Molecular Mechanisms Underlying Oseltamivir Resistance Mediated by an I117V Substitution in the Neuraminidase of Subtype H5N1 Avian Influenza A Viruses

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Background. The neuraminidase (NA) inhibitor oseltamivir is widely used to treat patients infected with influenza viruses. An Ile-to-Val change at position 117 in influenza A virus subtype H5N1 NA (NA-I117V) confers a reduction in susceptibility to oseltamivir carboxylate. However, the in vivo relevance and molecular basis of the decreased sensitivity mediated by this mutation are poorly understood.

Methods. We created single-point-mutant viruses with 3 genetically different backgrounds (ie, 1 belonging to clade 1 and 2 belonging to clade 2.3.4) and evaluated the effects of the I117V mutation on oseltamivir susceptibility in vitro, in vivo, and in silico.

Results. The NA-I117V mutation conferred a slight reduction in susceptibility to oseltamivir in vitro (1.3- to 6.3-fold changes), although it did not substantially compromise NA enzymatic activity. Mice infected with I117V virus exhibited reduced susceptibility to oseltamivir and decreased survival in 2 of 3 virus pairs tested. Molecular dynamics simulations revealed that I117V caused the loss of hydrogen bonds between an arginine at position 118 and the carboxyl group of oseltamivir, leading to a lower binding affinity for oseltamivir.

Conclusions. Our findings provide new insight into the mechanism of NA-I117V-mediated oseltamivir resistance in highly pathogenic H5N1 avian influenza viruses.

Keywords. Influenza A virus; H5N1; Oseltamivir; Neuraminidase; I117V mutation; Molecular dynamics simulation.

Since 2003, highly pathogenic subtype H5N1 avian influenza A viruses have circulated among poultry and wild birds in Asia, Africa, and Europe [1]. As of 10 August 2012, in addition to frequent avian outbreaks, >600 cases of H5N1 infections in humans have been reported [2]. Since these viruses are currently unable

to transmit efficiently among humans, human infections have been limited to close contact with infected poultry, with a few exceptions [3]. Yet, unlike seasonal subtype H1N1 influenza A virus, seasonal subtype H3N2 influenza A virus, or 2009 pandemic subtype H1N1 influenza A virus, the illness associated with H5N1 viruses in humans follows an unusually aggressive clinical course, with rapid deterioration and high mortality (approximately 60%) [2].

The neuraminidase (NA) inhibitor oseltamivir is commonly used in clinical practice to treat influenza virus infections in patients. NA inhibitors interrupt the virus replication cycle by preventing the release of viruses from infected cells. They were designed to target the sialic acid-binding pocket of NA, which is composed of 19 conserved residues (8 functional

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residues [R118, D151, R152, R224, E276, R292, R371, and Y406; N2 numbering] and 11 framework residues [E119, R156, W178, S179, D198, I222, E227, H274, E277, N294, and E425; N2 numbering]) [4, 5]. Therefore, mutations at these positions can confer drug resistance [6, 7].

A previous report raised the possibility that genetic variation, represented by E248G and Y252H, in the absence of any drug-selective pressure can lead to significant changes in NA inhibitor sensitivity [8]. McKimm-Breschkin et al [9] reported that some H5N1 viruses isolated in Cambodia and Indonesia are less sensitive than 2004 clade 1 viruses to oseltamivir, yet none of the NA amino acid changes detected in these viruses was known to confer oseltamivir resistance. These findings suggest that the decrease in sensitivity may be due to drift mutations rather than to selective pressure posed by oseltamivir per se. Thus, viruses could acquire reduced anti-NA drug sensitivity by not only drug-selective pressure but also natural genetic variation [10]. In either case, with increasing clinical use and stockpiling of NA inhibitors for pandemic preparedness, it is important to define all mutations that could confer resistance to NA inhibitors.

Recently, a novel substitution, isoleucine to valine at position 117 (I117V) in the NA protein (NA-I117V) of an avian isolate was reported to slightly increase the half maximal inhibitory concentration (IC_{50}) of oseltamivir carboxylate in vitro (from 5.1-fold to approximately 16-fold) [11, 12]. However, the clinical relevance of this approximate 10-fold decrease in sensitivity is unknown. Although variations at this position (I117V/M) have been reported to reduce susceptibility to oseltamivir in 2009 pandemic H1N1 viruses [13, 14], the molecular mechanisms underlying the reduced sensitivity to oseltamivir caused by NA-I117V remain poorly understood. Here, to elucidate the effects of the NA-I117V substitution on susceptibility to oseltamivir, we generated recombinant viruses with this substitution in 3 genetically different virus backgrounds and compared their NA enzymatic activity and susceptibility to oseltamivir in vitro and in vivo. To reveal the mechanisms underlying any conformational changes in the NA enzymatic site induced by the I117V mutation, we also performed molecular dynamics analysis to assess the binding stability of NA to oseltamivir carboxylate.

MATERIALS AND METHODS

Details of the cells, viruses, plasmid-based reverse genetics, statistical analysis, and other experimental procedures can be found in the [Supplementary materials](#).

NA Inhibition Assay

The sensitivity of viral NA to oseltamivir carboxylate was evaluated by using an NA enzyme inhibition assay based on the method of MUNANA. Virus dilutions containing between 800

and 1200 fluorescence units were used in this assay. Diluted virus and the drug (0.01 nM–1 mM in 33 mM 2-[N-morpholino] ethanesulfonic acid [pH 6.0] containing 4 mM $CaCl_2$) were mixed and incubated for 30 minutes at 37°C, followed by addition of the substrate. After 1 hours at 37°C, the reaction was stopped, and fluorescence was quantified. The relationship between the concentration of inhibitor and the percentage of fluorescence inhibition was determined, and IC_{50} values for NA activity were obtained by extrapolating those findings.

Therapeutic Efficacy of Oseltamivir in Mice

Female BALB/c mice aged 5–6 weeks (Japan SLC, Shizuoka, Japan) were used in the experiment. Prior to evaluating the efficacy of oseltamivir in mice, we anesthetized 4 mice per group by using sevoflurane inhalation and intranasally inoculated them with serial 10-fold diluted virus in 50 mL of phosphate-buffered saline to calculate the 50% mouse lethal dose (MLD_{50}). To study the efficacy of oseltamivir, 16 mice per group were infected with 10 MLD_{50} of viruses and then orally mock treated or treated with 30, 100, or 300 mg/kg oseltamivir phosphate 2 hours after infection and then twice daily for 5 days. Mice were monitored daily for morbidity and mortality for 21 days after infection. On days 3 and 6 after infection, 3 mice per group were euthanized, and brain, nasal turbinate, lung, spleen, liver, kidney, and jejunum were collected. These tissue samples were subsequently homogenized in Eagle's minimal essential medium containing 0.3% bovine serum albumin at a final concentration of 10%, and cellular debris was removed by centrifugation at 5000 g for 5 minutes. Virus in the supernatant was then determined by using plaque assays involving Madin-Darby canine kidney cells.

Molecular Dynamics Simulations

The initial coordinates of wild-type VN1203 NA with oseltamivir were taken from the cocrystal structure (PDB code 2HU4). The structure of the NA-I117V mutant with oseltamivir was generated by replacing isoleucine 117 in the wild-type complex with valine by using the LEAP module in the AMBER 11 software suite [15]. Protonation states of the ionizable residues were assigned at pH 6.5 by using the PDB2PQR web server [16]. All missing hydrogen atoms were added by using LEAP. The geometry and electrostatic potential of oseltamivir were calculated at the HF/6-31G(d) level with Gaussian 03 [17]. On the basis of the results of these calculations, partial charges of oseltamivir were determined with the restrained electrostatic potential procedure [18], using the Antechamber program of AMBER 11 [19]. The FF99SB force field and the generalized AMBER force field were applied for each NA and for oseltamivir, respectively [20, 21]. The total charges of the NA-oseltamivir complexes were neutralized by the addition of sodium counterions. Then, the systems were solvated in a truncated octahedral box of TIP3P water molecules with a

distance of at least 10 Å around the complex. The total atoms were 30 400 for both the wild-type and mutant NA systems. All energy minimization and molecular dynamics (MD) simulations were performed by using the PMEMD module in AMBER 11, with a cutoff radius of 12 Å for the nonbonded interactions. The locations of hydrogen atoms, water molecules, and counterions were optimized to remove bad contacts. Then, each system was energy minimized without any constraints by using the steepest descent method for 500 steps, followed by the conjugate gradient method for 1500 steps. After minimization, the system was gradually heated from 0 K to 300 K at 5 ps per 50 K with a force constant of 1.0 kcal/mol-Å². An additional 2 rounds of MD (50 ps each at 300 K) were performed with decreasing restraint weight reduced from 0.5 to 0.1 kcal/mol-Å². Then, 2.5 ns of unrestrained MD at 300 K was run to equilibrate the system. Finally, a 2.5-ns production run was performed, and the production trajectories were collected every 1 ps. All MD simulations were performed using the NPT ensemble and the Berendsen algorithm [22] to control temperature and pressure. The time step was 2 fs, and the SHAKE algorithm [23] was used to constrain all bond lengths involving hydrogen atoms. Long-range electrostatic interactions were treated using the particle mesh Ewald method [24]. Binding free energies were calculated using the script of the molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) method in AMBER 11 (MM/PBSA.py), which uses a single-trajectory approach. Snapshots were taken every 5 ps for the enthalpy estimates and every 350 ps for the entropy estimates on the free calculations. Calculation of the binding free energy between an NA mutant and oseltamivir by using the MM/PBSA method was described in detail previously [25–28].

RESULTS

NA Enzymatic Activity of H5N1 Viruses Possessing a Single Point Mutation at Position 117

To investigate the prevalence of H5N1 virus strains that possess the NA-I117V mutation, we reviewed the H5N1 NA sequences in the public database. Of the 2691 virus strains reviewed, variation at this position was found in at least 58 strains (approximately 2%), the HAs of which differed such that they covered a broad range of clades, including 0, 1, 2.1.1, 2.2, 2.3.2, 2.3.4, and 9 (Supplementary Table 1). In our surveillance study in Vietnam from 2007–2008, we found the NA-I117V substitution in 2 poultry isolates: A/duck/Vietnam/TY103/2007 (Bacgiang province isolate) and A/duck/Vietnam/TY114/2007 (Thaibinh province isolate). This substitution was also found in a clinical specimen from a patient infected with an H5N1 virus in Hai Duong province, Vietnam, in 2008 (A/Vietnam/UT31412II/2008) [29]. We then studied the contribution of NA-117 to oseltamivir resistance by using the following 3 genetically different viruses: A/Vietnam/1203/2004

(VN1203, clade 1), A/duck/Vietnam/TY114/2007 (TY114, clade 2.3.4), and A/Vietnam/UT31412II/2008 (VN31412, clade 2.3.4), none of which have any known oseltamivir resistance-conferring mutations. Of these 3 viruses, VN1203 possessed NA-117I, whereas TY114 and VN31412 had NA-117V, as stated above. We therefore generated 3 virus pairs, each differing by only the amino acid at position NA-117: VN1203 (wtNA-117I) and VN1203 (NA-117V), TY114 (wtNA-117V) and TY114 (NA-117I), and VN31412 (wtNA-117V) and VN31412 (NA-117I).

We then evaluated the effect of the NA-I117V substitution on NA enzymatic activity by using MUNANA substrate (Figure 1A–C). We found that viruses possessing valine at position 117 in NA exhibited statistically significantly higher enzymatic activity than did those possessing isoleucine at this position, regardless of the virus backbone. Next, we tested the biologic effect of the NA-I117V substitution in the hemagglutination-elution assay with chicken erythrocytes [30] and found that the NA-I117V substitution did not influence the ability of NA to elute virus from erythrocytes in any of the 3 virus backbones tested (Figure 1D). Thus, the NA-I117V mutation did not compromise the NA enzymatic activity; rather, it increased it regardless of the virus backbone tested.

Effects of NA-I117V on Oseltamivir Carboxylate Susceptibility In Vitro

To evaluate the effect of the NA-I117V substitution on susceptibility to oseltamivir in vitro, the IC₅₀ values of oseltamivir carboxylate were measured by using a fluorometric NA inhibition assay (Figure 1E–G; Table 1). Clade 2.3.4 viruses (TY114 and VN31412 pairs) exhibited higher IC₅₀ values than did the clade 1 virus (VN1203 pair), as previously reported [9]. The VN1203 NA-117V and wild-type VN31412 (wtNA-117V) viruses exhibited 6.3- and 3.4-fold higher IC₅₀ values, respectively, than those of viruses possessing isoleucine, whereas wild-type TY114 (wtNA-117V) showed a mere 1.3-fold higher IC₅₀ value than its 117I mutant. These results indicate that the I117V substitution confers slightly reduced susceptibility to oseltamivir carboxylate in vitro, the degree of which was dependent on the virus backbone.

Effects of NA-I117V on the Therapeutic Efficiency of Oseltamivir Phosphate in a Mouse Model

We next assessed the effect of the NA-I117V substitution on the therapeutic efficacy of oseltamivir in mice. Prior to treatment, we calculated the MLD₅₀ values to compare the pathogenicity of the virus pairs in mice (Table 1). Although the VN31412 pair was more virulent in mice than were the other 2 virus pairs, there were no substantial differences in MLD₅₀ values within virus pairs, suggesting a limited contribution of the I117V substitution to pathogenicity in mice.

To evaluate the effect of the NA-I117V substitution on oseltamivir susceptibility in mice, mice were infected with 10

Figure 1. Neuraminidase (NA) activity of subtype H5N1 influenza A viruses and the inhibitory effect of oseltamivir carboxylate. Three H5N1 virus pairs were evaluated for NA enzymatic activity, hemagglutination-elution activity, and NA sensitivity to oseltamivir carboxylate. *A–C*, NA enzymatic activity was measured by the method of MUNANA substrates; the values shown represent the means of triplicate experiments. **P* < .05, significant difference compared with the I117I viruses. *D*, Serial 2-fold dilutions of the viruses were incubated with equal volumes of 0.5% chicken erythrocytes at 4°C for 1 hour (top panel), followed by incubation at 37°C for 8 hours (bottom panel). *E–G*, The sensitivity of NA to oseltamivir carboxylate was determined by using MUNANA as a substrate; the values shown represent the means of duplicate experiments.

MLD₅₀ of the VN1203 pair and then mock treated or treated with oseltamivir phosphate at a dosage of 30, 100, or 300 mg/kg twice daily for 5 days after infection (Figure 2*A* and 2*B*). In the mice infected with the wild-type VN1203 (wtNA-117I) virus (Figure 2*A*), oseltamivir treatment produced a dose-dependent protective effect against virus dissemination into multiple organs at days 3 and 6 after infection (Table 2). Virus titers in lungs were significantly reduced by the administration of 300 mg/kg oseltamivir at days 3 and 6 after infection (Figure 2*G*). Of the mice infected with the VN1203 NA-117V mutant virus (Figure 2*B*), however, 60% died even when treated with the highest oseltamivir dose, even though oseltamivir reduced viral growth and dissemination (Table 2 and Figure 2*G*). Because a difference in oseltamivir efficacy was observed at the high dose of oseltamivir in mice infected with the VN1203 pair, we also investigated mouse survival for the TY114 and VN31412 pairs at the high dose of

oseltamivir. The reduced efficacy of oseltamivir was also observed for the TY114 pair (Figure 2*C*, 2*D*, and 2*H* and Table 2). In mice infected with the VN31412 pair, the therapeutic efficacy was the lowest of all of the viruses tested, and there was no difference in the effect of the I117V substitution on the survival of the mice (Figure 2*E* and 2*F*). These results indicate that, depending on the genetic background of the virus, the NA-I117V substitution confers reduced susceptibility to oseltamivir in mice.

Effects of the NA-I117V on the Interaction Between R118 and Oseltamivir

The cocrystal structure of wild-type VN1203 NA with oseltamivir carboxylate (PDB code 2HU4) shows that the isoleucine at position 117 does not directly interact with oseltamivir (Figure 3*A*). To elucidate the molecular basis of how the I117V substitution causes reduced susceptibility to

Table 1. Fifty Percent Mouse Lethal Dose (MLD₅₀) and Half Maximal Inhibitory Concentration (IC₅₀) Values for Oseltamivir Carboxylate to Wild-Type and Neuraminidase (NA)–Mutant Subtype H5N1 Influenza A Viruses

Virus	Clade	Recombinant Virus	Amino Acid 117 in NA	MLD ₅₀ (PFU)	IC ₅₀ (nM) ^a	Ratio of IC ₅₀ (117V/117I)
A/Vietnam/1203/2004	1	VN1203 wtNA-117I	I	21	0.32	...
		VN1203 NA-117V	V	32	2.0	6.3
A/duck/Vietnam/TY114/2007	2.3.4	TY114 wtNA-117V	V	30	68	1.3
		TY114 NA-117I	I	30	52	...
A/Vietnam/UT31412II/2008	2.3.4	VN31412 wtNA-117V	V	0.59	22	3.4
		VN31412 NA-117I	I	2.1	6.5	...

Abbreviation: PFU, plaque-forming units.

^a Calculated from the duplicate experiments described in Figure 1E–G.

Figure 2. Therapeutic efficacy of oseltamivir phosphate against subtype H5N1 influenza A viruses in mice. Sixteen mice per group were intranasally infected with ten 50% mouse lethal doses of viruses and then given 30, 100, and 300 mg/kg oseltamivir phosphate orally 2 hours after infection and twice daily thereafter for 5 days. Three mice per group were euthanized on days 3 and 6 after infection, and their lungs were collected for virus growth assays. A–F, Survival was monitored daily for 21 days, and survival curves were obtained from 10 mice per group. G and H, Virus titers in lungs are shown; the values are mean $\pm$ SD ($n=3$). * $P<.05$, significant difference between levels in the lungs of mice infected with virus possessing 117I and those infected with virus possessing 117V.; # $P<.05$, significant difference between levels in the lungs of mice, mock-treated and treated with oseltamivir phosphate. (1-way analysis of variance with the Tukey multiple comparisons posttest).

Table 2. Virus Titers in Mice Infected With Wild-Type and Neuraminidase 117 Mutant Viruses

Time After Infection	Virus	Treatment ^a	Virus Titer (log ₁₀ PFU/g)					
			Brain	Liver	Spleen	Kidney	Jejunum	Nose
Day 3	VN1203 wtNA-117I	Mock	... ^b	...	4.5 ± 0.7	2.3	...	...
		Oseltamivir	...	...	...	...	...	...
	VN1203 NA-117V	Mock	...	...	3.7 ± 0.2	2.0	...	...
		Oseltamivir	...	...	...	...	...	...
	TY114 wtNA-117V	Mock	...	...	...	...	...	...
		Oseltamivir	...	...	...	...	...	...
Day 6	VN1203 wtNA-117I	Mock	6.1 ± 1.3	2.5 ± 0.7	3.1 ± 0.2	5.8 ± 0.3	...	4.7 ± 0.1
		Oseltamivir	...	...	...	...	...	...
	VN1203 NA-117V	Mock	7.4 ± 1.1	4.6 ± 0.9	4.3 ± 0.6	7.8 ± 0.7	3.3, 5.6	6.5 ± 2.0
		Oseltamivir	...	...	2.5	...	...	7.2
	TY114 wtNA-117V	Mock	...	...	2.6, 2.5	...	...	2.6, 2.8
		Oseltamivir	...	...	2.8	...	...	2.8
	TY114 NA-117I	Mock	2.9	...	3.3 ± 0.1	...	3.2	2.6 ± 0.2
		Oseltamivir	...	...	...	...	...	...
		Mock	...	...	...	...	...	...
		Oseltamivir	...	...	...	...	...	...
		Mock	...	...	...	...	...	...
		Oseltamivir	...	...	...	...	...	...

Data are mean ± SD (n = 3).

Abbreviation: PFU, plaque-forming units.

^a Mice were intranasally infected with ten 50% mouse lethal doses of virus and then mock treated (with distilled water) or treated with oseltamivir carboxylate (300mg/kg) 2 h after infection and then twice daily for 5 d.

^b Virus not detected (detection limit, <2.0 log₁₀ PFU/g).

oseltamivir, we performed molecular dynamics simulations. First, we assessed the equilibration of the trajectories by monitoring the root-mean-square deviations of all backbone heavy atoms from the initial structure as a function of simulation time (Supplementary Figure 1). Both the wild-type and the NA-117V systems reached equilibrium within 2.5 ns. Therefore, the trajectories extracted from the 2.5 to 5.0 ns simulations were used in subsequent analyses. Second, we compared the binding free energy difference between the wild-type and I117V mutant, which was calculated using the MM/PBSA method [31] (Table 3). This method has previously provided useful information regarding the binding affinity of oseltamivir to various NAs of influenza viruses [25–28]. The estimated binding free energies ($\Delta G_{\text{binding}}$) were −19.73 and −16.05 kcal/mol for the wild-type and I117V mutant, respectively. These values indicate qualitatively good agreement with the experimental binding free energies ($\Delta G_{\text{binding,exp}}$), which were estimated from the IC₅₀ values. Thus, our energy analysis shows that the I117V substitution causes a lower binding affinity of oseltamivir carboxylate for the I117V mutant.

To further investigate the effect of the I117V substitution on the interaction with oseltamivir, we analyzed the percentage occupation of hydrogen bonds between oseltamivir and NA during the simulation (Figure 3B and Supplementary Table 2). Hydrogen bonds were assigned by using the ptraj

program in AMBER 11 on the basis of the following criteria [26]: the distance between the proton donor and acceptor atoms was ≤ 3.5 Å, and the angle formed by the donor, hydrogen, and acceptor was $\geq 120^\circ$. Unlike the wild-type simulation, no hydrogen bonds were observed between R118 and the carboxyl group of oseltamivir in the simulation of the I117V mutant (Figure 3B). The preferential distances of O₂-NH₁ (R118) and O₂-NH₂ (R118) in the wild-type were 3.80 and 2.85 Å, respectively, whereas those in the I117V mutant were 5.45 and 5.30 Å, respectively (Figure 3C). Of note, the preferential distances of N₂-OE₁ (E119) were not different between the wild-type and mutant viruses. Since valine is smaller than isoleucine by 1 methylene group, the substitution of isoleucine for valine at position 117 could induce a slight distortion around residue 117 because of the loss of van der Waals contacts. This local change in molecular structure may be responsible for the change in orientation of R118 relative to oseltamivir. As a result, the loss of the hydrogen bond because of the I117V substitution would lead to the reduced susceptibility to oseltamivir.

DISCUSSIONS

Here, we found that an Ile-to-Val change at position 117 of NA substantially reduced the in vivo susceptibility to

Figure 3. Hydrogen bonds between residues in the binding pocket of neuraminidase (NA) and oseltamivir predicted by using molecular dynamics simulation. *A*, Amino acid residues analyzed for hydrogen bonding are shown in the structure of the N1 NA in complex with oseltamivir. *B*, Percentage occupation pattern of hydrogen bonds between residues of oseltamivir and NA (defined in Figure 3*A*) for wild-type and I117V NA. *C*, Interatomic distances of hydrogen bonds between oseltamivir and residues R118, E119, and E277 of NA.

oseltamivir for 2 of 3 virus pairs tested, although, in vitro, the decrease in susceptibility to oseltamivir carboxylate induced by this mutation was only a few fold (1.3- to 6.3-fold). Compared with substitutions such as H274Y or D292N, which are

Table 3. Free Energy of Binding Between Oseltamivir and Neuraminidase by Using the Molecular Mechanics/Poisson-Boltzmann Surface Area Method

Contribution	VN1203 wtNA-117I	VN1203 NA-117V
ΔE_{elec}	-185.68	-171.78
ΔE_{vdw}	-30.37	-31.42
ΔG_{sol}	175.09	167.54
$\Delta G_{\text{sol(ele)}} - E_{\text{ele}}$	-2.83	-2.78
$-T\Delta S(\text{total})$	24.06	22.39
$\Delta G_{\text{binding}}$	-19.73	-16.05
$\Delta G_{\text{binding,exp}}^a$	-13.10	-11.90

^a Calculated from the half maximal inhibitory concentration (IC_{50}) by using the following formula: $\Delta G_{\text{binding,exp}} = RT \ln K_{\text{dissociation}} = RT \ln(IC_{50} + 0.5 C_{\text{enzyme}}) \sim RT \ln IC_{50}$, where R is the ideal gas constant, T is the temperature in K, and C_{enzyme} is the concentration of the enzyme, which is very small after equilibration is reached and can be omitted in most cases. IC_{50} values of 0.32 nM (VN1203 wtNA-117I) and 2.0 nM (VN1203 NA-117V) were used to calculate $\Delta G_{\text{binding,exp}}$.

selected under drug pressure and increase IC_{50} values by at least 100-fold, the I117V mutation does not dramatically affect the in vitro susceptibility to oseltamivir. However, this mutation did affect the survival of the oseltamivir-treated mice (Figure 2). This discrepancy between in vitro and in vivo susceptibility to oseltamivir was previously reported by Govorkova et al [32], who found that strains that differ by only 2-fold in the IC_{50} of oseltamivir carboxylate in vitro differ substantially in their oseltamivir susceptibility in mice, as evaluated by lethality; our data are consistent with these findings.

The effects of the I117V substitution on susceptibility to oseltamivir also differed among the 3 virus backbones tested, both in vitro and in vivo. A previous study showed that clade 1 viruses are more sensitive to oseltamivir than are clade 2 viruses [9]. When we compared the VN1203 pair (clade 1), the NA-I117V mutation caused a 6.3-fold decrease in susceptibility to oseltamivir in vitro and a 60% decrease in the survival of mice treated with oseltamivir at the highest dose tested. On the other hand, for the TY114 and VN31412 pairs (both clade 2.3.4), modest increases in IC_{50} values induced by the I117V mutation were observed (1.3- and 3.4-fold decreases). In addition, although the increase in IC_{50} values caused by the

I117V mutation was smaller for the TY114 pair than for the VN31412 pair, decreases in the therapeutic effect of oseltamivir in mice were observed for TY114 but not for VN31412. There are 19 amino acid differences between the NA proteins of VN1203 and TY114. The NA of TY114 and that of VN31412 differ by 2 amino acids, at positions 16 and 42; however, these residues are located in the NA transmembrane and stalk domain, respectively, and are not thought to be involved in the binding between NA and oseltamivir [33, 34]. Given that the virulence of an H5N1 virus strain is one factor that determines the therapeutic efficacy of oseltamivir in mice [35], this strain difference in oseltamivir efficacy in mice may be due to differences in pathogenicity rather than to these amino acid differences, because VN31412 is more virulent than TY114 by 10-fold on the basis of MLD₅₀ values. Our results indicate that the NA-I117V mutation does not alter susceptibility to oseltamivir dramatically but compromises the effectiveness of the drug in virus strains that are more sensitive to oseltamivir or are of low pathogenicity in mice.

Molecular dynamics simulation demonstrated that NA-I117V induces the loss of hydrogen bonding between NA R118 and the carboxyl group of oseltamivir, although this did not lead to a global conformational change in the binding between oseltamivir and NA. This slight conformational change did, however, result in a lower binding affinity of the enzymatic site of NA for oseltamivir, providing a structural basis for the reduced sensitivity to oseltamivir caused by this mutation. A review of the influenza sequence database revealed that a few viruses possessing the mutation at position 117 in NA were isolated in East Asian countries, like Bangladesh, Bhutan, and India, from 2010 to 2011 (Supplementary Table 1). Remarkably, most of these viruses were avian isolates, suggesting that this mutation occurred sporadically during the course of the evolution of the viruses that prevail among poultry and not as a result of oseltamivir treatment. Given the wide range of H5N1 clades/subclades in which genetic variation has been observed at residue 117 of NA, it would be important to assess the effects of introducing this mutation into a more diverse set of H5N1 virus NAs, particularly clade 2.2 and 2.1, which have been implicated in a large number of human infections with H5N1 viruses.

In summary, we examined the effect of the NA-I117V mutation on susceptibility to oseltamivir in vitro, in vivo, and in silico and determined that the I117V mutation is a genetic variation that reduces susceptibility to oseltamivir slightly in vitro but dramatically in vivo. This mutation results in the loss of a hydrogen bond between residue R118 and oseltamivir carboxylate, resulting in a lower binding affinity between oseltamivir carboxylate and NA. Importantly, the I117V mutation appears to arise not as a result of oseltamivir treatment but because of genetic drift among poultry—a finding that justifies the continued global surveillance of H5N1 viruses among

poultry. Although an H5N1 virus with the I117V mutation was attenuated in a ferret model [12], given the spontaneous emergence of viruses with the I117V mutation and their potentially reduced sensitivity to oseltamivir, our findings are of value to those who treat patients infected with H5N1 viruses.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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