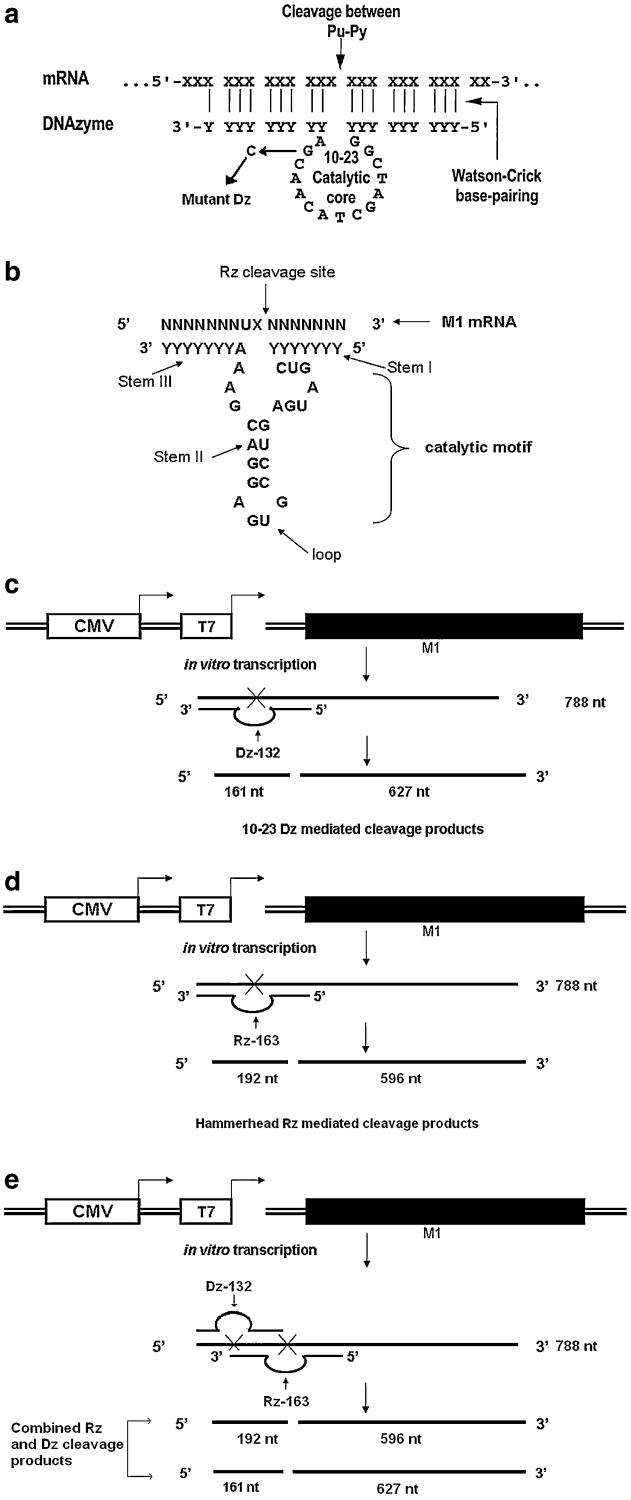

Fig. 1 The 10–23 DNAzymes and hammerhead Ribozymes were designed using the secondary structures of M1 RNA. **a** General structure of 10–23 DNAzyme. The G–C replacement of nucleotide in the catalytic motif leads to mutant Dz. **b** General structure of hammerhead Ribozyme. **c** Schematic representation of the cleavage of M1 RNA with Dz. **d** Schematic representation of the cleavage of M1 RNA with Rz. **e** Schematic representation of the combined cleavage of M1 RNA with Dz and Rz

Table 1 Sequence of the DNAzymes

M1-Dz 89	5′ AAA GAC AGG CTA GCT ACA ACG ACT TCA AG 3′
M1-Dz 132	5′ TTA GCC AGG CTA GCT ACA ACG ATC CAT GA 3′
M1-Dz 245	5′ AAG GGC AGG CTA GCT ACA ACG ATT TGG AC 3′
M1-Dz 272	5′ CAT GTT AGG CTA GCT ACA ACG ATT GGA 3′

AS2: 5′-GAACCTCAAGATCGGTGTTCT-3′ designed to hybridize downstream of Rz cleavage site) were analyzed [16]. The designed Dz and Rz that showed best cleavage results were analyzed for their synergistic effect (Fig. 1e), and the results were compared to the additive effects of the individual catalytic nucleic acid [16].

Virus Challenge, Cell Staining, FACS Analysis, and Real-time PCR

MDCK cells were seeded in six-well plates (0.5×10^6 cells per plate). After attaining 70–80% confluency, the cells were transfected with 3 μg each of Dz and Rz and 1.5 μg of AS complexed with lipofectamine-2000 (Invitrogen) as described earlier [5]. Twenty-four hours post-transfection, the cells were infected with 20 μl of A/PR/8/34 strain of influenza A virus (MOI of 0.1) in a total volume of 150 μl in 1× PBS containing 2 μg/ml TPCK-treated trypsin, for 1 h at 37°C and 5% CO_2. The cells were harvested 10 h post-transfection and subjected to FACS analysis using primary (1:100 dilutions) mouse monoclonal antibody influenza type A (cat. #VS2208), obtained from CDC, Atlanta, GA, USA and (1:100 dilution) of rabbit antimouse IgG-FITC labeled (Serotec, Oxford, UK) as secondary antibody. Unstained cells infected with virus were taken as negative control while stained cells infected with virus but without any Dz or Rz were used as a positive control. FACS results were analyzed by WinMDI software.

The inhibition of virus replication was also evaluated by a SYBR Green-based real-time PCR, and the specificity of the designed AS1 and AS2 was confirmed by comparing the results with a non-related oligo with a similar GC content as negative control. Briefly, a 25-μl reaction mixture containing One-Step SYBR Green RT–PCR mix (Bio-Rad Laboratories Inc.), forward and reverse primers specific for M1 gene, and 5 μl of RNA was used in each reaction. The PCR was performed in an iCycler iQ5 real-time PCR detection system (Bio-Rad Corp., Hercules, CA, USA) with the following conditions: 50°C for 30 min, 95°C for 5 min followed by 40 cycles at 95°C for 15 s and 55°C for 30 s. A melting curve analysis was performed after the PCR. The reactions were performed in triplicates for each sample.

Table 2 Sequence of the ribozymes

M1-Rz 60	Oligonucleotide	5′ TCGGCTTTCTGATGAGTCCGTGAGGACGAAAGGGGGCC 3′
	Sense terminal primer	5′ GGC AAG CTT TCG GCT TTC TGA TGA 3′
	Antisense terminal primer	5′ GGC GGA TCC GGC CCC CTT TCG TC 3′
M1-Rz 126	Oligonucleotide	5′ CAT TCC ATC TGA TGA GTC CGT GAG GAC GAA AGA ACC TC 3′
	Sense terminal primer	5′ GGCAAGCTTCATTCCATCTGATGAGTCC 3′
	Antisense terminal primer	5′ GGCGGATCCGAGGTTCTTTCGTCCTCA 3′
M1-Rz 163	Oligonucleotide	5′ CTTAGTCACTGATGAGTCCGTGAGGACGAAAGGTGACA 3′
	Sense terminal primer	5′ GGCAAGCTTCTTAGTCACTGATGAGTCC 3′
	Antisense terminal primer	5′ GGCGGATCCTGTCACCTTTCGTCCT 3′
M1-Rz 348	Oligonucleotide	5′ GAATAACTCTGATGAGTCCGTGAGGACGAAAGTGAGAT 3′
	Sense terminal primer	5′ GGCAAGCTTGAATAACTCTGATGAGTCC 3′
	Antisense terminal primer	5′ GGCGGATCCATCTCACTTTCGTCCTC 3′

Results

Designing of the Dz and Cloning of Rz

Thirty-one-nucleotide-long oligonucleotide containing the 15 bases long earlier characterized 10–23-Dz catalytic motif and eight nucleotides on either side of the AU target which hybridizes with the target mRNA by Watson–Crick base pairing was synthesized. A similar 38-nucleotide-long hammerhead motif Rz targeted for the NUX (where N ia any nucleotide and X is any nucleotide except guanosine) sequence was cloned successfully into pGEM-T-Easy, and the recombinant clones obtained for Rz were grown to large scale and purified on a Qiagen column. The purified clones were subjected to sequencing using forward and reverse primers commercially for confirmation. The designed catalytic Rz were cloned in 3′–5′ direction with respect to the T7 promoter, which was flanked by the *Eco*RI restriction sites. The ribozyme RNA could be synthesized using SP6 promoter of the vector. A set of four oligonucleotides for both the types of catalytic nucleic acid were designed for identification of the most efficient Dz and Rz (Figs. 2a, 3a).

Identification of Dz and Rz Having Best Cleavage Potential

Plasmid DNA encoding the M1 gene was linearized by *Eco*R1 and *Xho*I and subjected to in vitro transcription to generate a truncated (356 nt) and full-length M1 transcripts of 788 nt (Figs. 2a, 3a). The four Dz were tested for their ability to cleave the in vitro generated substrate RNA molecules. The cleavage reaction was carried out with the labeled substrate RNA and Dz used in equimolar amounts (100 pmol each) for 2 h at 37°C. The lane 1 and lane 6 in Fig. 2a shows the truncated and full-length M1 transcripts in the absence of any Dz, while the lanes 2–5 and lanes 7–10 show the effect of various Dz targeted against 89, 132, 245, and 272 base positions on truncated and full-length RNA transcripts, respectively. Among the several Dz, the Dz 132 showed best results with a cleavage product of 161 nt in both the types of RNA transcripts. Cleavage reaction was continued for the Dz 132 in the presence of varying concentrations (10–50 mM) of $MgCl_2$ (Fig. 2b).

Similarly, four hammerhead Rz targeted against 60, 126, 163, and 351 base positions on substrate RNA were tested, for their cleavage potential, on both the types (truncated and full length) of M1 transcripts at standardized cleavage conditions for the Dz. The lane 4 in Fig. 3a shows the cleavage products of 192 and 164 nt in truncated, and lane 9 shows 596 and 192 nt in full-length substrate RNA transcripts, respectively. The Fig. 3b shows the increasing cleavage potential of Rz 163 at increasing concentrations (1–100 mM) of $MgCl_2$ (Fig. 3b). The cleavage potential of both the Dz 132 and Rz 163 was found to be increasing with increasing concentrations of $MgCl_2$ in a dose-dependent manner.

Augmentation of the Hammerhead Rz-Mediated Cleavage by AS Molecules

We aimed to find out whether the cleavage potential of Rz 163 could also be enhanced by antisense molecules. We tested two sets of antisense molecules (AS1 and AS2), for their ability to enhance the Rz 163 mediated cleavage. The AS1 molecule was designed to hybridize just 10 nt upstream and AS2 to hybridize 10 nt downstream of the hybridizing arm of the Rz 163 cleavage site. The Fig. 4 shows that the AS2 molecule was better than AS1 in augmenting the Rz 163-mediated cleavage in both truncated and full-length M1-RNA. The synergistic cleavage

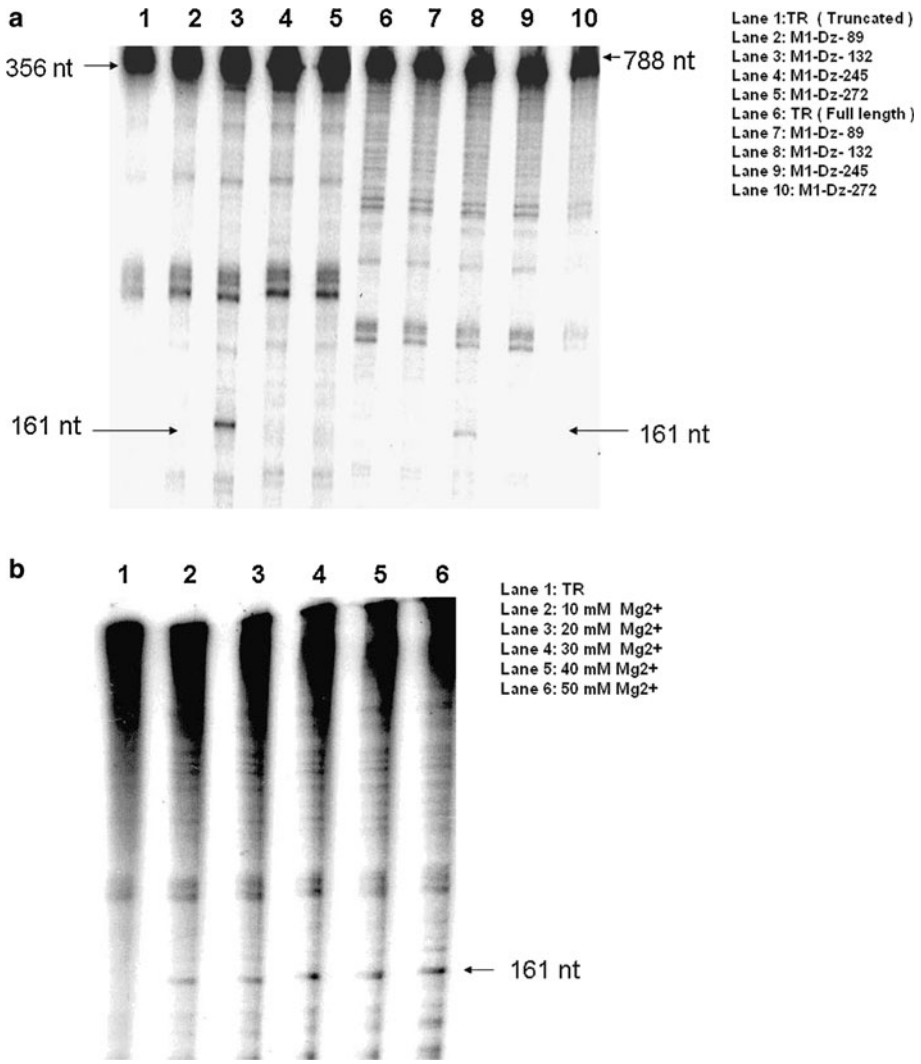

Fig. 2 The M1 gene was in vitro transcribed using T7 RiboMAX^{TM} Express Large-Scale RNA Production System (Promega). The truncated as well as full-length M1 transcripts were subjected to cleavage with different DNAzymes and in the presence of increasing concentration of $MgCl_2$. **a** *Lane 1* and *Lane 6* represent the truncated and full-length transcripts, respectively, loaded in the absence of any DNAzyme. *Lane 2–Lane 5* represent the truncated M1 transcript subjected to cleavage with Dz 89, Dz 132, Dz 245 and Dz 272 respectively. *Lane 3* shows the cleavage product of 161 nt. *Lane 7–Lane 10* represent the full-length M1 transcript subjected to cleavage with Dz 89, Dz 132, Dz 245 and Dz 272 respectively. *Lane 8* shows the cleavage product of 161 nt. **b** *Lane 1* represents the full-length M1 transcript. *Lane 2–Lane 6* represent the full-length M1 transcript subjected to cleavage with Dz 132 in the presence of 10, 20, 30, 40 and 50 mM $MgCl_2$ respectively

potential of AS molecules, however, was more than their individual effects.

Synergistic Effect of Dz and Rz, FACS Analysis

The substrate M1-RNA was subjected to cleavage by Dz 132 and Rz 163 individually and synergistically. The lane 4 in Fig. 5 shows the cleavage products of 161 and 627 nt due to Dz 132 and 192 and 596 nt due to Rz 163. The combined effect of Dz 132 and Rz 163 showed better results than their individual cleavage potentials.

To validate the cleavage potentials of these catalytic nucleic acids, FACS analysis was performed. Briefly, the MDCK cells were transfected with 3 μg each of Dz 132 and Rz 163 individually and synergistically for 24 h and subjected to virus challenge (MOI of 0.1) as described earlier in materials and methods. Significant inhibition of virus replication was observed when both the Dz 132 and Rz 163 were used synergistically (Fig. 6). The cleavage potential was found to be augmented when Rz 163 was co-transfected with two designed AS molecules, of which AS2 showed better results as seen in Fig. 7.

Real-time PCR Analysis

A real-time PCR assay was performed to confirm the cleavage potential of the catalytic nucleic acids in the presence of antisense molecules. The assay validated the specificity of AS molecules when compared to a non-related oligonucleotide of a similar GC content (Fig. 8). The data presented in Fig. 8 show that the virus replication was not at all affected when MDCK cells were transfected alone with AS molecules, ruling out the possibility of the antisense molecules to act through siRNA mechanism. When the amounts of AS molecules were doubled, the virus replication

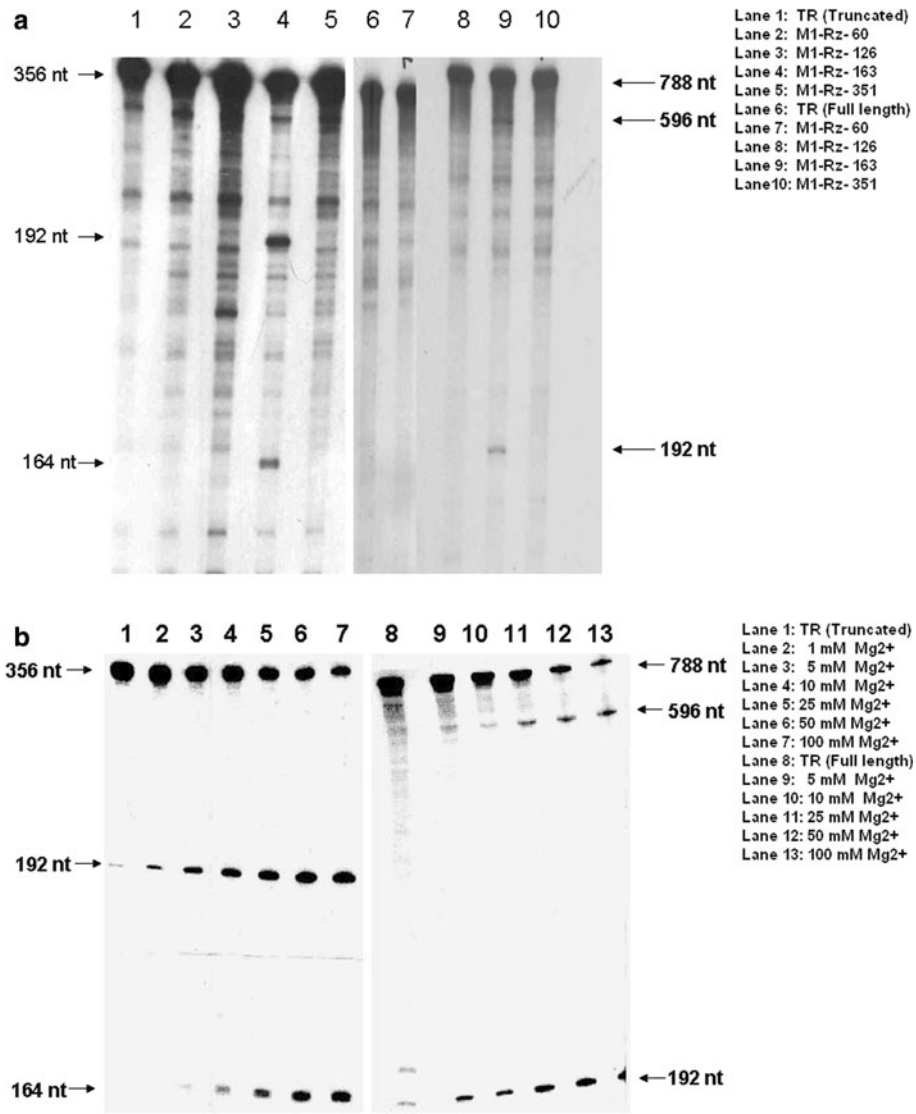

Fig. 3 The M1 gene was in vitro transcribed using T7 RiboMAX^{TM} Express Large-Scale RNA Production System (Promega). The truncated as well as full-length M1 transcripts were subjected to cleavage with different Ribozymes and in the presence of increasing concentration of $MgCl_2$. **a** *Lane 1* and *Lane 6* represent the truncated and full-length transcripts, respectively, loaded in the absence of any Ribozyme. *Lane 2–Lane 5* represent the truncated M1 transcript subjected to cleavage with Rz 60, Rz 126, Rz 163 and Rz 351 respectively. *Lane 4* shows the cleavage product of 192 and 164 nt. *Lane 7–Lane 10* represent the full-length M1 transcript subjected to cleavage with Rz 60, Dz 126, Rz 163 and Rz 351 respectively. *Lane 9* shows the cleavage product of 596 and 192 nt. **b** *Lane 1* and *Lane 8* represent the truncated and full-length M1 transcripts, respectively. *Lane 2–Lane 7* represent the truncated M1 transcript subjected to cleavage with Rz 163 in the presence of 1, 5, 10, 25, 50 and 100 mM $MgCl_2$ respectively. *Lane 9–Lane 13* represent the full-length M1 transcript subjected to cleavage with Rz 163 in the presence of 5, 10, 25, 50 and 100 mM $MgCl_2$ respectively

was almost similar to their single dose. This shows that the decrease in virus replication was due to synergistic effect of AS1 and AS2 molecules (~56%) and not due to increased dose of individual AS molecule (Fig. 8).

The real-time PCR study also showed that the Rz and Dz were able to inhibit the virus replication individually, and there was a significant decrease (~54%) in virus replication when they were used simultaneously (Fig. 9).

Discussion

With the purpose of selectively downregulating the replication of influenza virus, we have constructed hammerhead Rz and synthesized 10–23 Dz targeted against the M1 gene, which plays crucial role in the virus life cycle. The RNAi technology has been exploited against several RNA viruses including the influenza virus which is very prone to mutations, and hence, targeting a single region in the genome of the virus by one antiviral approach may result in rapid generation of variants that may be resistant to the specific Rz or Dz. Earlier investigators have shown downregulation of influenza virus gene expression using mono-specific siRNA [17] or Rz [10]. The efficient cleavage of a target RNA by Dz and Rz is important for biochemical and gene therapy approaches. Although there are several known sites for Dz and Rz activity on the mRNA, most of them do not work efficiently and cleave poorly or not at all when used against a longer mRNA length. In the present study, we have shown the downregulation of the target M1 gene of influenza A virus by novel Dz and Rz. These catalytic

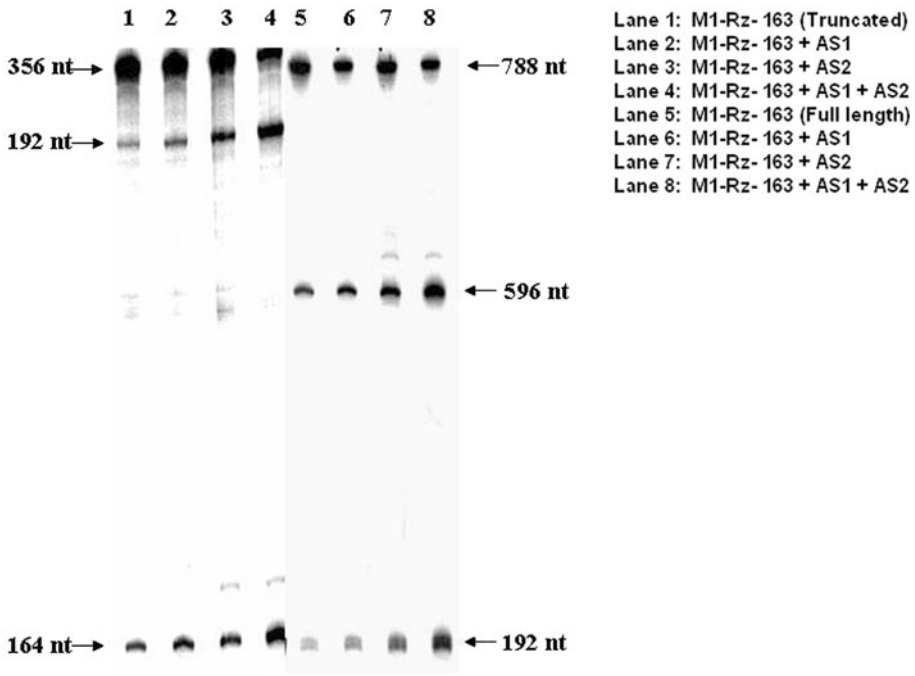

Fig. 4 The antisense oligonucleotides were developed both upstream and downstream to the cleavage sites of ribozymes. The truncated as well as full-length M1 transcripts were subjected to cleavage in the presence of different combinations of ribozymes and antisense oligonucleotides. *Lane 1* and *Lane 5* represent the cleavage of truncated and full-length M1 transcripts, respectively, with Rz 163. *Lane 2* and *3* represent the cleavage of truncated transcript in the presence of Rz 163 and AS1 (antisense upstream to cleavage site) and Rz 163 and AS2 (antisense downstream to cleavage site) respectively. *Lane 4* represents the cleavage of truncated transcript in the presence of Rz 163, AS1 and AS2. *Lane 6* and *7* represent the cleavage of full-length transcript in presence of Rz 163 & AS1 and Rz 163 & AS2 respectively. *Lane 8* represents the cleavage of full-length transcript in presence of Rz 163, AS1 and AS2

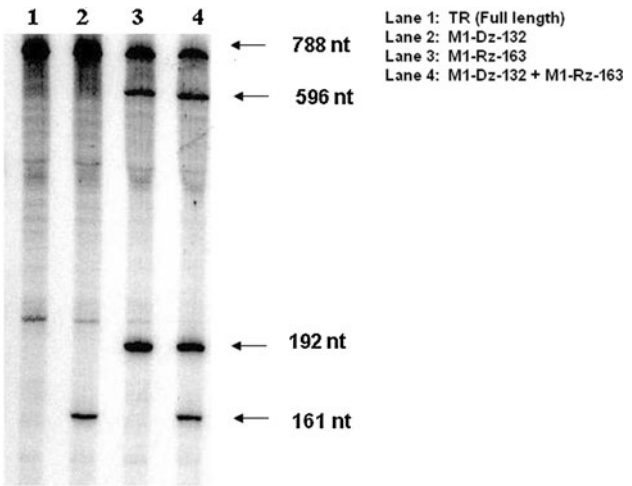

Fig. 5 The full-length M1 transcript was subjected to simultaneous cleavage with Rz 163 and Dz 132 to observe their synergistic effect. *Lane 1* represents the full-length transcript loaded in the absence of any catalytic nucleic acids. *Lane 2* and *Lane 3* represent the full-length M1 transcript cleaved with Dz 132 and Rz 163 respectively. *Lane 4* represents the full-length M1 transcript cleaved in the presence of Rz 163 and Dz 132

nucleic acids were designed with the aim of specifically interfering with the influenza virus gene expression. We chose to target the M1 gene of influenza virus with the hope that the selective downregulation of the M1 gene will possibly reduce the infectivity of the virus. The DNAzymes and ribozymes were targeted against the 788 nucleotides of the M1 gene at the AU and NUX sequences, respectively. This report has significance as most work on ribozymes or DNA enzymes have used very short either synthetic or in vitro synthesized RNA to test the cleavage potential [3, 18, 19], but in this study, we have tested the cleavage potential of our DNA enzyme (Dz) and ribozyme (Rz) on in vitro synthesized M1-RNA, which was either full length (788 nt) or greater than 50% of its authentic size. The same Dz and Rz cleaved both the truncated and full-length substrate RNA, and as expected, the cleavage was better with the truncated substrate RNA. Of all the designed Dz and Rz, the Dz 132 showed better results while the Rz-163 worked best as compared to other designed Rz. The cleavage efficiency of both the Dz and Rz was greatly influenced by the concentration of $MgCl_2$ as already proved for other Rz in literature [5]. Dz 132 with 50 mM concentration of $MgCl_2$ showed significant cleavage while almost complete cleavage was observed when Rz 163 was used at 100 mM concentration of $MgCl_2$. Similar results have been cited in literature for other unrelated ribozymes [20]. The positive results of Dz 132 and Rz 163 encouraged us to use them

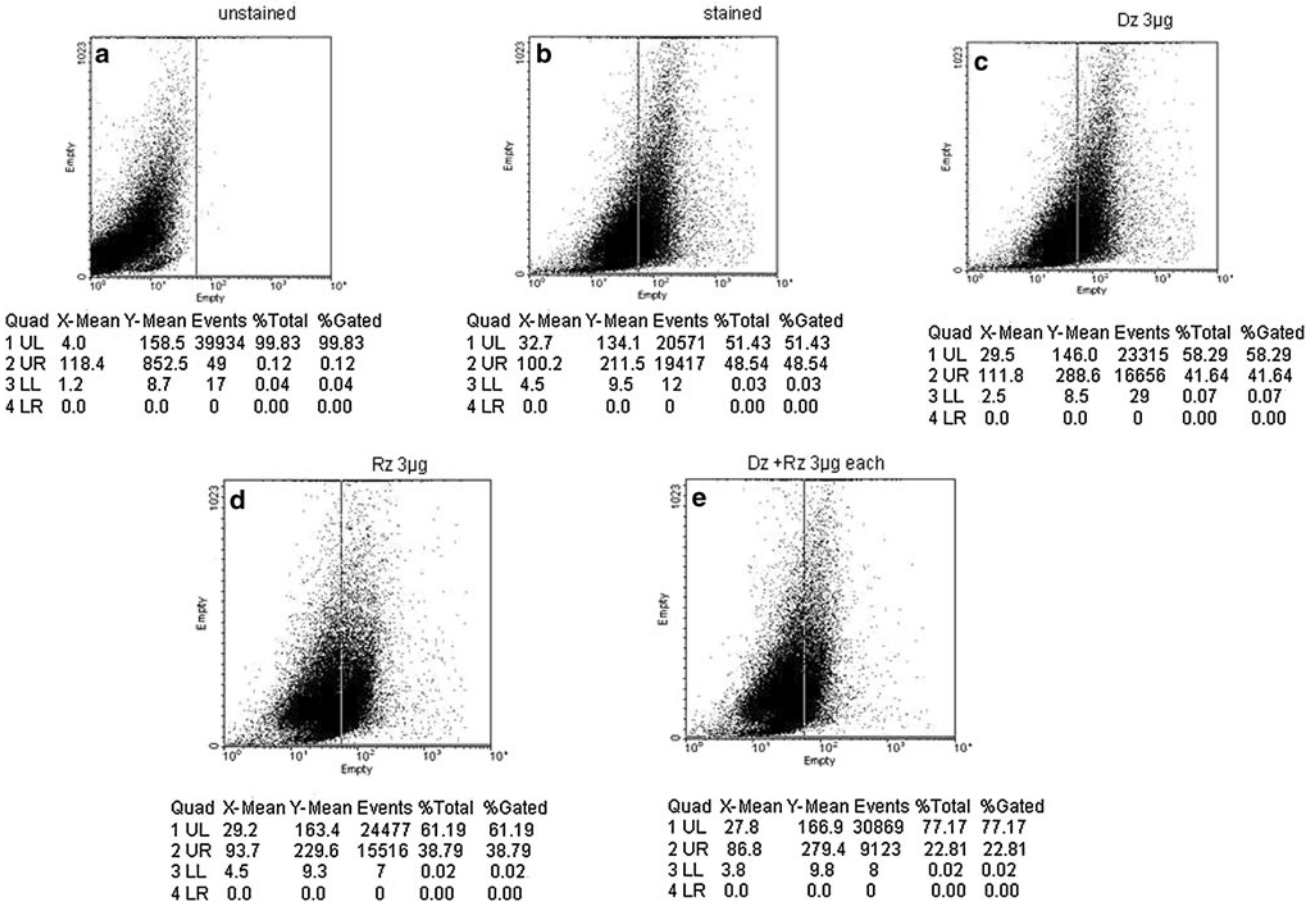

Fig. 6 The MDCK cells were transfected with or without the DNAzymes and Ribozymes and subjected to virus challenge (MOI of 0.1) at 24 h post-transfection. The cells were then subjected to FACS analysis. Panel **a** represents the uninfected cells. Panel **b** represents the MDCK cells infected with the influenza A virus at a MOI of 0.1. Panel **c** represents the infected MDCK cells treated with 3 µg of Dz 132. Panel **d** represents the infected MDCK cells treated with 3 µg of Rz 163. Panel **e** represents the infected MDCK cells treated with 3 µg each of Dz 132 and Rz 163

in combination, to analyze their synergistic cleavage effect on the substrate RNA. The synergistic effect was found better then their individual effects; however, direct comparisons could not be made between the Dz and Rz since they were targeted to cleave at different positions on the M1-RNA; nevertheless, Rz was found to be more efficient.

We then addressed the question whether the use of AS molecules could augment the cleavage potentials of Rz. For this, we used two AS molecules designed to hybridize both upstream (AS1) and downstream (AS2) of the substrate binding arms of the Rz 163, and as expected, the activity of Rz significantly got enhanced when used synergistically with antisense molecules to achieve maximum downregulation of the target gene.

A direct correlation of increasing concentration of $MgCl_2$ and the cleavage activity of Rz with AS molecules was observed. To analyze whether these designed molecules provided protection against live influenza virus, MDCK monolayer cells were transfected with Rz and Dz, both individually and synergistically, and after 24 h, it was infected with influenza A virus. The FACS analysis was performed to show that these Dz and Rz gave best results when used in combination, and a significant inhibition of the virus replication was also observed. The real-time PCR studies with a non-related oligonucleotide allowed us to conclude that both Dz and Rz played an important role toward the inhibition of gene expression, the major role being played by the ribozyme in the presence of specific antisense molecule. These results also confirmed that the designed AS molecules did not act through independent siRNA mechanism to downregulate the M1 gene. It was observed that the synergistic effect of AS1 and AS2 was better than the synergistic effect of Dz and Rz. Thus, our findings suggest that these novel molecules can be potently used as antiviral therapy to control the virus replication inside mammalian host cells.

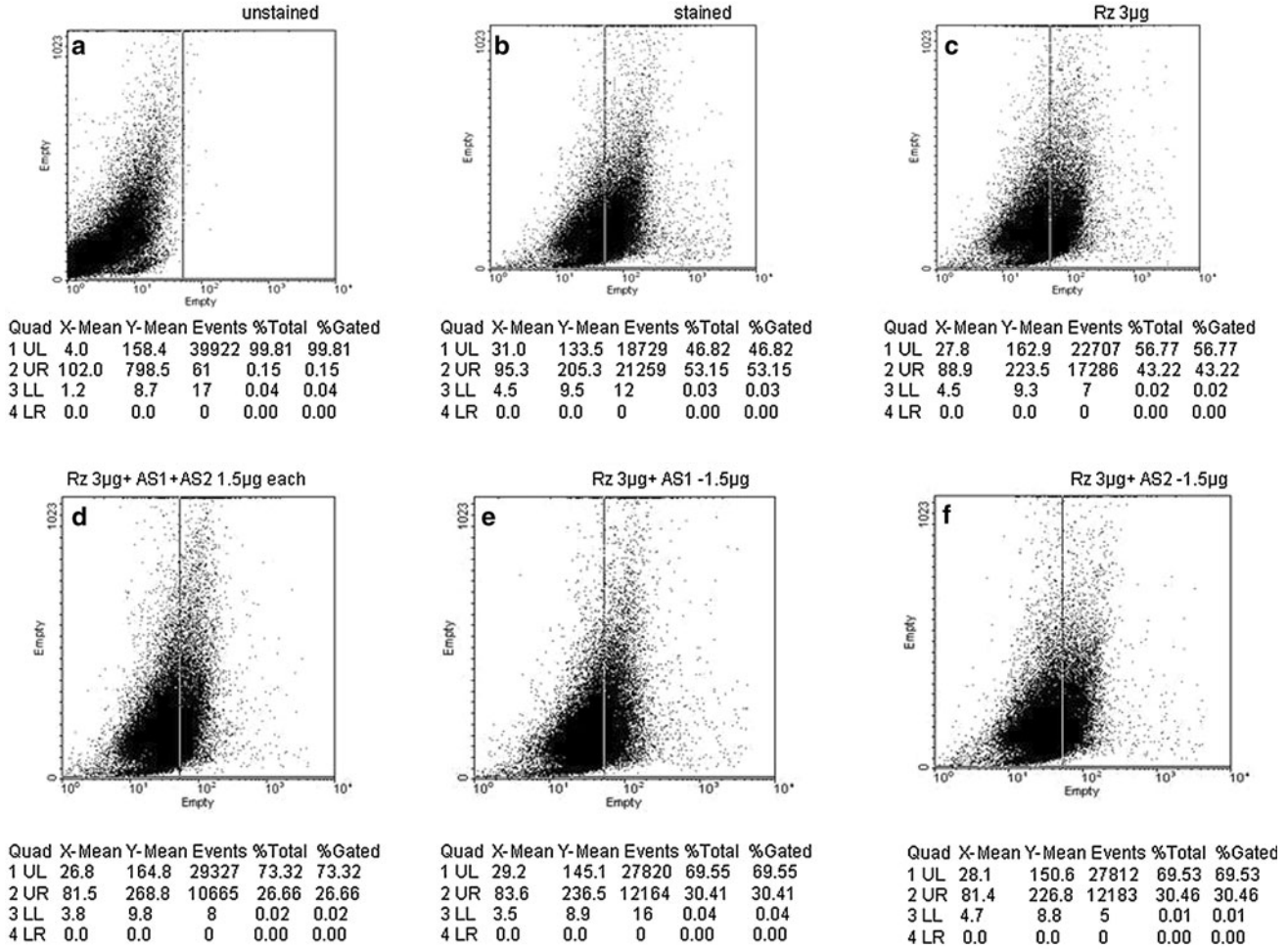

Fig. 7 The MDCK cells were transfected with or without the antisense molecules and subjected to virus challenge (MOI of 0.1) at 24 h post-transfection. The cells were then subjected to FACS analysis. Panel **a** represents the uninfected cells. Panel **b** represents the MDCK cells infected with the influenza A virus at a MOI of 0.1. Panel **c** represents the infected MDCK cells treated with 3 µg of ribozyme. Panel **d** represents the infected MDCK cells treated with 3 µg of ribozyme and 1.5 µg each of AS1 and AS2. Panel **e** represents the infected MDCK cells treated with 3 µg of ribozyme and 1.5 µg of AS1. Panel **f** represents the infected MDCK cells treated with 3 µg of ribozyme and 1.5 µg of AS2

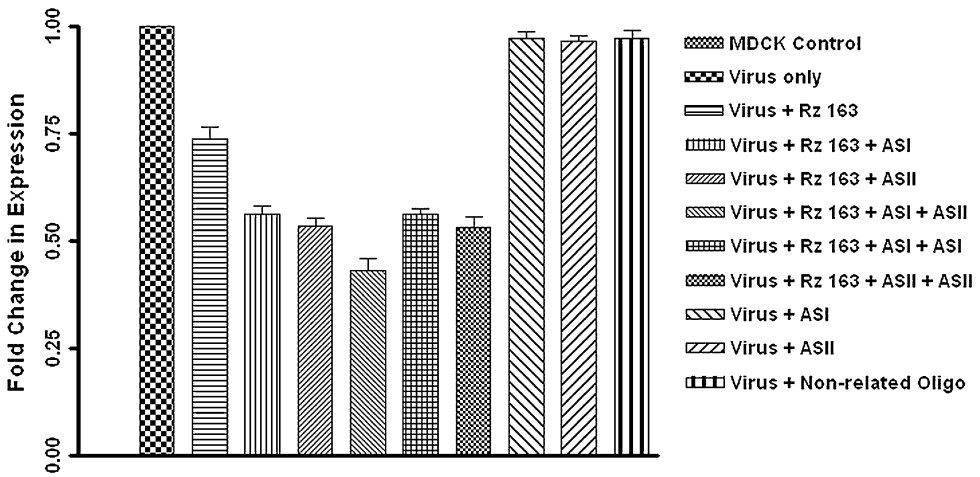

Fig. 8 The MDCK cells were transfected with 3 µg of ribozyme and 1.5 µg each of AS1 and AS2 molecules and subjected to virus challenge (MOI of 0.1) at 24 h post-transfection. Total RNA was isolated and subjected to real-time PCR analysis to detect the fold change in expression of M1 gene of influenza A virus

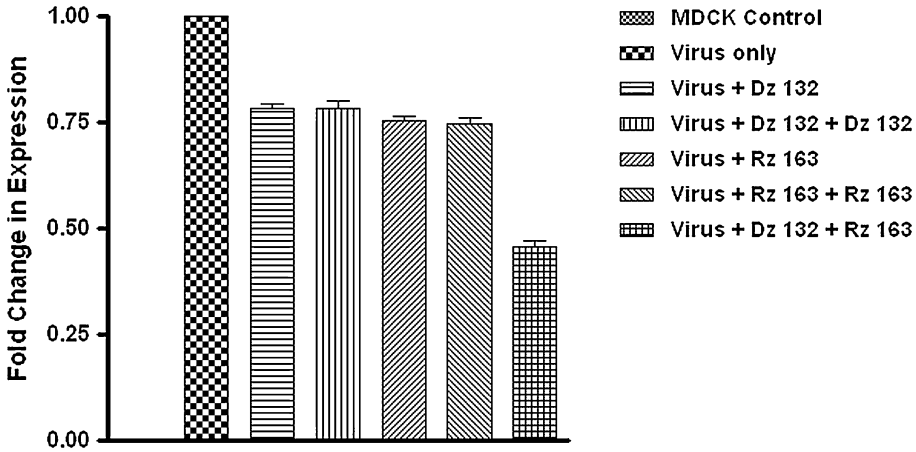

Fig. 9 The MDCK cells were transfected with 3 μg each of ribozyme and DNAzyme individually as well as synergistically and subjected to virus challenge (MOI of 0.1) at 24 h post-transfection. Total RNA was isolated and subjected to real-time PCR analysis to detect the fold change in expression of M1 gene of influenza A virus

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