PA<sub>N</sub> substitutions A37S, A37S/I61T and A37S/V63I attenuate the replication of H7N7 influenza A virus by impairing the polymerase and endonuclease activities

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Abstract

Substitutions in the PA N-terminus (PA<sub>N</sub>) of influenza A viruses are associated with viral pathogenicity. During our previous study, which identified PA<sub>N</sub>-V63I and -A37S/I61T/V63I/V100A substitutions as virulence determinants, we observed a severe decrease in virus growth and transcription/replication capacity posed by PA<sub>N</sub>-A37S/V100A substitution. To further delineate the significance of substitutions at these positions, we generated mutant H7N7 viruses bearing the substitutions PA<sub>N</sub>-A37S, -A37S/I61T, -A37S/V63I, -V100A, -I61T/V100A and -V63I/V100A by reverse genetics. Our results showed that all mutant viruses except PA<sub>N</sub>-V100A showed a significantly reduced growth capability in infected cells. At the same time, the PA<sub>N</sub>-A37S, -A37S/I61T and -A37S/V63I mutant viruses displayed decreased viral transcription and replication by diminishing virus RNA synthesis activity. Biochemical assays indicated that the substitutions PA<sub>N</sub>-A37S, -A37S/I61T and -A37S/V63I suppressed the polymerase and endonuclease activities when compared with those of the wild-type. Together, our results demonstrated that the PA<sub>N</sub>-A37S, -A37S/I61T and -A37S/V63I substitutions contributed to a decreased pathogenicity of avian H7N7 influenza A virus.

INTRODUCTION

The influenza A virus (IAV) is an important pathogen that causes diseases in avian, mammalian and human hosts [1]. The natural reservoirs of IAVs are wild aquatic birds, and all subtypes of IAVs can be detected in them [2]. IAVs have caused recurring or sporadic outbreaks in human populations at global or local scales [1, 3], including pandemics characterized by human-to-human transmission [4–6] and clusters of human infections transmitted directly from avian hosts [7–9]. In the light of threats posed to human health and the economy by circulating and future avian IAVs, elucidating virus molecular determinants and the underlying mechanisms that restrict or favour virus replication and/or pathogenicity are paramount.

IAV is a single-stranded, negative-sense RNA virus with a segmented genome [10]. The molecular basis of IAV pathogenicity and/or adaptation to mammalian and human hosts is complex and polygenic, and remains incompletely understood [10, 11]. However, increasing evidence has determined the critical roles of viral ribonucleoproteins (RNPs) in virus pathogenicity and/or host adaptation. The RNPs are composed of virus RNAs associated with NP and the heterotrimeric PB1–PB2–PA polymerase complex, which drive viral transcription and replication [2, 12]. Briefly, PB1 serves as the RNA-dependent RNA polymerase, while PB2 is required for the cap-binding during virus mRNA synthesis. PA has been determined to have multiple functions in viral transcription and replication, including cap-dependent endonuclease activity, proteolytic activity, promoter binding activity and potential roles in the elongation during virus RNA synthesis [10]. Importantly, the PA N-terminus (PA<sub>N</sub>) functions as an endonuclease and performs endonucleolytic cleavage of the host mRNA, which is critical for virus mRNA synthesis in viral transcription [13–16]. Due to its important roles in the IAV infection cycle, amino acid substitutions in PA<sub>N</sub> have been implicated to be associated with IAV pathogenicity and/or host-range [17–19]. For example, PA<sub>N</sub>-E80A, -R84A,
Substitutions PA<sub>N</sub>-A37S, -A37S/I61T, -A37S/V63I, -I61T/V100A and -V63I/V100A decreased virus growth

In a previous report, we found that the growth capacity of the PA<sub>N</sub>-A37S/V100A mutant virus was substantially lower than that of the wild-type H7N7 IAV (wt-H7N7) in Madin–Darby canine kidney (MDCK) cells [25]. We asked whether the decrease was caused by one of the single substitutions or the effect of double substitutions. To determine this, we generated mutant viruses carrying double substitutions, including PA<sub>N</sub>-A37S/I61T, -A37S/V63I, -I61T/V100A and -V63I/V100A.

To explore the potential impact of substitutions on virus growth, we inoculated wild-type and mutant viruses to MDCK cells at a multiplicity of infection (m.o.i.) of 1, and harvested cell culture supernatants at 8 h post-infection (p.i.). The mutant virus PA<sub>N</sub>-A37S/V100A was included as a control for the virus growth assay and the following experiments. Virus titres were titrated in MDCK cells by plaque assay. Our results showed that the virus titres of the mutant viruses PA<sub>N</sub>-A37S, -A37S/I61T, -A37S/V63I, -I61T/V100A and -V63I/V100A were substantially lower than that of wt-H7N7 (P<0.001) (Fig. 1a). In contrast, the virus titre of the PA<sub>N</sub>-V100A mutant virus was similar to that of wt-H7N7 (P=0.9975) (Fig. 1a). Additionally, the above samples were quantified by RT-qPCR, and the results were consistent with those of the plaque assay (Fig. 1b). Collectively, our results demonstrated that the substitutions PA<sub>N</sub>-A37S, -A37S/I61T, -A37S/V63I, -I61T/V100A and -V63I/V100A decreased virus growth, while the substitution PA<sub>N</sub>-V100A presented a similar growth capacity to wt-H7N7.

Substitutions PA<sub>N</sub>-A37S, -A37S/I61T, -A37S/V63I, -I61T/V100A and -V63I/V100A suppressed virus mRNA synthesis

Next, we investigated whether the PA<sub>N</sub> substitutions might alter viral transcription and replication by examining the synthesis of virus haemagglutinin (HA) mRNAs, HA cRNAs and HA vRNAs. MDCK cells were infected with wild-type and mutant viruses at an m.o.i. of 2. Cell lysates were collected at 4 and 6 h p.i., and were then subjected to RNA extraction and quantification. Our results showed that the mRNA levels of the mutant viruses PA<sub>N</sub>-A37S, -A37S/I61T, -A37S/V63I, -I61T/V100A and -V63I/V100A were lower than that of wt-H7N7 (P<0.001) (Fig. 2a). Therefore, these PA<sub>N</sub> substitutions contributed to a decrease in virus transcription capacity. Moreover, the PA<sub>N</sub>-A37S, -A37S/I61T and -A37S/V63I mutant viruses produced fewer virus mRNAs (Fig. 2a), cRNAs (Fig. 2b) and vRNAs (Fig. 2c) compared with those of wt-H7N7 at 4 h p.i. (P<0.05), indicating a reduced virus transcription and replication capacity. To our surprise, the PA<sub>N</sub>-V100A mutant virus showed modestly higher mRNA levels (P<0.001 at 4 and 6 h p.i.) but a lower cRNA level (P=0.0023 at 4 h p.i.) when compared with those of wt-H7N7. This indicated that the PA<sub>N</sub>-V100A substitution might confer an increased virus transcription but a decreased replication. Taken together, our results suggested that the substitutions PA<sub>N</sub>-A37S, -A37S/I61T, -A37S/V63I, -I61T/V100A and -V63I/V100A suppressed viral transcription by reducing the synthesis of virus mRNAs.

Substitutions PA<sub>N</sub>-A37S, -A37S/I61T and -A37S/V63I attenuated polymerase activity

To determine the contributions of these PA<sub>N</sub> substitutions to polymerase activity, we performed minireplicon assays in human 293 T cells. As shown in Fig. 3, the PA<sub>N</sub>-A37S, -A37S/I61T and -A37S/V63I mutant polymerase complex presented a substantially lower polymerase activity when compared with that of wt-H7N7 (P<0.05). Intriguingly, the mutants with substitutions PA<sub>N</sub>-V100A, -I61T/V100A and -V63I/V100A did not exhibit appreciable effects on the
polymerase activity compared with wt-H7N7 (Fig. 3). This suggested that the polymerase activity did not account for the changes observed in virus transcription of the PAN

V100A, -I61T/V100A and -V63I/V100A mutants. Collectively, our results indicated that the PAN-A37S, -A37S/I61T and -A37S/V63I substitutions conferred attenuated polymerase activity in human 293 T cells.

Substitutions PAN-A37S, -A37S/I61T and -A37S/V63I impaired endonuclease activity

The substitutions identified above are all located at the PAN domain, which acts as an endonuclease that cleaves cellular mRNAs during the cap-snatching process of virus transcription [13, 14]. Our earlier data demonstrated that PAN proteins (amino acids 1–196) of wild-type H7N7 IAV expressed in Escherichia coli retained endonuclease activity [25]. Given that virus transcription was suppressed or instability conferred by the substitution in the context of the PAN domain expressed in E. coli. The vacant vector pET-32a (+) without virus PAN inserts was also expressed and purified as the negative control, which was termed Vector. We then evaluated the endonuclease activity of the PAN proteins by fluorescence-based endonuclease assays. Our results showed that the mutants with substitutions PAN-A37S, -A37S/I61T, -A37S/V63I and -V63I/V100A presented lower endonuclease activity than wt-H7N7 (P<0.001) (Fig. 4c). The PAN-I61T/V100A mutant virus presented a small decrease in the endonuclease activity, but the difference was not statistically significant (P=0.0795) (Fig. 4c).

Interestingly, the mutant with the PAN-V100A substitution exhibited higher endonuclease activity than wt-H7N7 (P<0.001) (Fig. 4c), which might account for the increased mRNA synthesis (Fig. 2a). The in vitro comparison of the endonuclease activity confirmed that the substitutions PAN-A37S, -A37S/I61T and -A37S/V63I might decrease viral transcription by suppressing the endonuclease activity.

Substitutions PAN-A37S, -A37S/I61T and -A37S/V63I conferred a decreased binding affinity towards the nucleic acid substrate as well as a destabilized structure

To identify the effect of the A37S, A37S/I61T and A37S/V63I substitutions on the PAN structure, 3D models of PAN proteins of wt-H7N7 and those of the mutant viruses carrying the substitutions were generated and docked with a nucleic acid substrate. Our results showed that the binding energy changes of PAN-A37S, -A37S/I61T and -A37S/V63I were −15.30, −8.51 and −15.80, respectively (Fig. 5). However, the binding energy change of wt-H7N7 PAN was −21.66, which was substantially lower than those of the proteins with substitutions PAN-A37S, -A37S/I61T and -A37S/V63I (Fig. 5). This suggested that the PAN-A37S, -A37S/I61T and -A37S/V63I substitutions resulted in a decreased binding affinity towards the nucleic acid substrate. Additionally, the effects of the substitutions on PAN models were evaluated. Our data showed that the substitutions PAN-A37S, -A37S/I61T and -A37S/V63I resulted in reduced protein stability changes when the wt-H7N7 PAN model was used as a native structure. This suggested that the substitutions PAN-A37S, -A37S/I61T and -A37S/V63I introduced a destabilizing effect on the PAN structure.

![Fig. 1. Comparison of virus growth in MDCK cells. MDCK cells were infected with wt-H7N7 and mutant viruses PAN-A37S, -A37S/I61T, -A37S/V63I, -V100A, -I61T/V100A, -V63I/V100A and -A37S/V100A at an m.o.i. of 1. Cell culture supernatants were harvested at 8 h p.i., and viral titres were determined in MDCK cells by plaque assay (a) and real-time RT-qPCR (b).](https://www.microbiologyresearch.org/article/364-373)
models. Our results showed that the substitutions PA-N-A37S, -A37S/I61T and -A37S/V63I might lead to a decreased binding affinity towards the nucleic acid substrate as well as a destabilized structure.

**DISCUSSION**

In this study, we demonstrated that the substitutions PA-N-A37S, -A37S/I61T and -A37S/V63I decreased virus pathogenicity. Our results showed that all of the mutant viruses except PA-N-V100A decreased virus growth (Fig. 1) and virus mRNA synthesis (Fig. 2). Moreover, minireplicon assays showed that the substitutions PA-N-A37S, -A37S/I61T and -A37S/V63I decreased polymerase activity (Fig. 3). These substitutions also suppressed endonuclease activity, as revealed by endonuclease assays (Fig. 4). Collectively, we demonstrated that the PA-N-A37S, -A37S/I61T and -A37S/V63I substitutions contributed to decreased virus pathogenicity through decreasing the polymerase and endonuclease activities.

We previously found that almost all of the recently emerged IAVs, including H7N9, H5N6 since 2015 and H10N8 and H9N2 since 2014, presented amino acid substitutions PA-N-A37S/I61T/V63I or -A37S/I61T/V63I/V100A [25]. It was demonstrated that the substitutions PA-N-V63I and -A37S/I61T/V63I/V100A increased virus virulence in the background of avian H7N7 IAV [25]. Interestingly, we found that the PA-N-A37S/V100A substitution substantially decreased virus growth and transcription/replication capacity [25]. This raised questions about the contributions of the single substitutions PA-N-A37S and -V100A to viral pathogenicity. Using structural analysis, Kowalinski et al. found that the PA-N-37A residue is critical for the conformation and placement of a PA-N endonuclease inhibitor [26]. This suggested that the residue PA-N-37A might be essential for endonuclease activity. Neumann et al. also proposed that

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**Fig. 2.** Comparison of virus RNA synthesis in MDCK cells. The wt-H7N7 and mutant viruses PA-N-A37S, -A37S/I61T, -A37S/V63I, -V100A, -I61T/V100A, -V63I/V100A and -A37S/V100A were inoculated into MDCK cells at an m.o.i. of 2. The infected cells were then subjected to lysis and harvested at 4 and 6 h p.i. Viral mRNAs (a), cRNAs (b) and vRNAs (c) were extracted and quantified. Three independent experiments were performed. Data shown are the amount of virus RNAs normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (mean ± SD). *P<0.05, **P<0.01 and ***P<0.001, as compared with those of wt-H7N7 using a one-way ANOVA followed by Dunnett’s test.
PA\textsubscript{N}-A37S might affect endonuclease activity [27]. In the present study, our results demonstrated that the PA\textsubscript{N}-A37S substitution reduced the endonuclease activity. In addition to reducing polymerase activity, the substitution PA\textsubscript{N}-A37S reduced the virus pathogenicity of avian H7N7 IAV, which added to our evolving understanding of IAV pathogenicity. The presence of the PA\textsubscript{N}-A37S substitution in the recently emerged avian IAVs might help to counterbalance the enhancing effect posed by other virus substitutions, as Gabriel et al. reported that excessive polymerase activity might be incompatible with the high pathogenicity of IAVs in mammals [28].

Comprehensive studies have shown that IAV transcription is carried out through the process of vRNA into mRNA, while the virus replication involves the processes of vRNA into cRNA and cRNA into vRNA [29, 30]. The PA\textsubscript{N}-V100A has been determined to be the human host marker of IAVs [31–33]. Our study demonstrated that the PA\textsubscript{N}-V100A substitution elevated viral transcription but suppressed viral replication by producing more mRNAs and slightly fewer cRNAs. This might account for the virus vRNA synthesis activity and virus growth of the mutant virus carrying the PA\textsubscript{N}-V100A substitution being similar to those of wt-H7N7.

In our previous research, the PA\textsubscript{N}-I61T substitution presented a slight suppression in virus growth, whereas the PA\textsubscript{N}-V63I substitution contributed to an obvious increase [25]. In the present study, we evaluated the role of the double substitutions PA\textsubscript{N}-A37S/I61T and PA\textsubscript{N}-A37S/V63I in virus pathogenicity, as the substitutions at these positions might occur simultaneously in nature. The results showed that the mutant virus carrying the PA\textsubscript{N}-A37S/I61T substitution exhibited a more severe decrease in virus growth when compared with those carrying the PA\textsubscript{N}-A37S and -I61T substitutions. This indicated a synergistic effect between the substitutions at positions 37 and 61. Song et al. also determined the synergistic effect between PA substitutions at positions 224 and 383 for enhancement of the virulence of H5N1 IAV [34]. Note that we might not be able to arbitrarily infer the effect of double substitutions merely based on the effect of single substitutions on viral pathogenicity. The combination of the substitutions might lead to some unexpected impact on virus pathogenicity. For instance, the mutant virus carrying the PA\textsubscript{N}-A37S/V63I substitution showed more severe attenuation in virus growth and pathogenicity than the one carrying PA\textsubscript{N}-A37S, even though PA\textsubscript{N}-V63I was demonstrated to enhance virus virulence. Our present data also indicated that the PA\textsubscript{N}-A37S substitution conferred a dominant suppression effect on the PA\textsubscript{N}-V63I substitution. Intriguingly, our previous data demonstrated that the PA\textsubscript{N}-A37S/I61T/V63I/V100A substitution increased virus pathogenicity [25]. It is not yet known why PA\textsubscript{N}-A37S did not result in a predominant decrease in the PA\textsubscript{N}-A37S/I61T/V63I/V100A substitution. Some interactions among viral factors and/or cellular factors might account for this variation.

Similarly, the PA\textsubscript{N}-I61T/V100A and -V63I/V100A substitutions were found to decrease virus growth and transcription capacity. Of note, the PA\textsubscript{N}-I61T/V100A and -V63I/V100A substitutions did not significantly alter the polymerase activity, even though a correlation between polymerase activity and virus growth was observed by Gabriel et al. [28, 35], in addition to being seen in our studies. Yamaji et al. also found that the PA-V44I, PA-V127A, PA-C241Y, PA-A343T and PA-I573V substitutions did not contribute to variations in polymerase activity, while they enhanced virus growth and pathogenicity [36]. However, Yamaji et al. did not further clarify the molecular mechanisms that underlay the enhancement. In the present study, we demonstrated that the PA\textsubscript{N}-I61T/V100A and -V63I/V100A substitutions reduced the endonuclease activity. This might help to explain the decrease in viral transcription and virus growth.

It is essential to test the significance of substitutions in the background of different IAV subtypes, as the substitutions may be strain-dependent and thereby result in variations in viral pathogenicity. This view is supported by the observations that the well-characterized PB2-E627K substitution attenuates the pathogenicity of A(H1N1)pdm09 [37], whereas the substitution potentiates virus pathogenicity in H7N9 [38, 39], H5N1 [40, 41] and H7N7 IAVs [28] in mammalian hosts. Recently, Yamayoshi et al. tested the significance of PA\textsubscript{N}-S37A and -A100V in the backbone of the A/Anhui/1 (H7N9) virus [19]. In our study, we further extended the initial observations and identified the suppressing role of PA\textsubscript{N}-A37S and the enhancing effect of PA\textsubscript{N}-V100A on endonuclease activity.

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**Fig. 3.** Comparison of the polymerase activity. Human 293 T cells were transfected with plasmids pHW2000-PA (with or without the substitutions), -PB1, -PB2 and -NP, together with a firefly luciferase reporter plasmid flanked by a fragment of the IAV noncoding region and an eGFP plasmid. Luciferase activity was determined at 24 h p.i. At least three independent experiments were performed. Data shown are the relative polymerase activity of the mutants indicated to that of wt-H7N7 (mean±SD). *P<0.05 and **P<0.001, as compared with that of wt-H7N7 using a one-way ANOVA followed by Dunnett's test.
In summary, our findings demonstrated that the substitutions PA\textsubscript{N}-A37S, -A37S/I61T and -A37S/V63I suppressed virus growth and transcription/replication \textit{in vitro} through impairment of the polymerase and endonuclease activities. Our data will help to improve our understanding of the roles of PA\textsubscript{N} in IAV pathogenicity, which may be beneficial for both surveillance and for the development of potential therapeutics against the viruses.

**METHODS**

**Cells and viruses**

Madin–Darby canine kidney (MDCK) cells and human embryonic kidney 293 T cells were maintained in Eagle’s minimum essential medium (MEM; Life Technologies) and Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies), respectively. All media were supplemented with 10 % FBS (Life Technologies) and 1 % penicillin/streptomycin (P/S) at 37 °C in 5 % CO\textsubscript{2} [42]. Avian influenza A virus (IAV) strain A/Netherlands/219/2003 (H7N7) and mutant viruses were propagated in MDCK cells supplemented with 1 % P/S [43]. All experiments with H7N7 IAVs were conducted in biosafety level 3 (BSL-3) laboratories [44].

**Site-directed mutagenesis and virus rescue**

The reverse genetics of avian IAV A/Netherlands/219/2003 (H7N7) was established, as reported previously [25]. Site-directed mutagenesis was conducted to introduce PA\textsubscript{N}-A37S, -A37S/I61T, -A37S/V63I, -V100A, -I61T/V100A and -V63I/V100A substitutions into the plasmid pHW2000-PA using a QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). Primers possessing the specific substitutions are available upon request. The desired mutations were confirmed by sequencing in the Centre for
Genomic Sciences, the University of Hong Kong. Avian H7N7 IAVs, with or without PA$_N$ substitutions, were rescued by reverse genetics [25].

**Virus replication assay**

To evaluate virus growth competence, wild-type and mutant viruses of H7N7 IAVs were inoculated into confluent MDCK cells in 24-well plates at a m.o.i. of 1. The inocula were aspirated and washed away by using PBS at 1 h p.i. Afterwards, the infected MDCK cells were cultured in MEM medium at 37°C. Cell culture supernatants were harvested at 8 h p.i. The samples were titrated in MDCK cells by plaque assay and real-time RT-qPCR by targeting virus HA fragments [42, 45].

**Virus mRNA, cRNA and vRNA quantification**

A modified real-time RT-qPCR was carried out for the quantification of virus mRNAs, cRNAs and vRNAs, which could reflect viral transcription and replication capacity [46]. Briefly, wild-type and mutant viruses of H7N7 IAVs were inoculated into confluent MDCK cells in 24-well plates at an m.o.i. of 2 and washed away by using PBS at 1 h p.i. The infected MDCK cells were cultured in MEM medium at 37°C, and cell lysates were harvested at 4 and 6 h p.i., respectively. Total RNAs were extracted using the RNeasy Mini kit (Qiagen). Viral cDNA was synthesized using primers targeting the viral HA gene and containing specific genetic tags (HA mRNA primer 5'-CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTCCAACTTATA-3', HA cRNA primer 5'-GCTAGCTTCAGCTAGGCATCAGTA-GAAACAGGGGTGGTTTTCCAA-3' and HA vRNA primer 5'-GGCCGTCATGGGTCGGATACCTACAGCGGAATAGAACTAATG-3'). The tags are shown in italics. A Transcriptor First Strand cDNA Synthesis kit (Roche) was used for RT-PCR reactions according to the manufacturer's protocol. The expression of cellular glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) mRNA was included as an internal control.

Real-time qPCR was performed on the LightCycler 96 (Roche) using primers targeting the specific tags and HA gene fragments [HA mRNA forward primer (FP) 5'-GCC TTGCTCATTATGTTGGAAGAA-3', reverse primer (RP) 5'-CCAGATCGTCCAGTCTG-3']. HA cRNA FP 5'-TG TTCATGATCTGGAATCGG-3' and HA vRNA FP 5'-GGCGG GCATGCTTC-3'; and HA mRNA reverse primer (RP) 5'-CCGTTGCGGAAGAA-3'. The reactions contained 5 µl cDNAs (1:10 diluted), 1 µl FPs (10 µM) or 1 µl RPs (10 µM), 10 µl FastStart SYBR Green I Master and 3 µl sterile water. Viral HA RNA levels were normalized to the GAPDH mRNA level [47].

**Minireplicon assay**

Human 293 T cells were transfected by four pHW2000 plasmids harbouring virus PA (+) with and without the substitutions), PB2, PB1 and NP (0.05 µg each), together with a firefly luciferase reporter plasmid flanked with IAV 5' noncoding regions (0.05 µg), and a plasmid expressing eGFP (0.10 µg) [25, 48]. The transfected 293 T cells were incubated at 37°C. At 24 h post-transfection, the cells were lysed and the luciferase activity was measured using the Bright-Glo Luciferase Assay System (Promega). The fluorescence signals released from the DNA cleavage were determined, and the values of the luciferase activity was measured using they the Minireplicon assay.

**Cloning, expression and purification of PA<sub>N</sub> proteins**

PA<sub>N</sub> proteins were expressed and purified, as reported previously [25, 49]. Briefly, virus PA<sub>N</sub> fragments (amino acids 1–196) were amplified by PCR using the pHW2000-PA plasmids (with or without the indicated substitutions) as templates. Specific primers were used in the amplification process (FP 5'-GGTACCATGGAAGACTTTGTGCGA-3' and RP 5'-CATGTTTCAGGGAGGAAGCAGCAGATGTGC-3'). The reactions contained 5 µl cDNAs (1:10 diluted), 1 µl FPs (10 µM) or 1 µl RPs (10 µM), 10 µl FastStart SYBR Green I Master and 3 µl sterile water. Viral HA mRNA levels were normalized to that of the luciferase activity.

**Fluorescence-based endonuclease assay**

Fluorescence-based endonuclease assays were performed *in vitro* to detect the endonuclease activity of the wild-type and mutants [25, 49]. Briefly, 0.2 µg PA<sub>N</sub> proteins were incubated with the short nucleic acid substrates (5' FAM-TCTC TAGCAGTGGCC-3' BHQ) in a final volume of 100 µl at 37°C. The DNA cleavage was carried out by the PA<sub>N</sub> proteins via the endonuclease activity. The reaction buffer was 50 mM HEPES, 130 mM NaCl and 1 mM MnCl<sub>2</sub> (pH 7.8). Fluorescence signals released from the DNA cleavage were subjected to quantification by using a Victor X3 Multilabel plate reader (Perkin Elmer) after 30 min.

**Structure analyses**

The 3D model of the PA<sub>N</sub> protein of wt-H7N7 was generated by the Swiss Model algorithm and validated (Protein Data Bank accession code 2W69) [50]. The wt-H7N7 PA<sub>N</sub> model was subjected to simulation with a single-stranded nucleic acid sequence (5'–CTCTAGCA-3'), and the binding energy change was calculated using the Patchdock/Firedock algorithm [51–53]. Similar approaches were applied to the substitutions indicated to calculate their binding energy changes. Additionally, to investigate the impact of the substitutions on PA<sub>N</sub> protein stability, the DUET server was utilized to obtain the overall protein stability changes for the substitutions indicated when using the wt-H7N7 PA<sub>N</sub> model as a native structure [54].

**Statistical analysis**

Statistical significance was evaluated using one-way ANOVA followed by Dunnett's test with the GraphPad Prism 6 software (GraphPad Software). *P*<0.05 was considered significant.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

The study did not contain animal experiments.

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