The significance of naturally occurring neuraminidase quasispecies of H5N1 avian influenza virus on resistance to oseltamivir: a point of concern

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Viral adaptability and survival arise due to the presence of quasispecies populations that are able to escape the immune response or produce drug-resistant variants. However, the presence of H5N1 virus with natural mutations acquired without any drug selection pressure poses a great threat. Cloacal samples collected from the 2004–2005 epidemics in Thailand from Asian open-billed storks revealed one major and several minor quasispecies populations with mutations on the oseltamivir (OTV)-binding site of the neuraminidase gene (NA) without prior exposure to a drug. Therefore, this study investigated the binding between the NA-containing novel mutations and OTV drug using molecular dynamic simulations and plaque inhibition assay. The results revealed that the mutant populations, S236F mutant, S236F/C278Y mutant, A250V/V266A/P271H/G285S mutant and C278Y mutant, had a lower binding affinity with OTV as compared with the WT virus due to rearrangement of amino acid residues and increased flexibility in the 150-loop. This result was further emphasized through the IC50 values obtained for the major population and WT virus, 104.74 nM and 18.30 nM, respectively. Taken together, these data suggest that H5N1 viruses isolated from wild birds have already acquired OTV-resistant point mutations without any exposure to a drug.

INTRODUCTION

Influenza A viruses are highly communicable and are responsible for seasonal epidemics and the occasional pandemics (Horimoto & Kawaoka, 2005; Palese, 2004). However, the emergence and re-emergence of the highly pathogenic avian influenza (HPAI) H5N1 virus with the potential of causing a pandemic has raised the need for meticulous study of the virus worldwide.

Three supplementary figures are available with the online Supplementary Material.
virus depends on its ability to enter a certain host cell, replicate within it, and release new viral progenies. Sometimes, these occurrences cause occasional eruptions of HPAI H5N1 virus transmission to humans resulting in a mortality of more than 50% (Neumann et al., 2010). Although person-to-person transmission of H5N1 avian influenza virus (AIV) appears to be very rare, its high fatality rate among those infected is a cause of great concern. Furthermore, the wide range of birds infected not only provided opportunity for efficient spread of the virus to new areas but also may have caused positive selection in newer host species (Vandegrift et al., 2010). Migratory birds, including storks, are widely blamed for the spreading of H5N1 viruses. The recent outbreaks of HPAI H5N1 in Egypt with 10 deaths out of 22 identified human patients and the culling of poultry in Japan due to the outbreak highlight the continued risk of human infections (Kyodo, 2014; Reuters, 2014). Therefore, monitoring of the phenotypic characteristics of H5N1 avian influenza virus isolated from reservoir birds is important due to sporadic outbreaks of H5N1 virus occurring in various locations.

As an RNA virus, the H5N1 HPAI can evolve rapidly in a variety of hosts, mainly due to the error-prone nature of its RNA-dependent RNA polymerase causing the accumulation of spontaneous point mutations that can eventually lead to amino acid substitutions in haemagglutinin (HA) and neuraminidase (NA) proteins (Lewis, 2006). This mechanism leads to the production of numerous, closely related RNA variants termed ‘quasispecies’. The diversity of quasispecies in RNA viruses is essential for their survival and adaptation under new unfavourable or pressured environments. The error-prone nature of RNA polymerase generates different phenotypic variants such as immune escape variants and drug-resistant mutants that may become dominant in the viral population if they harbour a selectable phenotype (Lewis, 2006). Seasonal influenza vaccines do not provide protection against H5N1 influenza viruses and H5N1 vaccine is not yet available. Therefore, we must rely solely on antiviral drugs for now. So far, the only antiviral drugs approved by the FDA which effectively inhibit release of progeny virions are NAIs (neuraminidase inhibitors). The two effective drugs present in this class are oseltamivir (OTV) and zanamivir, with the availability of newer drugs, peramivir and laninamivir, in Japan and South Korea since 2010 (McKimm-Breschkin & Barrett, 2013; Singh & Soliman, 2015). These drugs have been designed based on the crystal structures of group-2 NA (N2, N3, N6, N7 and N9) (von Itzstein, 2007). N1 NA belongs to group-1 NA (N1, N4, N5 and N8), which differs from group-2 based on the highly conserved amino acid residues in the 150-loop (residues 147–152) and the formation of an adjacent 150-cavity only found in group-1 NAs (Russell et al., 2006; Wang et al., 2011). Thus, the emergence of resistant variants is a huge concern with the use of antiviral drugs (Nitsch-Osuch & Brydak, 2014). In fact, depending on the detection method, a variety of NA-resistant mutants isolated from clinical samples have been identified (Bloom et al., 2010; Ferraris & Lina, 2008; Ho et al., 2007; Hurt et al., 2007, 2009; Ilyushina et al., 2010; Richard et al., 2008; Yen et al., 2005, 2006, 2007). Some of these resistant variants have undiminished viral fitness, highlighting the need for continued surveillance of antigenic variants of NA (Baek et al., 2015). Computational approaches including molecular dynamic (MD) simulations are used in an attempt to determine the impact of OTV resistance (Baek et al., 2015).

NA plays an important role in liberating the bond between the virus and the host cell by specifically cleaving N-acetyl neuraminic acid (Neu5Ac) from its attachment to glycoconjugates on the cell surface (Colman, 1994). Thus, it is of interest to investigate natural viral isolates for variant NAs and also the influences of such variants on the susceptibility of H5N1 to NAIs. In the present study, we investigated the significance of novel quasispecies of NA found in naturally infected Asian open-billed storks. MD simulations revealed that natural drift quasispecies from those birds bind with a lower affinity to OTV resulting in reduction of susceptibility to OTV, which was confirmed by functional test. Our finding indicates the presence of OTV-resistant variants in nature, which will be harder to control if they become a dominant strain.

RESULTS

Three variants of the NA gene found at the OTV-binding site of H5N1 viruses from naturally infected Asian open-billed storks

Seven cloacal swabs from naturally infected storks of the 2004 and 2005 epidemics in Thailand were subjected to RNA purification. The purified RNAs then served as templates for cDNA synthesis. A total of 90 clones were sequenced, aligned and analysed using the BioEdit program (De Clercq, 2006) and MEGA version 4 (Hayden, 2006). Amongst the 90 clones sequenced, one major population and several minor variants were detected. However, in the present study, only three populations, one major population and two minor variants, were present in all cloacal swabs tested, suggesting the significance of these three populations. Therefore, they were selected for further investigation. The sequences of the major population and two minor variants are shown in Fig. 1(a) in comparison with a sequence of the same domain of WT N1 neuraminidase obtained from the protein database (PDB 2HU4). Out of the 90 clones, one major population with an S236F mutation and two minor populations, one with a double mutation at S236F/C278Y and one with a quadruple mutation at A250V/V266A/P271H/G285S, were observed at 30.54%, 4.19% and 1.20%, respectively. In addition, Fig. 1(b) demonstrates the relative positions of these mutations found on the NA protein. As can be seen, these natural drift mutations do not lie within the active site or drug-binding site but are instead located at the back of it. Whether their proximity to the active site and the important loops of the NA
protein affect drug binding within the active site is what we aimed to determine in this study.

**Significance of the quasispecies observed on OTV susceptibility**

The wide usage of OTV has given rise to the emergence of various drug-resistant strains. Genetic drifts related to such resistance have been identified which are shown to be different from our reported mutations. Whether NA mutations shown in our present study interfere with OTV activity or not has not been investigated. To answer this question, the mutant populations with a single S236F mutation, double S236F and C278Y mutations and quadruple A250V, V266A, P271H and G285S mutations were rescued via a plasmid-based reverse genetics system to reveal the viability of naturally occurring quasispecies populations. We successfully rescued the WT and S236F populations but not the double mutant and quadruple mutant populations (results not shown). The inability to rescue the double mutant even though it differed from the S236F mutant by only one amino acid mutation at position C278Y brought about the generation of the single mutant population of C278Y. The C278Y population was not observed in nature but was instead generated to test the single point mutation at C278Y. Unfortunately, reverse genetics of the C278Y variant was also unsuccessful. These data suggest

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**Fig. 1.** (a) Deduced amino acid sequences obtained from 90 clones were aligned and analysed using the BioEdit program (De Clercq, 2006) and MEGA version 4 (Hayden, 2006). Amongst the 90 clones sequenced, one major population and several variants were revealed. The sequences of the major population (S236F) and two minor variants (double mutant and quadruple mutant) are shown in comparison with a sequence of the same domain of WT N1 NA obtained from the protein database (PDB2 HU4). Out of the 90 clones, the S236F mutant, S236F/C278Y mutant and A250V/V266A/P271H/G285S mutant populations were observed at 30.54 %, 4.19 % and 1.20 %, respectively. (b) The locations of the quasispecies mutation points are illustrated using UCSF Chimera (a molecular visualization software; Pettersen, 2004) version 1.8 (www.cgl.ucsf.edu/chimera) in order to understand their relative positions from the active site on the NA gene. In addition, the active site and drug-binding site residues are also depicted along with the loops of the NA protein.
the significance of NA variants on the survival of H5N1 virus. Therefore, in vitro experiments proceeded only for the WT and S236F populations.

The rescued recombinant WT and S236F variant viruses replicated well in MDCK (Madin–Darby canine kidney) cells and peaked at 1.2 ± 0.03 \times 10^7 p.f.u. ml^{-1} and 1.5 ± 0.13 \times 10^6 p.f.u. ml^{-1} for WT and S236F variant, respectively. A plaque reduction assay was then employed to determine the IC_{50} values of OTV when infected with either the WT or S236F population. The results showed the percentage plaque inhibition of the WT and S236F variant that occurred at varying concentrations of OTV (Table 1).

The IC_{50} values calculated were 18.30 ± 3.08 and 104.74 ± 32.55 nM for the WT and S236F population, respectively. This illustrates that the IC_{50} value of the S236F mutant is approximately sixfold higher than that of the WT virus. An IC_{50} value of greater than 50 nM can be considered as having reduced effect on the virus while an IC_{50} value of less than 50 nM can be considered as susceptible (Stoner et al., 2010). Therefore, the S236F mutant is less susceptible to OTV than the WT virus.

**MD simulations of the quasispecies populations**

MD simulations can be applied to examine the dynamic nature of biological systems. In the present study, the interactions of OTV embedded in the active site of NA of the WT and quasispecies populations were investigated to understand the effect of natural mutations in H5N1 NA on drug binding.

The inability to rescue the C278Y variant left unanswered the question of the importance of this mutation. Furthermore, the reduced sensitivity of the S236F mutant to OTV also demanded more detailed explanation into the binding of drug–protein. Therefore, MD simulation was applied to all four quasispecies populations as well as the WT virus to explore the effect of those variants for influenza NA stability and OTV binding. To obtain reliable conformational space sampling, long MD simulations of 100 ns were performed for all those systems. The structures obtained from the MD simulations were then analysed at the molecular level to

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**Table 1. Percentage inhibition of WT and major population virus against varying concentrations of OTV drug**

<table>
<thead>
<tr>
<th>Oseltamivir carboxylate (nM)</th>
<th>Percentage inhibition (mean ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
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<tr>
<td>200</td>
<td>90.19 ± 4.15</td>
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<tr>
<td>100</td>
<td>78.04 ± 2.39</td>
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<tr>
<td>50</td>
<td>68.61 ± 5.90</td>
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<tr>
<td>25</td>
<td>55.60 ± 3.16</td>
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<tr>
<td>12.5</td>
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<td>6.25</td>
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<tr>
<td>3.125</td>
<td>7.60 ± 0.67</td>
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<tr>
<td>IC_{50}</td>
<td>18.30 ± 3.08</td>
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**Fig. 2.** The RMSDs relative to the initial structure (PDB code 2HU4) obtained for all five systems complexed with OTV obtained during a 100 ns simulation in order to determine the stability of the overall MD simulations. The WT complex (black) was taken as the deciding factor for the stability of the system. The quasispecies populations – S236F population (red), S236F/C278Y population (blue), A250V/V266A/P271H/G285S population (green) and C278Y population (grey), were then compared with the WT in order to judge the system stability of each quasispecies simulation over the 100 ns time period. (a–c) The figure is further divided into all atom (each system complexed with OTV), backbone (protein structure without OTV) and OTV. The systems were seen to reach equilibrium at about 50 ns and thus the production phase (50–100 ns) was used for subsequent analyses. (d–f) The RMSD of important NA loops such as the 150–loop, the 270–loop and the 430–loop were monitored in respect to their initial structure to discover the structural dynamics of the loops.
discover the effects of natural quasispecies on conformational change of NA and OTV binding.

**Stability of system.** In general, the stability of the simulated systems was assessed by considering the plot of root-mean square displacement (RMSD) relative to the initial structure along the simulation time. The RMSD is a measure of the difference in distance between two structures providing conformational change during the simulation where the maintained fluctuation of RMSD value can point to the stability of the MD structures. Here, the RMSD analysis of each system consisting of OTV complexed with WT NA, OTV-S236F NA, OTV-S236F/C278Y NA, OTV-A250V/V266A/P271H/G285S NA and OTV-C278Y NA was calculated in terms of all atoms, protein backbone atoms and ligand atoms as illustrated in Fig. 2(a–c). The results suggest that all systems were stable after 50 ns as can be seen from the maintained fluctuation of RMSD values; hence, the MD structures during 50–100 ns of each complex were adopted for conclusive analysis of drug–protein interaction. Analysis of RMSDs of all systems showed convergence with values below 3 Å. However, quasispecies complexes (except for single C278Y variant) showed higher fluctuation than those of the WT complex, suggesting a difference in the binding of OTV-WT and OTV-NA quasispecies.

In addition, a two-dimensional heat map (2D-RMSD) analysis can illustrate the conformational flexibility by comparing the structures with one another (von Graffenstein et al., 2015). In this study, 2D-RMSD observed the structural comparison between WT and quasispecies complexes depicted in Fig. 3, which was computed on the basis of all NA residues. The different conformation of NA during the simulation was represented as the change in colour from blue to red. Blue zones represent areas of conserved conformation (0.00–0.75 Å), green colour indicates slight differences (0.75–1.50 Å), whereas yellow, orange and red areas denote substantial differences of sampling conformation (1.50–2.25 Å). The WT and single C278Y mutation complexes showed slight differences in conformations with the green areas throughout the heat map, which indicates a predominantly conserved structure of NA protein during the simulation (Fig. 3a, e). In contrast, the other complexes showed higher fluctuation by representation of substantially different conformations, which can be seen from the appearance of yellow contour and orange/red areas after 40 ns (Fig. 3b–d). Although the single mutation of C278Y is not different from the WT complex, the double mutation of S236F/C278Y shows more fluctuation than the single S236F mutation as can be seen from the red areas of MD structure after 50 ns compared with the structure at 30 ns (Fig. 3c). This suggests that the C278Y mutation has an effect on

![Fig. 3. Two-dimensional heat map (2D-RMSD) comparing structures with one another during the entire simulation time. This heat map shows the complex stability in terms of structural comparison. The different conformations of all NA residues (RMSD) were observed as the change in colour from blue to red. Blue zones represent areas of conserved conformation (0.00–0.75 Å), green colour indicates slight differences (0.75–1.50 Å), whereas yellow, orange and red areas denote substantial differences of sampling conformation (1.50–2.25 Å).](http://jgv.microbiologyresearch.org)
the conformational flexibility of the double S236F/C278Y mutant NA.

Protein dynamics and conformational change. IAV NAs are divided into two phylogenetically distinct groups based on the highly conserved amino acid residues of the 150-loop (residues 147–152) (Russell et al., 2006). Besides the 150-loop, which has been exceedingly studied, the NA contains other structural motifs that harbour important residues. These motifs include the 110-helix (residues 105–110), the 270-loop (267–276), the 380-loop (380–390) and the 430-loop (429–433) (Li et al., 2013) as depicted in Fig. 1(b). As known, the 150-loop plays an important role for substrate and/or drug binding because this loop is flexible, revealing a closed and open cavity of the NA binding pocket (Amaro et al., 2007, 2009; Han & Mu, 2013). The motion of this loop is coupled with the motion of the 430-loop; hence, the 150-cavity width was monitored as the distance between V149 and P431 residues (Amaro et al., 2011). In addition, the residues D151 and R152 belonging to the 150-loop can form hydrogen bonds with functional groups of NAIs (Le et al., 2010). Furthermore, the conformation of the 270-loop has implications on the rotation of E276 towards R224 to form a hydrophobic pocket that allows for high-avidity OTV binding (Li et al., 2013). This loop lies in close proximity to the novel C278Y mutation. Therefore, this section will elucidate the dynamics and structural change of the 150-, 270- and 430-loops in terms of their RMSD, plot of distance and secondary structure change along the simulation time.

Firstly, the RMSD of these interesting loops were monitored in respect to their initial structure to discover the structural dynamics of loops, illustrated in Fig. 2(d–f). The WT complex shows conserved fluctuation along the simulation time for all these loops. In contrast, the other mutant systems exhibit fluctuations with different points. Although the RMSD of C278Y is mostly conserved with that of the WT complex (Fig. 2a–c), its 270-loop RMSD appears to be higher than that found in the WT. This suggests that the mutation of C278Y can affect the conformation of the 270-loop. The S236F and quadruple mutant complexes exhibit high fluctuations at the 150- and 270-loops, whilst the double mutant (S236F/C278Y) shows some fluctuation of the 150-loop during 50–60 ns and tend to fluctuate after 90 ns.

Subsequently, the time series analysis of distance concerning the 150-loop was considered, which is shown in Fig. 4 including the distances of (a) V149 and P431, (b) D151 and OTV and (c) R152 and OTV. To consider the 150-loop width in Fig. 4(a), it can be seen that all quasispecies complexes, except for the C278Y variant, display a larger cavity compared with the WT, which suggests the transition of the 150-loop from closed to open form as evidenced in the previous study of Amaro et al. (2011). These data support that mutations of the residues located at the back of the active site can affect the conformation of the 150- and 270-loops, such as driving the 150-loop from closed configuration into open form.

The dynamic change of the 150-loop was further monitored to discriminate the closed/open conformation by plotting the distance between two binding residues belonging to the 150-loop (D151 and R152) and heteroatoms of their interaction side chain (Fig. 4b, c). A distance between the CG atom belonging to the D151 catalytic residue and the ammonium N2 atom of OTV of 3–4 Å or >5 Å was indicated as closed or open conformation, respectively (Udommaneethanakit et al., 2009). The initial structure of the NA complex (2HU4.pdb) shows a closed conformation. From the simulation, the 150-loop of the WT complex seems to favour a closed conformation, which is consistent with a previous study (Kar & Knecht, 2012). In contrast, the S236F variant and the double mutation variant are shown to switch conformations with a distance of more than 5 Å at the first 20 ns for both complexes and 70–90 ns for the S236F complex. Whilst the C278Y variant shows a somewhat open configuration at nearly 20 ns, it reaches stabilization after that. Although the D151 distance in the quadruple mutant is frequently conserved with that of the WT complex, R152 cannot make hydrogen bond to stabilize the –NHAc side chain of OTV (distance >3.5 Å in Fig. 4c). These data suggest that the 150-loop of the NA variants bind to OTV with less avidity than the NA WT.

To investigate how these mutations drive conformational changes of the major loops of NA, the secondary structure of the protein was determined using the DSSP algorithm (Kabsch & Sander, 1983) illustrated in Fig. 5. All types of secondary structure were depicted with different colours; white, red, blue, green and black representing coil, alpha-helix, beta-sheet, bend and turn, respectively. In general, the main secondary structure of NA protein is predominated by beta-sheet. Since the main areas of structural change during the simulation were found at the major loops of NA, i.e. the 150-, 270- and 430-loops in Fig. 5, these changes of structure will be discussed in depth and compared with the WT complex.

The secondary structure of 150-loop shows different conformations for all systems. The WT complex displays a frequent switching of alpha-helical/bend/beta-sheet structures during the simulation. The secondary structure of the two single S236F and C278Y mutations likely confers to the structures of bend and alpha-helix, respectively. It is worth noting that the secondary structure of residue 151 of the C278Y variant completely changes from alpha-helix to bend structure after 60 ns. The double mutation of S236F/C278Y represents a combination of bend and alpha-helix structures with a somewhat appearance of turn conformation, while the quadruple mutation tends to present coil and beta-sheet structures after 60 ns.

For the 270-loop, the secondary structure of WT, S236F variant and double mutant complexes show the same trend of beta-sheet/helix/coil structures. The beta-sheet structure is seen to disappear in the quadruple mutant, while the secondary structure of the C278Y variant differs from those systems by the representation of beta-sheet or hydrogen-bonded
turn, instead of coil structure as found in the WT system. In contrast, the 430-loop of all systems except for the C278Y variant entirely displays the bend form of secondary structure. The C278Y variant, however, prefers beta-sheet/bend/hydrogen-bonded turn structures. This suggests that the C278Y substitution directly affects the conformation change of the 270- and 430-loops. These data indicate that mutations of residues outside the major loops of NA play a significant role in the secondary structures of these loops.

Protein–ligand interactions OTV has three polar moieties (−COO−, −NH3 and −NHAc), which are able to form hydrogen bonds with NA residues, while a bulky group (−OCH2CH3) side chain is accommodated in the hydrophobic pocket of R224 and E276 residues (Fig. 1). In the WT system, the distance of those hydrophobic-forming residues was intensely conserved at 3 Å during 10–100 ns (Fig. S1, available in the online Supplementary Material). This proves that the initial structure of Y252 NA does not affect the orientation of the E276 residue as also found in previous studies (Ilyushina et al., 2010).

The reduced susceptibility to OTV in variants conferring the S236F mutation was discovered by plaque reduction assay as described above. To seek for the effect of drug binding corresponding to the natural quasispecies in H5N1 NA, OTV binding towards NA for both WT and quasispecies was evaluated in terms of hydrogen bond interactions (Fig. 6) and change in dihedral angles of bulky group (Fig. S2). The percentage occupation of hydrogen bonds between residues in the NA binding pocket and three side chains of OTV was determined over the stable MD structures (50–100 ns) based on the criteria of a maximum distance of 3.5 Å between hydrogen donor and acceptor, and a minimum angle of 120° for donor-H-acceptor. In addition, the conformation of NA and OTV complexes taken every 20 ns from 0 to 100 ns is shown in Fig. S3.

In the WT complex (Fig. 6a), the OTV formed hydrogen bonds with binding site residues of WT H5N1 NA including R292, R371, E119, D151 and R152 as found in previous studies (Malaisree et al., 2008; Rungrotmongkol et al., 2009a). The drug-binding pattern of the single C278Y
mutation variant was considerably conserved with that of the WT. However, a dramatic reduction in D151 interaction was observed (Fig. 6e). Similar to the single S236F variant, its double mutation variant (S236F/C278Y) showed a noteworthy reduced interaction at $\text{NH}_3^+$ and $\text{NHAc}$ side chains compared with the WT (Fig. 6b, c). These decreased interactions are related to the formation of a wider 150-cavity (Fig. 4a) and somewhat fluctuation of the D151-OTV distance (Fig. 4b). In contrast, in the quadruple mutant a dramatic reduction of hydrogen bond interaction (~70 % of R292 and R371 occupations and less than 35 % of the rest of the residues) against all binding residues was found (Fig. 6d). This directly correlates to the loss of interactions with the $\text{NHAc}$ side chains (Fig. 4c) which affects the ill-fitting of OTV in the binding pocket. The dihedral angles of bulky groups of OTV are shown to be conserved for all systems except for the quadruple mutant (Fig. S2). The dihedral angles of this mutant show the changes in rotation for $\tau_1$ and angular distribution for $\tau_2$ and $\tau_3$. This could possibly have caused the decreased hydrogen bonding interactions of the quadruple mutant with OTV.

**DISCUSSION**

Although most resistant mutants arise in response to NAI treatments, several other reports have also documented naturally occurring antigenic drift mutations that alter the susceptibility of H5N1 viruses to NAIs in the absence of any drug selection pressure (Baranovich et al., 2011; Rameix-Welti et al., 2006; Stoner et al., 2010). The contamination of OTV in the environment (water or sewage) has been reported in Japan (Ghosh et al., 2010; Söderström et al., 2009). Furthermore, there is the potential that wild water birds and gallinaceous birds may encounter water contaminated with the drug (Fick et al., 2007; Gillman et al., 2015). Whether the concentration of contamination is high enough to induce the emergence of OTV-resistant mutants in infected birds is unclear. However, the chance of this being the cause of natural resistance in our study is unlikely because our samples were obtained from the 2004–2005 epidemics when widespread use of OTV in poultry in Thailand did not occur. The infected poultry and free-grazing birds were culled to stop the spread of the virus (Songserm et al., 2006; Walker et al., 2012). Therefore, it is pertinent to investigate these naturally occurring mutations, and in this study the focus was on the genetic diversity found outside the NA drug-binding site of H5N1 viruses collected from naturally infected Asian open-billed storks during the outbreak in 2004–2005 in Thailand.

From our sequencing data, three dominant quasispecies, which are the S236F, the double and the quadruple mutants, were found. These three quasispecies were subjected to reverse genetics. Only the S236F mutant could be rescued and quantified via plaque assay. The inability to rescue the double and quadruple mutant populations, despite their relatively high quasispecies percentages (4.19 % and 1.20 %, respectively), indicates the possibly important role of host factors that may be absent in 293T- and MDCK-based cell systems. Several factors associated with the survival and persistence of HPAI
H5N1 viruses still need to be elucidated. The interplay between host (reservoir birds) and viral factors could have promoted the survival of the quasispecies populations found in this study in their natural habitat while the cell-based systems could not (Horm et al., 2012).

Furthermore, the NA sequences of quasispecies populations were subjected to MD simulations compared with WT N1 structure (PDB code 2HU4) to determine the pattern of OTV binding. We found dramatic changes on OTV binding comparing between WT and NA variants tested. For the three variants tested, the hydrophobic groups of the OTV side chains rotated to provide a better binding angle with the NA structures. Moreover, the 150-loop of all variants tested changed from open to closed and then to open again when bound to OTV during the simulation time. Furthermore, this conformational change led to the rearrangement of environmental amino acid residues of NA in terms of hydrogen bonding and binding free energy. From these findings we inferred that OTV did not fit well into the NA cavity of all the variants, and this may thus account for the reduction in susceptibility to OTV.

The NA catalytic site consists of eight residues (R118, R371, R224, E276, D151, R152, R292 and Y406) that directly interact with OTV and 11 residues that provide structural support to the active site residues (Yen et al., 2006). The involvement of catalytic and framework residues of N1 NA in its binding to OTV has only been documented for the more well-known mutations (H252Y, H274Y and N294S). Mutations located at the binding site can affect the structural and/or functional behaviour of the protein. However, mutations located outside the binding site could possibly alter the structure of the binding pocket and, thus, could be associated with drug resistance (Ramírez-Salinas et al., 2015). Kar & Knecht (2012) suggested that the binding pockets of the mutated N8 NAs were less flexible as compared with the binding pocket of the WT protein. These behaviours enabled the WT structure to exhibit stronger binding affinity to OTV than the mutated structures. In addition, when OTV binds with NA, amino acids within the active site rearrange to accommodate the drug’s hydrophobic side pocket created by residues R224 and E276 (Karthick & Ramanathan, 2014). Any mutations that affect this rearrangement may result in resistance to the drug.

In addition, it was shown that H274Y and N294S mutants confer resistance to OTV but do not compromise the ability of A/Vietnam/1203/04 (H5N1) and A/PR/8/34 (H1N1) viruses to replicate in vitro (Yen et al., 2007). Therefore, it is clear that the disruption of hydrogen bonds, which directly contributes to the binding affinity of protein–ligand complex, leads to the rearrangement of side chain conformations and results in reduced binding affinity of NA to its inhibitor. In accordance with these reports, the hydrogen bonding results indicated that the switching of the 150-loop between the open and closed conformations disrupted the hydrogen bonds between key active site residues allowing for reduced binding affinity of the drug and ligand as

![Fig. 6.](http://jgv.microbiologyresearch.org) The percentage occupation of hydrogen bonds formed between OTV and the active-site residues for each of the protein systems was determined using the Ptraj suite of programs from the AMBER12 package software.
observed in all the quasispecies. Furthermore, the 150-loop residue D151 has previously been reported to help stabilize the 150-loop in the closed conformation with its hydrogen bonding (Amaro et al., 2007). Therefore, the loss of hydrogen bonds in the variant populations, especially the major population, can be attributed to the increased plasticity of the 150-loop during the 100 ns simulation time. Eventually, this evidence supports that the fluctuation of the 150-loop in the NA protein can affect the binding interactions of drugs (Wang & Zhang, 2010). Furthermore, the loss of hydrogen bonding may further contribute to the decreased binding affinity of OTV to N1 NA, based on the IC₅₀ values, causing reduced susceptibility to the virus. In addition, we also found that the free binding energy reiterated the reduction in binding affinity of mutant NAs towards OTV (data not shown).

Even though the IC₅₀ concentration of the S236F variant (104.74 nM) might be less pronounced as compared with other NA-resistant mutants already studied, it is of interest to note that this particular resistance has occurred without previous exposure to OTV, unlike the other NA-resistant mutants, and could favour the emergence of fully resistant viruses. In addition, the IC₅₀ value of the WT (18.30 nM) is a great deal lower than that of the S236F variant, which is in accordance with our MD simulation results that the S236F variant has lower affinity to OTV than the WT virus. Taken together, these data suggest that OTV might not be efficient enough to control infections of these NA variants.

Upon detailed examination by various authors it was discovered that non-active site residues have subtle but important effects on the activity and level of expression of the NA enzyme (Baranovich et al., 2011; McKimm-Breschkin, 2013). For example, the V234M and R222Q mutations help to decrease NA folding and transport defects by increasing the amount of NA reaching the cell surface and thus compensate for the active site mutation H274Y (Baranovich et al., 2011). These findings highlight the possibility of mutations on residues other than catalytic or framework residues causing resistance to NAIs. In addition, the residue E276 is part of the hydrophobic pocket in the active site of NA needed to accommodate the pentyl side chain of OTV and this residue is highly conserved across all influenza A viruses (Hung et al., 2009). Furthermore, Tolentino-Lopez et al., (2013) showed that mutations outside the active site could affect the binding pattern of OTV and thus influence its susceptibility and on other biological properties of rapidly evolving, potentially pandemic H5N1 influenza viruses.

To conclude, the present study highlighted the significance of pre-existing mutations in natural H5N1 quasispecies during the 2004 and 2005 epidemics in Thailand on drug resistance. Our observation suggests that H5N1 viruses from infected wild birds have already acquired point mutations without any requirement of selective pressure to facilitate viral species survival, OTV resistance and adaptation to the ongoing war between the virus and antiviral drugs.

METHODS

Cells. MDCK (Madin–Darby canine kidney) cells and 293T (human embryonic kidney) cells were kindly provided by Dr Pilaipan Puthavathana at Siriraj Hospital, Mahidol University. The cells were maintained in modified Eagle medium (MEM; Gibco, USA) and Dulbeco’s modified eagle medium (DMEM; Gibco, USA) for MDCK and 293T cells, respectively. Both media were supplemented with 10% FBS and incubated at 37 °C. Cells were passaged regularly and passages 12–14 of MDCK and passages 6–8 of 293T cells were used in this study.

Avian H5N1 viruses. Seven cloacal swabs from naturally infected Asian open-bill storks were used in our study. These swabs were obtained from dead, infected Asian open-bill storks in Bung Boraphet, NakhonSawan province, Thailand, during the 2004 and 2005 epidemics. The swabs were collected in a virus transport medium containing medium M199, 1000 U penicillin G ml⁻¹, 1000 mg streptomycin ml⁻¹, 400 mg gentamicin ml⁻¹, 2.5 mg Fungizone ml⁻¹ and bovine serum albumin and kept at −80 °C. The number of viruses in each cloacal swab was quantified and expressed as H5N1 RNA copy number. These RNAs were subjected directly to clonal sequencing.

Viral RNA extraction and RT-PCR amplification. Viral RNA was isolated from cloacal swab samples with an RNAasy Mini kit (Qiagen) according to the manufacturer’s instructions. The viral RNA was reverse transcribed into cDNA by reverse transcription (RT) reaction at 42 °C for 1 h. The RT reaction contained 1 μg of total RNA template, 1 × AMV RT buffer (Promega), DTT, dNTP, RNase inhibitor, random primers and AMV reverse transcriptase (Promega). PCR amplification of the NA gene segment was carried out as previously described with minor modification (Ubol et al., 2011). This amplification was performed using a pair of specific primers: 5’-ATCTGGAAGACTCAAGATC-3’ (sense) and 5’-TTATCCCTGCACACATG-3’ (anti-sense) for amplification of a fragment at nucleotide positions from 625 to 843. Platinum Taq DNA Polymerase High Fidelity was used to reduce mutations generated during the amplification process (Invitrogen).

Clonal sequencing. Purified PCR products were cloned into sequencing vector pCR2.1-TOPO as recommended by the manufacturer (TA Cloning kit; Invitrogen). At least 30 well-isolated white colonies were picked and subsequently cultivated in 5 ml Luria–Bertani broth containing 100 μg ampicillin (Invitrogen) ml⁻¹ at 37 °C overnight. Plasmid DNAs were extracted from harvested, transformed Escherichia coli by the alkaline lysis method before being subjected to sequencing. The nucleotide sequences and putative amino acid sequences of the H5N1 variants pool were compared using BioEdit version 7.0.1.2 (Hall, 1999). Pairwise comparison of each nucleotide sequence was performed using MEGA version 4 (Tamura et al., 2007).

Generation of mutants. A H5N1 NA gene covering the OTV binding site (218 base pairs) cloned into the reverse genetics plasmid pHW2000 was kindly provided by Dr Pilaipan Puthavathana at Siriraj Hospital, Thailand. Taking that as the WT, PCR was used to perform site-directed
mutagenesis to obtain the quasispecies populations detected earlier through sequencing. Briefly, 1 µg of plasmid template was denatured in 40 µl water and 10 µl 1 M NaOH/1 mM EDTA solution and incubated at 37 °C for 15 min. Five microlitres of 3 M sodium acetate (pH 4.8) was then added to neutralize the solution. DNA was precipitated with 150 µl ice-cold ethanol and centrifuged at 8000 g for 10 min at 4 °C. Once all the ethanol had evaporated, 40 µl water was used to resuspend the DNA. A series of PCRs were then set using primers containing the desired mutation designed specifically for each quasispecies population (Macrogen). Once the mutants were generated, sequencing was done to check for presence of the mutation required to make it a quasispecies. The mixture of cDNAs containing the WT and each mutant was separately transfected into a co-culture of 293T and MDCK cells in the background of the other seven genomic segments of A/Puerto Rico/8/34 (H1N1) to generate the viruses via reverse genetics (Hoffmann et al., 2000).

Plaque reduction assay. MDCK cells were seeded in six-well plates to form a confluent monolayer and infected with 50 plaque-forming units of influenza virus per well. Virus adsorption was carried out for a period of 1 h at 37 °C in an incubator with 5 % CO₂. Following the 1 h incubation period, the viral inoculum was removed from the cell monolayer, and the cells were washed twice with modified Eagle medium (MEM) (Gibco). Tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (0.25 %; Gibco) was added at a concentration of 2 µg ml⁻¹ to the MEM to provide support for replication. Low-melting agar (1 %) was added in a 1:1 concentration with the medium containing varying concentrations of oseltamivir carboxylate (provided by Dr Pilai(Kornkruay)) into each well. One millilitre of Gram crystal violet stain (5 g of crystal violet in 200 ml of ethanol and 200 ml of PBS; MERCK) was added to each well for a period of 30 min at room temperature. The crystal violet stain was then removed from the wells, and cells were washed with water. Plaques were then visualized and counted. Excel (Microsoft) was used to determine the percentage of plaque inhibition and IC₅₀.

MD simulations of NA. The initial structure of WT H5N1 NA bound with OTV was prepared on the basis of the crystal structure taken from the Protein Data Bank (PDB), code 2HU4 (Russell et al., 2006). Consequently, this WT complex was adopted for preparing the four complexes of quasispecies including (i) S236F, (ii) S236F+C278Y, (iii) A250V and (iv) C278Y through the LEaP module in AMBER12 (Case et al., 2005, 2012). All water molecules present in the crystal structure were conserved, while a calcium ion was imported from another NA complex with PDB code 2HTY (Russell et al., 2006). All complex preparations and MD simulations were carried out using AMBER12 package simulation. To further prepare the complex for investigations by MD simulation, the protonation state of all ionizable residues (arginine, lysine, histidine, aspartic acid and glutamic acid) was determined at pH 7 by PROPKA 3.1 (Olsson et al., 2011) together with manual verification. Disulfide bonds between cysteine residues were assigned in order to keep protein stability. All hydrogen atoms were added to the NA-OTV complex using the LEaP module to compensate for those missing in the X-ray structure. Then, the systems were neutralized by the addition of sodium cations. In order to demonstrate a realistic aqueous environment representation, each system was finally soaked in a cubic box of explicit TIP3P water molecules, which is a three-point water model containing a point charge of each atom (Jorgensen et al., 1986), with a distance of 10 Å from the protein surface to the edge. The force field parameter of protein atoms is based on ff12SB, while the oseltamivir parameter and their RESP atomic charges were obtained from previous studies (Phanich et al., 2016; Rungrotmongkol et al., 2009b).

MD simulations were performed for each system of OTV complex under periodic boundary conditions. An integration time step of 2 fs was allowed by applying the SHAKE algorithm to all bonds involving hydrogen atoms (Ryckaert et al., 1977). In order to reduce the computing time of non-bonded interactions, a distance cut-off of 10 Å was assigned for non-bonded interactions, while the particle mesh Ewald (PME) summation method was adopted for calculating long-range electrostatic interactions (York et al., 1993).

To remove bad contacts and optimize the structure position, energy minimization was subsequently applied to all hydrogen atoms and water molecules using 1000 steps of steepest descents, followed by 2000 steps of conjugated gradient with position restraints of 500 kcal mol⁻¹Å⁻³ on protein and ligand atoms. Finally, the entire system was freely minimized without positional restraints.

After minimization, all MD simulations were carried out using the pmemd.CUDA module in AMBER12. Each system was heated from 10 to 298 K for 100 ps. After reaching the target temperature, the systems were further equilibrated at this temperature and 1 atm of pressure for a further 100 ps. Finally, each system was simulated for 100 ns by collecting all of the systems with canonical ensemble (NVT), for which the thermodynamic state is characterized by fixing of a number of atoms (N), volume (V) and temperature (T). Temperature was controlled at 298 K using the weak-coupling algorithm (Berendsen et al., 1984). The trajectories obtained from MD simulations were analysed in terms of root-mean-square displacement (RMSD), two dimensional RMSD (2D-RMSD), distance of interests and hydrogen bond interaction using the ptraj module in AMBER12. The change in secondary structure of NA protein during the simulation time was monitored using the DSSP program (Kabsch & Sander, 1983).

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