Competitive Fitness of Oseltamivir-Sensitive and -Resistant Highly Pathogenic H5N1 Influenza Viruses in a Ferret Model

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ABSTRACT

The fitness of oseltamivir-resistant highly pathogenic H5N1 influenza viruses has important clinical implications. We generated recombinant human A/Vietnam/1203/04 (VN, clade 1) and A/Turkey/15/06 (TK, clade 2.2) influenza viruses containing the H274Y neuraminidase (NA) mutation, which confers resistance to NA inhibitors, and compared the fitness of the wild-type (WT) and resistant virus pairs in ferrets. The VN-H274Y and VN-WT viruses replicated to similar titers in the upper respiratory tract (URT) and caused comparable diseases signs, none of the animals survived. On days 1-3 post-inoculation, disease signs caused by oseltamivir-resistant TK-H274Y virus were milder than those caused by TK-WT virus, and all animals survived. We then studied fitness by using a novel approach. We co-inoculated ferrets with different ratios of oseltamivir-resistant and -sensitive H5N1 viruses and measured the proportion of clones in day-6 nasal washes that contained the H274Y NA mutation. Although the proportion of VN-H274Y clones increased consistently, that of TK-H274Y virus decreased. Mutations within NA catalytic (R292K) and framework (E119A/K, I222L, H274L, N294S) sites or near the NA active enzyme site (V116I, I117T/V, Q136H, K150N, A250T) emerged spontaneously (without drug pressure) in both pairs of viruses. The NA substitutions I254V and E276A could exert compensatory effect on the fitness of VN-H274Y and TK-H274Y viruses. NA enzymatic function was reduced in both drug-resistant H5N1 viruses. These results show that the H274Y NA mutation affects the fitness of two H5N1 influenza viruses differently. Our novel method of assessing viral fitness accounts for both virus-host interactions and virus-virus interactions within the host.
INTRODUCTION

The neuraminidase (NA) inhibitors (orally administered oseltamivir and inhaled zanamivir) are currently an important class of antiviral drugs available for the treatment of seasonal and pandemic influenza. Although administration of NA inhibitors may significantly reduce influenza virus transmission, it risks the emergence of drug-resistant variants (16, 32).

The impact of drug resistance would depend on the fitness (i.e. infectivity in vitro, virulence and transmissibility in vivo) of the resistant virus. If the resistance mutation only modestly reduces the virus’ biological fitness and does not impair its replication efficiency and transmissibility, the effectiveness of antiviral treatment can be significantly impaired. The unexpected natural emergence and spread of oseltamivir-resistant variants (carrying the H274Y NA amino acid substitution) among seasonal H1N1 influenza viruses of A/Brisbane/59/07 lineage demonstrated that drug-resistant viruses can be highly fit and transmissible in humans (11, 20, 29), although the fitness of these variants is not completely understood. They are hypothesized to have lower NA receptor affinity and more optimal NA and HA functional balance than do wild-type viruses (38). Fortunately, oseltamivir-resistant variants have rarely been reported among the novel pandemic H1N1 influenza viruses that emerged in April 2009; therefore, initial data suggests that currently circulating wild-type viruses possibly possess greater fitness than drug-resistant viruses (45), although only retrospective epidemiological data can provide a conclusive answer. The key question is whether the risk posed by NA inhibitor resistant viruses can be assessed experimentally and what the most reliable approach may be.

All NA inhibitor-resistant influenza viruses characterized to date have contained specific mutations in the NA molecule. Clinically derived drug-resistant viruses have carried mutations that are NA subtype–specific and differ with the NA inhibitor used (12, 35). The most commonly
observed mutations are H274Y and N294S in the influenza A N1 NA subtype; E119A/G/D/V and R292K in the N2 NA subtype; and R152K and D198N in influenza B viruses (35, 36). The fitness of NA inhibitor-resistant viruses has been studied in vitro and in vivo. Many groups have assessed their replicative capacity in MDCK cells, but this assay system can yield anomalous results (49), particularly in the case of low-passage clinical isolates. The mismatch between virus specificity and cellular receptors can be overcome by using cell lines engineered to express human-like α-2,6-linked sialyl cell surface receptors (MDCK-SIAT1) (15, 34) or a novel cell culture-based system that morphologically and functionally recapitulates differentiated normal human bronchial epithelial (NHBE) cells (24). Investigations in vivo typically compare replication efficiency, clinical signs, and transmissibility between oseltamivir-resistant viruses and the corresponding wild-type virus. Initial studies found that NA inhibitor-resistant influenza viruses were severely compromised in vitro and in animal models (6, 17, 26), and thus led to the idea that resistant viruses will unlikely have an impact on epidemic and pandemic influenza. However, clinically derived H1N1 virus with the H274Y NA mutation (18) and reverse genetics-derived H3N2 virus with the E119V NA mutation (46) were subsequently found to possess biological fitness and transmissibility similar to that of drug-sensitive virus in direct contact ferrets. Recent studies in a guinea pig model showed that recombinant human H3N2 influenza viruses carrying either a single E119V NA mutation or the double NA mutations E119V+I222V were transmitted efficiently by direct contact but not by aerosol (5).

There is limited information about the fitness of NA inhibitor-resistant H5N1 influenza viruses. Although they are not efficiently transmitted human-to-human, their pandemic potential remains a serious public health concern because of their virulence in humans (3, 4, 7). H5N1 viruses isolated from untreated patients are susceptible to the NA inhibitors oseltamivir and
zanamivir (19), although oseltamivir-resistant variants with the H274Y NA mutation have been reported in five patients after (9, 30) or before (41) treatment with oseltamivir. The World Health Organization reported the isolation of two oseltamivir-resistant H5N1 viruses from an Egyptian girl and her uncle (44) after oseltamivir treatment. The virus was moderately resistant and possessed an N294S NA mutation. Preliminary evidence suggests that the resistance mutation existed before transmission of the virus from birds to the patients and thus before initiation of treatment (41). We previously showed that wild-type A/Vietnam/1203/04 (H5N1) influenza virus and recombinants carrying either the H274Y or N294S NA mutation reached comparable titers in MDCK and MDCK-SIAT1 cells and caused comparable mortality in BALB/c mice (48). In contrast, clinically-derived A/Hanoi/30408/05 (H5N1) influenza virus with the H274Y NA mutation reproduced to lower titers than the oseltamivir-sensitive virus in the lungs of inoculated ferrets (30).

In a ferret model, we compared the fitness of two pairs of H5N1 viruses in the absence of selective drug pressure. One virus of each pair was wild-type while the other carried the H274Y NA mutation conferring oseltamivir resistance. The two viruses used, A/Vietnam/1203/04 (HA clade 1) and A/Turkey/15/06 (HA clade 2.2), differ in their pathogenicity to ferrets. Virus fitness was evaluated by two approaches. Using the traditional approach, we compared clinical disease signs, relative inactivity index, weight and temperature change, and virus replication in the upper respiratory tract (URT). We then used a novel competitive fitness approach in which we genetically analyzed individual virus clones after co-infection of ferrets with mixtures of oseltamivir-sensitive and -resistant H5N1 viruses; thus, we determined virus-virus interactions within the host. We observed no difference between the resistant and sensitive virus of each pair in clinical signs or virus replication in the URT; however, analysis of virus-virus interactions...
within the host showed that the H274Y NA mutation affected the fitness of the two viruses differently. The oseltamivir-resistant A/Vietnam/1203/04-like virus outgrew its wild-type counterpart, while the oseltamivir-resistant A/Turkey/15/06-like virus showed less fitness than its wild-type counterpart.
MATERIALS AND METHODS

Viruses, cells and compounds. The recombinant H5N1 influenza A/Vietnam/1203/04 (VN-WT) and A/Turkey/15/06 (TK-WT) viruses were generated by using the 8-plasmid reverse genetics system (22). A mutation encoding a substitution at conserved NA residue 274 (H274Y) was introduced into the NA plasmids by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The plasmids were sequenced to ensure their identity to the field strains and recombinant H5N1 viruses were rescued by transfecting human embryonic kidney cells (ATCC, Manassas, VA). The HA and NA gene sequences of all stock recombinant H5N1 influenza viruses were verified. Monolayers of MDCK cells were grown in minimum essential medium (MEM) supplemented with 5% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin. The NA inhibitors oseltamivir carboxylate ([3R,4R,5S]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexene-1-carboxylic acid) and zanamivir (4-guanidino-Neu5Ac2en) were provided by Hoffmann-La Roche, Ltd. (Basel, Switzerland). All experiments with recombinant H5N1 viruses were conducted in a biosafety level (BSL) 3+ containment facility approved for use by the U.S. Department of Agriculture.

Virus growth in MDCK cells. Virus titers were determined by plaque assay in MDCK cells. Briefly, confluent MDCK cells were incubated for 1 h at 37°C with 10-fold serial dilutions of virus in 1 ml of infection medium. The cells were then washed and overlaid with freshly prepared MEM containing 0.3% BSA and 0.9% Bacto agar. After incubation at 37°C for 3 days, plaques were stained with 0.1% crystal violet solution containing 10% formaldehyde. Mean plaque diameter as measured by using the Finescale® comparator (Los Angeles, CA).

Virus susceptibility to NA inhibitors in vitro. Susceptibility was tested by using a fluorescence-based NA enzyme inhibition assay (14, 37). H5N1 viruses were standardized to
equivalent NA activity and incubated with NA inhibitors at concentrations of 0.0005–10 µM at 37°C for 30 min before addition of the substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma, St. Louis, MO) at a final concentration of 100 µM. The virus/inhibitor/substrate mixture was incubated at 37°C for an additional 30 min and the reaction was terminated by adding a stop solution of 25% ethanol and 12.5% glycine (Fisher Scientific, Rochester, NY) in distilled water. The fluorescence of the released 4-methylumbelliferone was measured on a Synergy 2 (BioTek Instruments, Winooski, VT) fluorimeter using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The concentration of NA inhibitor that reduced NA activity by 50% relative to a control mixture with no inhibitor (IC50) was calculated by nonlinear regression using Graph Pad Prism 4 software (Graph Pad Software, La Jolla, CA). Results are the means of 2-3 independent determinations.

**Relative NA activity.** The recombinant H5N1 influenza viruses were standardized to different PFUs/ml and their NA enzyme activity was expressed as the quantity (µM) of 4-methylumbelliferone sodium salt (4-MUSS, Sigma, St. Louis, MO) generated during a 30-min incubation at 37°C with MUNANA substrate (Sigma, St. Louis, MO) at a final concentration of 100 µM.

**Inoculation of ferrets.** Adult male ferrets (Marshall’s Farms, North Rose, NY) 3 to 5 months of age and seronegative by HI testing for exposure to currently circulating influenza B viruses and H1N1, H3N2, and H5N1 influenza A viruses were used in the study. Groups of three ferrets were lightly anesthetized with isoflurane and inoculated intranasally with either homogenous populations of virus or mixtures of oseltamivir-sensitive and -resistant virus pairs at different ratios (10% : 90%; 50% : 50%; 90% : 10%). Ferrets were inoculated with VN-WT, VN-H274Y, or mixtures of the two at a dose of 10^2 PFU in 0.5 ml PBS; they were inoculated with
TK-WT, TK-H274Y, or mixtures of the two at a dose of $10^6$ PFU in 0.5 ml PBS. Animals that showed severe disease signs were euthanized. Respiratory signs (labored breathing, sneezing, wheezing, and nasal discharge), neurologic signs (hind-limb paresis, ataxia, torticollis, and tremor), relative inactivity index (40), weight, and body temperature were recorded daily. Body temperature was measured by using subcutaneous implantable temperature transponders (Bio Medic Data Systems Inc.). All studies were conducted under applicable laws and guidelines after approval by the St. Jude Children’s Research Hospital Animal Care and Use Committee.

**Titration of virus in upper respiratory tract.** On days 2, 4, and 6 p.i, ferrets were anesthetized by intramuscular injection of ketamine (25 mg/kg), and 0.5 ml sterile PBS containing antibiotics was introduced into each nostril and collected in containers. Virus was titrated in embryonated chicken eggs and expressed as $\log_{10}EID_{50}/ml$ (39).

**Sequence analysis.** Viral RNA was isolated directly from nasal washes obtained on day 6 post-inoculation (p.i.) by using an RNA isolation kit (RNeasy, Qiagen, Valencia, CA). The universal primer set for influenza A virus was used for cDNA preparation and PCR (23). To characterize the virus population *in vivo*, individual virus clones in each nasal wash sample were sequenced by using a TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Briefly, viral RNA was extracted from nasal washes and one-step RT-PCR was performed. PCR products were purified with the QIAquick PCR purification kit (Qiagen), ligated to the pCR2.1-TOPO vector (Invitrogen), and used to transform TOP10-competent cells (Invitrogen). Plasmid DNAs were prepared by using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing was performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital. The DNA template was sequenced by using rhodamine or dRhodamine dye terminator cycle-sequencing Ready Reaction kits with AmpliTaq DNA polymerase FS (Perkin-Elmer,
Applied Biosystems, Inc., Foster City, CA) and synthetic oligonucleotides. Samples were analyzed in a Perkin-Elmer Applied Biosystems DNA sequencer (model 373 or 377). DNA sequences were completed and edited by using the Lasergene sequence analysis software package (DNASTAR, Madison, WI).

**Statistical analysis.** Virus titers in ferret organs and nasal wash samples and changes in ferret temperature and weight were compared by using the unpaired two-tailed t-test. The trend towards an increase/decrease in the frequency of the H274Y NA mutation relative to the wild-type was analyzed by unpaired two-tailed t-test with Welch’s correction. A probability value of 0.05 was prospectively chosen to indicate that the findings were not the result of chance alone.
RESULTS

NA inhibitor susceptibility, infectivity, and NA activity of the recombinant H5N1 viruses. An NA enzyme inhibition assay confirmed that both recombinant wild-type (VN-WT and TK-WT) influenza viruses were susceptible to oseltamivir carboxylate, the active form of oseltamivir (mean IC_{50} 0.3 and 4.9 nM, respectively). Introduction of the H274Y NA mutation conferred high resistance to oseltamivir carboxylate in vitro; the mean IC_{50} of the VN-H274Y and TK-H274Y viruses was 3375 and 1208 times, respectively, that of the corresponding wild-type viruses (Table 1). The oseltamivir IC_{50} of the TK-WT virus was ~16 times that of the VN-WT virus. All four recombinant H5N1 viruses were susceptible to zanamivir.

After multiple replication cycles in MDCK cells, the oseltamivir-sensitive and -resistant variants of each pair grew to comparable titers, although the TK pair grew to somewhat lower titers than the VN pair (Table 1). Each of the oseltamivir-sensitive viruses formed significantly larger plaques (P<0.02) than did its resistant counterpart, indicating that the H274Y NA mutation altered plaque morphology. Reduction of the TK-H274Y virus plaque size was more pronounced (0.3 mm).

To assess the impact of the H274Y mutation on the viruses’ NA enzymatic activity, we standardized virus preparations to similar growth in MDCK cells (PFU/mL) and measured their NA activity at different virus doses (Fig. 1). Introduction of the H274Y NA mutation reduced the NA activity of both recombinant H5N1 viruses (P<0.01; two-tailed t-test), but the loss was more pronounced in the VN-H274Y virus.

Fitness of oseltamivir-sensitive and -resistant H5N1 influenza viruses in ferrets. To evaluate the effect of the H274Y NA mutation on virus fitness in a ferret model, we compared clinical signs, survival, and virus replication in the URT in ferrets inoculated with oseltamivir-
sensitive and –resistant H5N1 viruses. All ferrets inoculated with $10^2$ PFU of either VN-WT or VN-H274Y virus exhibited acute disease signs (high fever, marked weight loss, anorexia, extreme lethargy), rapid progression, and death by day 6-7 p.i. (Table 2). To compare the fitness of the oseltamivir-sensitive and -resistant H5N1 viruses, we co-inoculated animals with mixtures of the viruses at different ratios (10% : 90%; 50% : 50%; 90% : 10%) based on virus infectivity (PFU). Sequence analysis of individual virus clones in these mixtures revealed the actual ratios of oseltamivir-sensitive and -resistant viruses that were achieved (Table 3); the ratios were not consistently predicted by infectivity. We do have different ratios for both pairs of H5N1 viruses (we did not change initial designations for the groups after verification of number of clones by sequence analysis).

Ferrets co-inoculated with the different ratios of VN-WT and VN-H274Y viruses showed similar clinical signs and virus shedding, and none survived (Table 2). At least 1 animal in each group exhibited neurologic signs. Virus titers in the URT were comparable in ferrets inoculated with VN-WT and VN-H274Y viruses and in the 3 groups co-infected with different ratios of VN-WT and VN-H274Y viruses (Table 2). Both of the TK viruses caused milder illness than did the VN viruses despite a much higher dose ($10^6$ PFU/ferret), and the TK-H274Y virus caused less weight loss and fever than the TK-WT virus (Table 2). In co-infected ferrets, clinical signs were less pronounced with inoculation of a higher percentage of TK-H274Y virus. Animals in all TK virus groups recovered fully and survived at day 21 p.i. Virus titers in the nasal washes were comparable across all study groups on days 2, 4, and 6 p.i. (Table 2).

Overall, the oseltamivir–resistant VN-H274Y virus retained the high virulence of the wild-type virus in ferrets. At the infectious dose studied, the virulence of the drug-resistant variant of the less pathogenic H5N1 virus, TK-H274Y, was slightly compromised on 1-3 days.
p.i. on the basis of weight and temperature changes, although virus replication in the URT was
comparable with that of TK-WT virus.

Genetic and phenotypic stability of virus populations in infected and co-infected
ferrets. We assessed the predominance of the oseltamivir-sensitive vs. oseltamivir-resistant
genotype in virus populations isolated from the URT of inoculated and co-inoculated ferrets by
using both direct sequencing (dominant virus population, Fig. 2) and analysis of individual virus
clones (Table 3). No changes in the NA sequence were found in isolates from ferrets inoculated
with VN-WT or VN-H274Y virus; therefore, the H274Y NA mutation remained stable in the VN
virus background. Clonal analysis of the virus populations confirmed the virus dominance and
 genetic stability determined by sequencing. The NA enzyme inhibition assay showed that the
phenotypic pattern of susceptibility to oseltamivir carboxylate had not changed in the VN-WT
and VN-H274Y viruses on day 6 p.i. (mean IC\textsubscript{50}, 0.4 and 1008.2 nM, respectively).

Direct sequencing (Fig. 2) and clonal analysis (Table 3) of viruses isolated after co-
inoculation of ferrets with different ratios of VN-WT and VN-H274Y viruses showed a mixed
genotype in all animals and all groups. Although the clonal populations remained mixed on day 6
p.i., the proportion of oseltamivir-resistant VN-H274Y clones had increased from 67% to 80%,
from 50% to 72%, and from 7% to 52% in the respective co-inoculated groups (Table 3).
Importantly, the proportion of resistant clones isolated on day 6 p.i. paralleled the virus
population’s susceptibility to oseltamivir carboxylate (Fig. 2).

A different pattern determined in the TK virus groups. Oseltamivir-sensitive TK-WT
virus remained genetically stable in all 3 animals as determined by direct sequencing and clonal
analysis. However, in all 3 ferrets initially inoculated with 100% oseltamivir-resistant TK-
H274Y virus, the proportion of clones carrying the H274Y mutation decreased (40%, 78%, and
The proportion of drug-resistant clones also decreased from 30% to 9% in one group of ferrets inoculated with a mixture of sensitive and resistant viruses and changed from 10% to 0%, 4%, and 18%, respectively, in the 3 ferrets in the other group (Table 3). NA enzyme activity paralleled the observed ratio of oseltamivir-sensitive and –resistant clones.

This detailed analysis of at least 20 clones in each ferret and of virus-virus interactions within each host (i.e., competitive fitness) established the uncompromised fitness of VN-H274Y virus and the impaired fitness of TK-H274Y virus. The trend towards an increase/decrease in the frequency of the H274Y NA mutation relative to the wild-type was a statistically significant trend (P>0.05) for 2 studied groups only (Table 3).

Host-dependent and compensatory NA mutations. The fitness of oseltamivir-resistant viruses can be improved by the presence of additional mutations in the NA gene or in other genes. We examined additional NA mutations that emerged in the infected ferrets and their relation to the presence of the H274Y NA mutation. All identified NA mutations were divided into 2 groups: (1) potential host-dependent NA mutations identified in clones with and without H274Y NA mutation that either occurred at residues 116, 117, 119, 136, 150, 222, 252, 292, or 294 or were detected with ≥20% frequency; and (2) potential compensatory NA mutations found with ≥20% frequency only in clones carrying the H274Y substitution (Table 3, Supplementary Fig. 1). We were interested in the existence of potential host-dependent NA mutations at the catalytic (R292), framework (E119, I222 and N294) sites and in close proximity to the NA enzyme active site (V116, I117, Q136, K150, and Y252) because mutations at these residues either confer resistance and cross-resistance to oseltamivir and zanamivir (12, 24, 26, 35) or were linked to reduced drug-susceptibility in avian and human viruses carrying N1 NA (10). In most groups, host-dependent NA mutations were detected in 2/3 or 3/3 animals. Potential host-
dependent mutations were detected in 1/3 ferrets in only 3 groups (those inoculated with VN-WT; with 10% VN-WT : 90% VN-H274Y; and with 90% TK-WT : 10% TK-H274Y). One to six host-dependent NA mutation(s) were identified per animal. The framework mutation N294S previously reported to be associated with oseltamivir resistance (12, 35) and the NA mutations V116I, I117V, and A250T were detected in the A/Vietnam/1203/04-like background (Table 3). The catalytic residue mutation R292K and mutations at framework residues (E119A, I222L, H274L, N294S) were detected in the A/Turkey/15/06-like background. Analysis of NA clones suggested the presence of 6 potential compensatory NA mutations: D103V, F132S, I254V, E276A, H296L, and F466S (Table 3). The I254V and E276A NA mutations are of particular interest in view of their high frequency of detection and/or their identification in numerous hosts (Supplementary Table 1).
DISCUSSION

The lethality and continuing circulation of H5N1 influenza viruses warrants an urgent search for an optimal therapy. While the NA inhibitors are currently our first line of defense against a pandemic threat, the potential emergence of virulent and transmissible drug-resistant variants is of concern. We compared the fitness of oseltamivir-sensitive and oseltamivir-resistant (carrying the H274Y NA mutation) virus pairs representing clades 1 and 2.2 in inoculated ferrets by examining their clinical manifestations and their replication efficiency and genetic stability in the URT. Ferret tracheal epithelial cells express primarily sialic acid (SA) α2,6-galactose receptor structures, and a lesser amount of SA α2,3-galactose receptors (31), and therefore, more closely represents the human airway epithelium and allows study the fitness of drug-resistant human influenza viruses. Within each pair, the oseltamivir-resistant and wild-type virus caused disease of equal severity (with an exception of TK-H274Y virus on days 1-3 p.i.) and replicated to comparable titers in the URT. In ferrets co-inoculated with mixtures of the oseltamivir-resistant and -sensitive virus pairs, the proportion of drug-resistant VN-H274Y clones tended to increase and the proportion of TK-H274Y clones tended to decrease. The NA enzymatic activity phenotype was consistent with the observed proportion of oseltamivir-resistant and -sensitive clones. These findings suggest that the H274Y NA mutation can affect the fitness of two H5N1 influenza viruses differently, although, in addition to NA mutation(s), it may depend on overall genetic composition of the viruses and contribution of other gene segments.

What factors may explain the observed differences in the fitness of H5N1 influenza viruses carrying the same H274Y NA mutation? Our use of viruses with an identical genetic background eliminated the possible effects of other genes on biological fitness within each pair of H5N1 viruses, but not between representatives of different HA clades. Although several
studies have used reverse genetics to generate recombinant viruses carrying NA mutations in the influenza A virus H1N1 (1, 2, 21) and H3N2 background (5, 48) and in the influenza B virus background (27), only one study, conducted by our laboratory, has used recombinant H5N1 influenza viruses to investigate the impact of NA inhibitor-resistant NA mutations in a homogeneous genetic background (46). That study found that neither the H274Y nor the N294S NA mutation compromises the lethality or pathogenicity of the A/Vietnam/1203/04 (H5N1) virus in BALB/c mice. The work reported here confirmed our previous observations and showed that A/Vietnam/1203/04 (H5N1) virus carrying the H274Y NA mutation is genetically stable in the URT of ferrets and retains an uncompromised phenotype. The A/Turkey/15/06 (H5N1) influenza virus carrying the H274Y NA mutation was less competitive with its wild-type counterpart and caused less pronounced weight and temperature change during days 1-3 p.i. The VN-WT and TK-WT viruses differed by 14 amino acids in their NA genes and by multiple amino acids in other genes (25). Both VN-H274Y and TK-H274Y viruses had less NA activity than their wild-type counterparts and it may in part explain the differences in plaque phenotype observed between drug-resistant and –sensitive viruses. The VN-H274Y and TK-H274Y viruses lost ~90% and 60% of the wild-type activity, respectively, and it could affect virus spread in MDCK cells, although we did not see differences in the final virus yields. Thus, virus fitness can be affected by antigenic and genetic diversity and by the degree of NA functional loss. However, the measurements of the NA activity could be affected by three processes: (1) the wild-type and mutant NAs could have different inherent catalytic activities per enzyme; (2) the particles of wild-type and mutant viruses could have different numbers of total NA proteins on their surface; and (3) the ratios of infectious particles to total particles could differ if H274Y NA substitution alters the infectivity of the virions or increases the number of defective particles.
We used two human H5N1 influenza viruses that differ dramatically in their virulence to ferrets. Previous studies showed that inoculation of ferrets with as little as 10 EID$_{50}$ of A/Vietnam/1203/04 (H5N1) virus caused systemic spread and death, whereas $10^6$ EID$_{50}$ of A/Turkey/15/06 (H5N1) virus was not lethal (13). A/Vietnam/1203/04 is a unique H5N1 virus that rapidly triggers multiple events in infected hosts and is characterized by broad tissue tropism, highly efficient replication, and rapid immune system dysregulation (13, 42). Virus-host interactions in A/Vietnam/1203/04 (H5N1) virus-ferret model could be affected by high virus virulence, and studies with A/Turkey/15/06 (H5N1) virus were undertaken to obtain experimental evidence on the fitness of oseltamivir-resistant vs. sensitive variants in the background of a less virulent virus that possesses lower efficiency of replication (13). The less virulent A/Hanoi/30408/05 (H5N1) influenza virus carrying the H274Y NA mutation was reported to yield reduced titers in ferret nasal washes (30). The number of virus particles in the VN and TK virus inocula differed by a factor of $\sim 10,000$. This difference in dose may affect the fitness of drug-resistant viruses. Inoculation of ferrets with a range of doses allows better detection of minor changes in clinical signs, such as those induced by low infectious doses of A/Texas/36/91 (H1N1) virus with the H274Y NA mutation (26) and A/Sydney/5/97 (H3N2) virus with the R292K NA mutation (6). We suggest that high virus virulence can affect virus-host interaction and thus affect the fitness of oseltamivir-resistant and -sensitive variants.

We used a novel approach to compare the fitness of oseltamivir-sensitive and –resistant influenza viruses that included analysis of virus-virus interactions within the host (competitive fitness) during co-infection with these viruses. Although mixed populations were present in the URT of ferrets on day 6 p.i., the fitness of VN-H274Y virus was uncompromised as compared to that of its drug-sensitive counterpart, while that of TK-H274Y virus was impaired. Mixtures of
oseltamivir-sensitive and -resistant clones have been found in clinical studies (9, 30) and in experimental animal models (13). Influenza virus replication is an error-prone process, resulting in a large number of variants (quasispecies) in the host. A minor population of NA inhibitor-resistant variants may gain a replication advantage under suboptimal therapy in two ways: (1) preexisting variants less sensitive to the drug are selected from the quasispecies population, leading to an increase of the number of resistant clones, and (2) outgrowing variants may acquire additional compensatory mutations that enhance their fitness. It is possible that use of antiviral drugs (particularly at suboptimal concentration) against mixtures of oseltamivir-resistant and sensitive viruses will promote the spread of drug-resistant variants by inhibiting drug-sensitive variants that are competing with them for the dominance in the infected host. Thus, resistant virulent and transmissible viruses that emerge at extremely low frequency during antiviral treatment could significantly alter the course of an epidemic or pandemic. The transmissibility of drug-resistant viruses carrying different NA mutations is of particular importance. The inefficient transmissibility of H5N1 influenza viruses in a ferret animal model (33, 47) (as in humans) restricted our studies to evaluation of pathogenicity and fitness. However, it was shown that oseltamivir-resistant NA mutations in H3N2 influenza viruses did not affect viral growth or contact transmission but did affect the aerosol transmission of the H3N2 virus in guinea pigs (5).

The influence of multiple genes on the fitness of viruses carrying H274 NA mutation cannot be excluded. In our study we focused on additional NA mutations, and sequence analysis of individual NA clones (13, 28) was done to identify potential host-dependent and compensatory NA mutations. We found that the NA mutations E119A and N294S, which confer cross-resistance to oseltamivir and zanamivir (12, 35), can emerge spontaneously in clade 2.2 H5N1 influenza virus in ferrets. Further, we observed that mutations at NA catalytic (R292K)
and framework (I222L and N294S) sites and in close proximity to the NA enzyme active site (V116I, I117T/V, Q136H, K150N, A250T) emerged without drug pressure in both pairs of H5N1 viruses. Compensatory mutations in NA or other genes may mitigate any fitness cost imposed by resistance mutations. Our study identified 6 potential compensatory NA changes (D103V, F132S, I254V, E276A, H296L, and F466S) that may affect the fitness of viruses with the H274Y NA mutation. We suggest that NA mutations at residues I254V and E276A are of importance. Interestingly, we observed differences in predominance of I254V and E276A NA mutations in different genetic backgrounds: I254V mutation was identified in A/Vietnam/1203/04 (H5N1)-like and E276A in A/Turkey/15/06 (H5N1)-like genetic background. Moreover, I254V NA mutation was identified only when ferrets were inoculated with the mixtures of VN-WT and VN-H274Y viruses but not in ferrets inoculated with VN-H274Y virus. None of the potential compensatory NA mutations was identified in the original inoculum used to infect ferrets. The H274Y NA mutation causes a large shift in the position of the side chain of the neighboring E276 residue (8), which must form a salt bridge with R224 to accommodate the large hydrophobic pentyl ether group of oseltamivir (43). The E276A substitution results in a shorter, nonpolar side chain at that residue and thus can affect interactions in this region. Residue I254 is located near the NA active site, and although it does not alter polarity, it results in a shorter side-chain and thus may indirectly affect the residues in the NA active site.

Overall, these preclinical studies highlight the complexity of virus fitness and the importance of experimental design in accurate assessment of the impact of drug-resistant mutations. The design of experiments and methodological approaches used to assess virus fitness can affect the conclusions. Our traditional virus fitness studies in a ferret model (based on virus-
host interaction) did not detect the differences in clinical signs and URT replication caused by oseltamivir-sensitive and –resistant H5N1 influenza viruses. However, competitive fitness experiments (based on virus-virus interaction within the host) revealed a disparity in the growth capacity of VN-H274Y and TK-H274Y viruses as compared to their wild-type counterparts. We suggest that antigenic and genetic diversity, virulence, the degree of NA functional loss, and differences in host immune response and genetic background can contribute to such differences. Therefore, the risk of emergence of drug-resistant influenza viruses with uncompromised fitness should be monitored closely and considered in pandemic planning.
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FIGURE LEGENDS

FIG. 1. NA enzyme activity of the recombinant H5N1 influenza viruses. The four recombinant H5N1 influenza viruses at infectious doses of $10^4$-$10^7$ PFU/ml were incubated at 37°C for 30 min with fluorogenic MUNANA substrate at a final concentration of 100 µM. NA activity is expressed in relative fluorescence units (RFU). The loss of NA activity was calculated as a RFU/PFU ratio between oseltamivir-sensitive and -resistant H5N1 viruses.

FIG. 2. Genetic and phenotypic stability of oseltamivir-sensitive and -resistant H5N1 influenza viruses in ferrets. Nasal washes were collected on day 6 p.i. from 3 ferrets/group inoculated with the indicated viruses or virus mixtures. Each panel of the chromatogram shows results from a single representative ferret. The sequence chromatogram represents results from individual ferrets inoculated with the VN (A) or TK (B) viruses or the indicated mixtures. The CAC codon represents histidine (oseltamivir-sensitive), and the TAC codon represents tyrosine (oseltamivir-resistant). Double peaks indicate the population mixture in vivo. IC$_{50}$ values (nM) were determined after a single passage of a nasal wash (obtained on day 6 p.i.) in MDCK cells. IC$_{50}$ values are the mean ± SD from 3 ferrets.

Supplementary Fig. 1. Detection of host-dependent and compensatory NA mutations in viruses isolated from the ferret URT. Uppermost panels show sequence data obtained from the virus inocula. Lower panels show mutation frequency on day 6 p.i. in nasal washes from individual ferrets (3 per group) inoculated with the indicated VN (A) and TK (B)
viruses and mixtures. At least 20 NA clones per animal were analyzed). F - ferret. NA
length is represented in amino acids.
REFERENCES


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<table>
<thead>
<tr>
<th>H5N1 recombinant virus</th>
<th>Growth in MDCK cells</th>
<th>NA enzyme inhibition assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean virus yield (± SD, log_{10} PFU/ml)</td>
<td>Mean plaque diameter (± SD, mm)</td>
</tr>
<tr>
<td></td>
<td>Oseltamivir carboxylate</td>
<td>Fold change</td>
</tr>
<tr>
<td>A/Vietnam/1203/04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VN-WT</td>
<td>7.9 ± 0.2</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>VN-H274Y</td>
<td>7.9 ± 0.3</td>
<td>2.0 ± 0.3*</td>
</tr>
<tr>
<td>A/Turkey/15/06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TK-WT</td>
<td>6.5 ± 0.4</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>TK-H274Y</td>
<td>7.1 ± 0.1</td>
<td>0.3 ± 0.03*</td>
</tr>
</tbody>
</table>

*The concentration of NA inhibitor that reduced NA activity by 50% relative to a control mixture with no inhibitor (IC_{50}). Values are from 2-3 independent determinations.

b Multiple of the value obtained for the recombinant wild-type virus.

* P < 0.01 compared to WT virus (unpaired two-tailed t-test).
<table>
<thead>
<tr>
<th>H5N1 virus or mixture</th>
<th>No. surviving/total no.</th>
<th>No. showing indicated signs(s) /total no.</th>
<th>RII a</th>
<th>Nasal wash titer (log_{10}EID_{50}/ml, mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weight loss (%; mean ± SD)</td>
<td>Temperature increase, (°C; mean ± SD)</td>
<td>Respiratory signs b</td>
</tr>
<tr>
<td>A/Vietnam/1203/04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VN-WT</td>
<td>0/3</td>
<td>3/3 (22.3 ± 6.7)</td>
<td>3/3 (1.4 ± 1.9)</td>
<td>3/3</td>
</tr>
<tr>
<td>VN-H274Y</td>
<td>0/3</td>
<td>3/3 (22.9 ± 1.0)</td>
<td>3/3 (2.1 ± 0.4)</td>
<td>3/3</td>
</tr>
<tr>
<td>10%VN-WT : 90% VN-H274Y</td>
<td>0/3</td>
<td>3/3 (21.9 ± 4.2)</td>
<td>3/3 (2.0 ± 0.3)</td>
<td>3/3</td>
</tr>
<tr>
<td>50%VN-WT : 50% VN-H274Y</td>
<td>0/3</td>
<td>3/3 (17.5 ± 1.7)</td>
<td>3/3 (1.9 ± 0.5)</td>
<td>3/3</td>
</tr>
<tr>
<td>90%VN-WT : 10% VN-H274Y</td>
<td>0/3</td>
<td>3/3 (19.3 ± 5.2)</td>
<td>3/3 (2.2 ± 0.5)</td>
<td>3/3</td>
</tr>
<tr>
<td>A/Turkey/15/06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TK-WT</td>
<td>3/3</td>
<td>3/3 (16.7 ± 5.8)</td>
<td>3/3 (2.8 ± 0.8)</td>
<td>1/3</td>
</tr>
<tr>
<td>TK-H274Y</td>
<td>3/3</td>
<td>3/3 (4.8 ± 5.0)</td>
<td>1/3 (1.7)</td>
<td>0/3</td>
</tr>
<tr>
<td>50%TK-WT : 50% TK-H274Y</td>
<td>3/3</td>
<td>3/3 (6.4 ± 3.3)</td>
<td>2/3 (1.8 ± 0.5)</td>
<td>0/3</td>
</tr>
<tr>
<td>90%TK-WT : 10% TK-H274Y</td>
<td>3/3</td>
<td>3/3 (19.2 ± 2.2)</td>
<td>3/3 (2.4 ± 0.5)</td>
<td>1/3</td>
</tr>
</tbody>
</table>

a Labored breathing, sneezing, wheezing, or nasal discharge.

b Hind-limb paresis, ataxia, torticollis, or tremors.

c Relative inactivity index (40) was calculated as the mean score per group of ferrets as compared to pre-inoculation value (0).

A/Vietnam/1203/04 virus groups were observed once daily for 7 days because of mortality; A/Turkey/15/06 virus groups were observed once daily for 14 days.

d Results obtained from 1 ferret.

<, below lower limit of detection (<0.75 log_{10}EID_{50}/ml).
TABLE 3. Sequence analysis of virus populations in the inocula and in inoculated and co-inoculated ferrets

<table>
<thead>
<tr>
<th>H5N1 virus or mixture</th>
<th>In inoculum</th>
<th>F0</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>Mean H274Y frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H274Y no. clones/ total no. (%)</td>
<td>Other mutations (%)</td>
<td>Host-dependent</td>
<td>Compen-satory</td>
<td>Host-dependent</td>
<td>Compen-satory</td>
</tr>
<tr>
<td>A/Vietnam/1203/04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VN-WT</td>
<td>0/18 (0)</td>
<td>H117T (6)</td>
<td>0/20 (0)</td>
<td>–</td>
<td>N/A</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>10%VN-WT : 90% VN-H274Y</td>
<td>20/30 (67)</td>
<td>K150R (3)</td>
<td>15/20 (75)</td>
<td>S388C (20)</td>
<td>D110V (20)</td>
<td>15/20 (75)</td>
</tr>
<tr>
<td>A/Turkey/15/06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TK-WT</td>
<td>0/20 (0)</td>
<td>–</td>
<td>0/20 (0)</td>
<td>K150N (10)</td>
<td>N/A</td>
<td>0/18 (0)</td>
</tr>
<tr>
<td>90% TK-WT : 10% TK-H274Y</td>
<td>2/20 (10)</td>
<td>–</td>
<td>4/22 (18)</td>
<td>–</td>
<td>E276A (50)</td>
<td>1/26 (4)</td>
</tr>
<tr>
<td>--------------------------</td>
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</tbody>
</table>

* NA mutations at residues 116, 117, 119, 136, 150, 222, 252, 292, or 294 or detected with ≥20% frequency.

b NA mutations detected with ≥20% frequency in clones carrying the H274Y substitution. Percentage is shown only for mixed and H274Y groups.

N/A - not applicable. –, no NA mutations were detected. F - ferret

* P<0.05, unpaired two-tailed t-test with Welch’s correction.
Figure 2

A

Before infection

VN-WT

IC_{50} 0.3 ± 0.1 nM

VN-H274Y

1012.6 ± 987.3 nM

10% VN-WT: 90% VN-H274Y

50% VN-WT: 50% VN-H274Y

90% VN-WT: 10% VN-H274Y

After infection

IC_{50} 0.4 ± 0.1 nM

VN-WT

1008.2 ± 933.3 nM

VN-H274Y

947.5 ± 315.7 nM

50% VN-WT: 50% VN-H274Y

90% VN-WT: 10% VN-H274Y

B

Before infection

TK-WT

IC_{50} 4.9 ± 0.7 nM

TK-H274Y

50% TK-WT: 50% TK-H274Y

90% TK-WT: 10% TK-H274Y

After infection

IC_{50} 4.5 ± 0.7 nM

TK-WT

1001.2 ± 478.2 nM

TK-H274Y

102.7 ± 33.2 nM

50% TK-WT: 50% TK-H274Y

90% TK-WT: 10% TK-H274Y

Figure 2