

Influenza A(H7N9) Virus Acquires Resistance-Related Neuraminidase I222T Substitution When Infected Mallards Are Exposed to Low Levels of Oseltamivir in Water

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Influenza A virus (IAV) has its natural reservoir in wild waterfowl, and new human IAVs often contain gene segments originating from avian IAVs. Treatment options for severe human influenza are principally restricted to neuraminidase inhibitors (NAIs), among which oseltamivir is stockpiled in preparedness for influenza pandemics. There is evolutionary pressure in the environment for resistance development to oseltamivir in avian IAVs, as the active metabolite oseltamivir carboxylate (OC) passes largely undegraded through sewage treatment to river water where waterfowl reside. In an *in vivo* mallard (*Anas platyrhynchos*) model, we tested if low-pathogenic avian influenza A(H7N9) virus might become resistant if the host was exposed to low levels of OC. Ducks were experimentally infected, and OC was added to their water, after which infection and transmission were maintained by successive introductions of uninfected birds. Daily fecal samples were tested for IAV excretion, genotype, and phenotype. Following mallard exposure to 2.5 µg/liter OC, the resistance-related neuraminidase (NA) I222T substitution, was detected within 2 days during the first passage and was found in all viruses sequenced from subsequently introduced ducks. The substitution generated 8-fold and 2.4-fold increases in the 50% inhibitory concentration (IC₅₀) for OC ($P < 0.001$) and zanamivir ($P = 0.016$), respectively. We conclude that OC exposure of IAV hosts, in the same concentration magnitude as found in the environment, may result in amino acid substitutions, leading to changed antiviral sensitivity in an IAV subtype that can be highly pathogenic to humans. Prudent use of oseltamivir and resistance surveillance of IAVs in wild birds are warranted.

Migratory waterfowl have a central role in the emergence of new human influenza A viruses (IAVs), as they are the reservoir hosts of IAV in the environment and as novel human-pathogenic IAVs often contain genetic material that originates from avian IAVs (1). In wild waterfowl, the IAV infection is intestinal with limited clinical signs (2, 3). Viruses are genetically diverse due to rapid rates of mutations and continuous reassortment of genetic segments (4) and are diffused along migratory routes (5). New human-pathogenic IAVs can evolve through reassortment of genetic segments from a human-adapted virus with segments from a virus originating from avian or other host species, a feature of all pandemic viruses of the last century (6). Avian IAVs can also infect humans by direct transmission following infection in poultry, which have both mammalian- and avian-type hemagglutinin receptors, and adaptations that enable viral crossing of the species barrier (7, 8). In March 2013, this type of transmission initiated an outbreak in China, resulting in severe human infection caused by a multiple reassortant avian A(H7N9) virus (9), with gene segments acquired from low-pathogenic avian influenza (LPAI) viruses of wild migratory birds and domesticated poultry (7, 10). Fortunately, to date no sustained human-to-human transmission of the virus has been seen (11), although it has become enzootic in China with establishment of multiple distinct regional lineages (12), and new human cases are being reported (13).

There are limited means to prevent and treat severe human IAV infections, particularly when a virus strain is novel and anti-

genically different from the circulating seasonal viruses, as little or no immunity exists in the population and vaccines are unavailable (14). New IAV drugs targeting different steps in the viral replication cycle and vaccines with broad immunogenicity might improve responses (15), but currently neuraminidase inhibitors (NAIs) are the best available initial means when in the face of a new human-pathogenic IAV (14). Therefore, global pandemic preparedness plans rely almost entirely on oseltamivir (Tamiflu;

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Roche) for the early-phase pandemic response, with large national stockpiles (16, 17). Indeed, NAIs are the primary treatment choice in the current Chinese A(H7N9) outbreak (18), and NAI resistance is associated with severe outcomes (19).

Resistant IAVs can arise in NAI-treated patients but might also be already inherent when a virus crosses the avian-human species barrier (20). Several lines of evidence suggest that wild waterfowl may be exposed to oseltamivir in their natural environment and that the IAVs infecting them might develop NAI resistance. The active metabolite, oseltamivir carboxylate (OC), is excreted by patients and is not removed during traditional sewage treatment (21, 22) or degraded by daylight exposure (23), thus ending up in sewage effluents and in river water where avian IAV hosts, such as different species of waterfowl, reside. OC measurements from river water have primarily been done in Japan, with collections from more than 40 river sample sites from the main river systems, where concentrations of up to 0.86 $\mu\text{g/liter}$ have been detected (24–27). In Europe (Germany from 12 sites [28], Spain from 11 sites [29], and England from 21 sites [30]), OC concentrations in river water of approximately 0.05 $\mu\text{g/liter}$ have been detected during the influenza season. The effect of environmental OC on IAV in the intestinal tract of wild birds is unknown. *In vivo* mallard experiments have demonstrated that IAVs of both the N1 and N2 subtypes (representing both phylogenetic neuraminidase [NA] groups N1 and N2) can develop NAI resistance when infected birds are exposed to OC concentrations ranging from 0.95 to 12 $\mu\text{g/liter}$ in their water (31–33). Additionally, it was demonstrated that an acquired resistance mutation in an avian IAV can persist without drug pressure, with maintained viral fitness (34). If an avian IAV acquires and retains NAI resistance, a novel pandemic virus may be resistant already when it crosses the species barrier. Stockpiles of oseltamivir would then lose their role in the response to a pandemic, and if the virus is virulent, the public health consequences would be dramatic with limited treatment options.

From this perspective, it is important to evaluate the propensity for different NA variants to acquire and maintain NAI resistance. Here, we investigated an LPAI A(H7N9) virus in an *in vivo* mallard (*Anas platyrhynchos*) model, in which infected birds were exposed to OC in drinking and swimming water.

MATERIALS AND METHODS

Virus. The influenza A/mallard/Sweden/1621/2002(H7N9) virus used in this study was isolated, as part of IAV surveillance, from a wild mallard in southern Sweden in December 2002, as described in reference 35. The virus was isolated in 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE); the virus was inoculated in the allantoic cavity, harvested after 2 days, and detected by hemagglutination with turkey erythrocytes. A second passage in SPF ECE was done if the hemagglutination assay was negative. The positive samples were hemagglutinin (HA) subtyped by a hemagglutination inhibition assay with turkey erythrocytes and hyperimmune rabbit antisera against all HA subtypes (36). NA was subtyped by reverse transcriptase (RT)-PCR and sequencing with universal primers as described by Hoffman et al. (37). Whole-genome sequencing was done through the Influenza Virus Genome Sequencing Project (GenBank accession numbers for all segments are AHZ41929 to AHZ41940).

The phylogenetic placement in the context of Eurasian viruses was determined utilizing Bayesian trees. Outgroups included all Eurasian H7 ($n = 531$), all Eurasian N9 ($n = 216$), and 10 North American sequences for each H7 and N9, downloaded from the Influenza Research Database (<http://www.fludb.org/>). The trees were built using resources provided by the Swedish National Infrastructure for Computing (SNIC) through the

Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX).

Drugs. OC and deuterium-labeled OC were obtained from F. Hoffmann-La Roche Ltd. (Basel, Switzerland) and zanamivir (ZA) (for use in the neuraminidase inhibition assay, as described in reference 38) was purchased locally as Relenza. The compounds were dissolved in double-distilled water, and stock solutions were stored at minus 20°C.

Mallard model. One-day-old male mallards were purchased and bred isolated indoors at the animal facilities of the Swedish Veterinary Institute. Breeding, housing, and experimental protocols were approved by the Ethics Committee on Animal Experiments in Uppsala, Sweden (permit C63/13), and complied with guidelines of the Swedish Board of Agriculture. IAV infection prior to experiments was ruled out by blood serology (FlockCheck avian influenza virus antibody test kit; IDEXX, Hoofddorp, The Netherlands) performed at 10 weeks of age, and by real-time reverse transcriptase (RRT)-PCR of the IAV matrix gene from fecal samples (described below) taken before the ducks entered the experiment at 4 months of age.

The experimental design was based on the dynamics of IAV infection in mallards, allowing continuous viral propagation by successive introduction of naive ducks to those infected and largely followed a previously described protocol (31, 32). Viral transmission between ducks is primarily by fecal excretion (1); infection and fecal shedding peak after 2 days and decrease markedly after approximately 5 to 7 days (39). At the start of the experiment, two mallards were inoculated in the esophagus with 1 ml of ECE allantoic fluid and placed in the experimental room (on day zero), which contained a single water source of 170 liters and food *ad libitum*. To sustain transmission with the most recently evolved viral variant, generations (two mallards) of naive ducks were introduced at day 3 postinoculation and housed together with the infected ducks during 48 h, until day 5 of the experiment, to allow transmission, after which the previous generation was euthanized (with intravenous injections of 100 mg/kg sodium pentobarbital [pentobarbital veterinary, 100 mg/ml]). On the following day (after 24 h and two changes of water), a new generation of two naive ducks was introduced and so forth, resulting in a total of seven generations of birds, each staying 5 days in the experiment, across 23 days. The water was changed daily and spiked with OC, initially to a concentration of 2.5 $\mu\text{g/liter}$, which was successively increased during the experiment, to 7.2 $\mu\text{g/liter}$ of OC from day 9 and to 24 $\mu\text{g/liter}$ of OC from day 18 (Fig. 1). Individual fecal samples were collected daily by swabbing fresh feces following defecation in single-use boxes. On a few occasions, cloacal swabbing replaced the fecal sampling if the birds did not defecate while in the boxes. Water samples (1 ml) were also taken daily for viral detection.

Viral detection and sequencing of NA. Viral RNA was extracted from all fecal samples, from water, and from isolates with an automated magnetic bead method using a Magnatrix 8000 extraction robot (Magnetic Biosolutions, Stockholm, Sweden) with a Vet viral RNA kit (NorDiag ASA, Oslo, Norway). Some samples with low yields of NA PCR amplicons (see below) were reextracted from fecal samples by organic extraction with TRIzol (Life Technologies) using 900 μl reagent per 100 μl sample. Detection and quantification of IAV from fecal samples and water were done by RRT-PCR with primers and probe targeting the influenza A matrix gene (40) and an iScript one-step RT-PCR kit for probes (Bio-Rad). The reaction volumes of 25 μl with 0.5 μl enzyme mix and final concentrations of primers and probe of 400 nM and 120 nM, respectively, were run in a Corbett Research Rotor-Gene 2000 real-time thermo cycler (Corbett Research). Samples with cycle threshold (C_T) values ≥ 45 were considered negative.

The NA gene was amplified from the RNA of fecal samples that were positive for IAV in the RRT-PCR by a one-step RT-PCR using specifically designed primers (see Table S1 in supplemental material) and a SuperScript III one-step RT-PCR system with Platinum *Taq* high fidelity polymerase (Life Technologies). Reaction volumes of 25 μl contained 0.25 μl enzyme mix, one forward and one reverse primer at a final concentration of 400 nM and 5 μl (1 pg to 1 μg) RNA sample. Thermocycling conditions

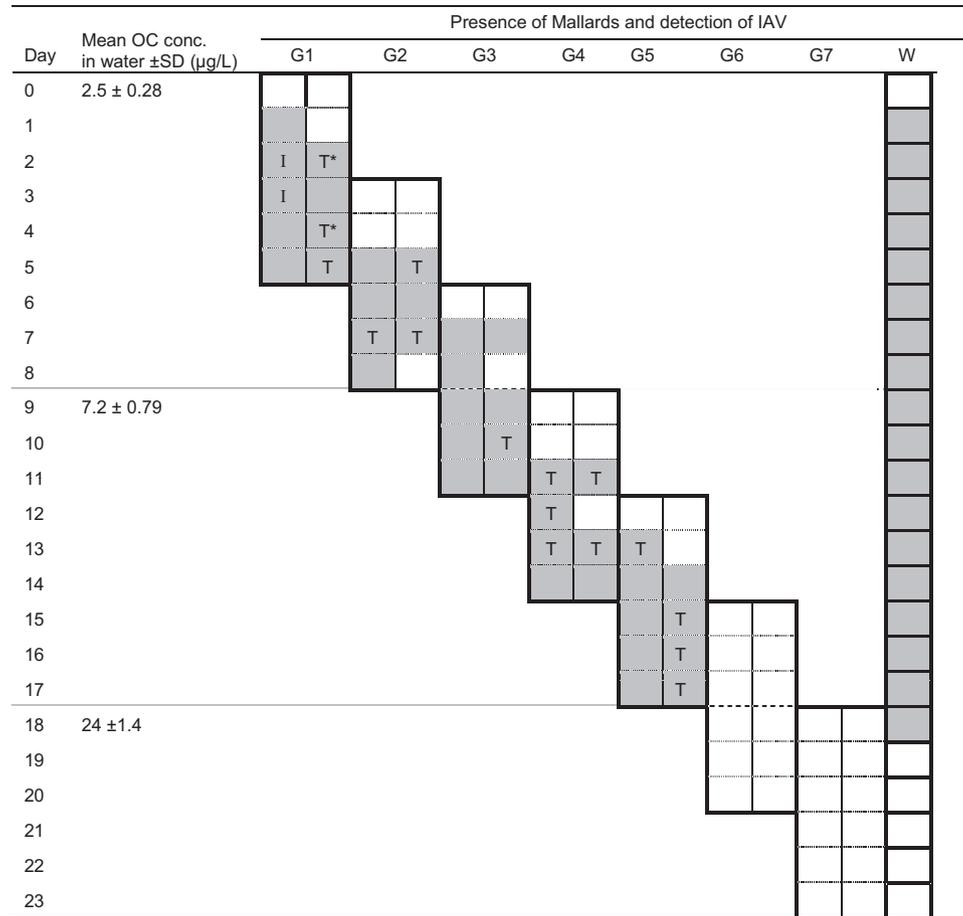


FIG 1 Experimental mallard model with IAV shedding and NA 222 amino acid residues. The values 2.5 \pm 0.28, 7.2 \pm 0.79, and 24 \pm 1.4 μ g/liter are the means \pm standard deviations of 11, 9, and 7 water samples, respectively. The dashed horizontal lines indicate changes in OC concentrations. G1 represents generation one consisting of two mallards, G2 represents the second pair of mallards introduced in the experimental room, etc. Closed rectangles represent the presence of mallards in the experimental room and dotted lines indicate days. Note that birds were introduced into and removed from the experiment room in the daytime such that each bird was part of the experiment during 5' 24 h, with a 24-h gap between every second generation. Shading indicates detection of IAV by RRT-PCR of the matrix gene from daily fecal samples (C_T values of ≥ 45 were determined to be negative). I, isoleucine at NA residue 222; T*, threonine in shared proportions with isoleucine at NA residue 222; T, threonine at NA residue 222, as determined by Sanger sequencing of fecal samples; W, water from the 170-liter pool in the experimental room. Closed rectangles represent change of water and shading indicates detection of IAV by RRT-PCR of the matrix gene.

were 30 min at 55°C, 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 57°C, and 4 min at 68°C. PCR products were confirmed by gel electrophoresis and purified by enzymatic treatment with ExoSAP-IT (Afymetrix Inc., CA, USA), using 2 μ l reagent to treat 24 μ l sample. PCR products were Sanger sequenced at Macrogen Inc. (The Netherlands) with four sequencing primers (see Table S1 in the supplemental material). The sequence results were analyzed in SeqScape v2.7 software (Applied Biosystems) with the original A/mallard/Sweden/1621/2002(H7N9) NA sequence as a reference. A sequence result was considered reliable if there were at least two high-quality electropherogram sequences at any given nucleotide position. The genotype was determined as "mixed" if two peaks representing different nucleotides were visible at a single position in the electropherograms.

Neuraminidase inhibition by OC and ZA. Prior to phenotypic NAI sensitivity testing, fecal samples were propagated in SPF ECE (Valo, Germany), as described above, to obtain a sufficient viral titer for the assay, followed by NA sequencing (as described above) to verify the genotype. Ten NA I222T isolates and three wild-type isolates were tested. Inhibition of NA by OC and ZA was determined on duplicate samples in a fluorogenic NA substrate assay using 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA) (Sigma) (41) with 10-step dilution series

(4,000 to 0.0015 nM) of OC and ZA in black 96-well flat-bottom plates, according to the protocol of the European Vigilance Network for the Management of Antiviral Drug Resistance (VIRGIL-CLINVIR) (42). Virus and drugs were preincubated in 37°C for 30 min followed by a 60-min incubation with MUNANA substrate. Fluorescent products were measured in an Infinite M1000 PRO (Tecan) microplate reader, and 50% inhibitory concentrations (IC_{50} s) were determined from the best-fit dose-response curves using Prism 6 software (GraphPad).

Detection and quantification of OC in water. Water samples (~40 ml) from the experimental water pool were collected daily, both before and after the water was changed (post- and preexposure samples, respectively). On the first and last days of each concentration level, triplicate samples were taken for evaluation of the method's variation. Ten milliliters of each water sample was prefiltered (0.45 μ m) and acidified (0.1% formic acid). Analysis for detection of OC was done on 1 ml of the filtrate using an on-line solid-phase extraction/liquid chromatography-tandem mass spectrometry (SPE/LC-MS/MS) method, as previously described (43). Results were quantified using deuterium-labeled OC as an internal standard, with three calibration points. For evaluation of OC degradation during 24 h, preexposure samples were compared to postexposure samples from the first and last days of the experiment.

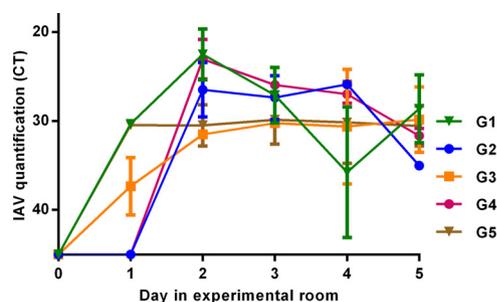


FIG 2 Shedding of A(H7N9) virus determined by RRT-PCR of the matrix gene from daily fecal samples. The x axis displays the number of days that each pair of birds had been present in the experimental room at the time of sampling. The y axis displays cycle threshold (C_T) values of the RRT-PCR as a semiquantitative measure of excreted IAV. C_T values of ≥ 45 were determined to be negative. G1 represents mallard generation one of the experiment, consisting of two mallards, G2 represents the second pair of mallards introduced in the experimental room, etc. Values in the graph indicate mean C_T values from two samples, error bars indicate standard errors of the mean (SEM). For clarity, if one of the two samples of a generation from 1 day was IAV negative (C_T of ≥ 45), only the positive sample was included in the graph, i.e., G1, day 1; G2, day 5; G3, day 2; G4, day 3; G5, day 1 (see also Fig. 1).

Screening of the NCBI Influenza Virus Resource database. All avian N9 sequences available from the NCBI Influenza Virus Resource database (44) (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>) were downloaded on 15 February 2015 and aligned with BioEdit v7.2.5 software. Screening was done for amino acid 222 variant sequences.

Statistical analysis. The hypothesis of no difference in mean IC_{50} s of OC and ZA between wild-type virus and NA-I222T mutated virus was tested with a two-sample *t* test for independent samples with calculations of 95% confidence intervals (95% CI) for differences in means using Statistica v12 software (StatSoft).

RESULTS

Evolutionary relationships of H7 and N9 segments. The LPAI A(H7N9) virus used in this study was phylogenetically nested in a clade with European wild bird viruses with regard to the H7 segment, which was also reflected by placement of the N9 segment. This virus was distantly related to poultry H7 sequences, including the Chinese A(H7N9) 2013 outbreak strain (45) (see Fig. S1 in the supplemental material).

Analysis of OC concentrations in water. The analysis had a limit of quantification (LOQ) of 2.5 ng/liter and linearity (R^2) of 0.9999. The relative standard deviations (RSD) between triplicate samples were 0 to 11%. The average daily OC concentrations (samples taken after 24 h of experiment before the change of water) were 2.5 $\mu\text{g/liter}$ ($n = 11$, SD, 0.28 $\mu\text{g/liter}$), 7.2 $\mu\text{g/liter}$ ($n = 9$; SD, 0.79 $\mu\text{g/liter}$), and 24 $\mu\text{g/liter}$ ($n = 7$; SD, 1.44 $\mu\text{g/liter}$) (Fig. 1). OC concentrations were stable within 24 h, as the measured increments of 5% and 8% are within the variation of the analytical method.

IAV infection and NA amino acid substitution. IAV infection was established and perpetuated in the first five of seven generations of mallards (across 17 days), when OC exposure was 2.5 $\mu\text{g/liter}$ (8.8 nM) and 7.2 $\mu\text{g/liter}$ (25 nM), as seen by fecal excretion from day 1 or 2 postinoculation or introduction to the experimental room, through day 5 (Fig. 2). During generations six and seven, when OC exposure was increased to 24 $\mu\text{g/liter}$ (84 nM), no viral shedding was detected (Fig. 1). In water samples, IAV was detected during the first 18 days but not the last 5 days of the

experiment, consistent with the negative fecal samples. Sequencing of NA demonstrated the amino acid substitution I222T (ATA for isoleucine changed to ACA for threonine) in a small proportion of virus from one mallard in generation one 2 days postinoculation, while OC exposure was 2.5 $\mu\text{g/liter}$. The proportion of the I222T variant increased over 3 days, and from day 5 all subsequently sequenced viruses had the I222T variant until the end of IAV transmission (Fig. 1; see also Fig. S2 in the supplemental material). No other amino acid substitutions were found in NA during the experiment.

Sensitivity to OC and ZA. NA inhibition by OC and ZA, tested on 10 NA I222T samples and 3 wild-type samples, showed increased IC_{50} s of the NA I222T variant compared to those of the wild-type virus for both OC ($P = 0.000011$; 95% CI for difference in means, 2.5 to 4.6 nM) and ZA ($P = 0.016$; 95% CI for difference in means, 0.16 to 1.28 nM) (Table 1). No difference in inhibition was found between wild-type samples from the experiment and the original virus (OC: $P = 0.62$; 95% CI for difference in means, -0.37 to 0.48 nM; ZA: $P = 0.77$; 95% CI for difference in means, -1.12 to 0.96 nM) (Table 1), with IC_{50} s in the same range as those for other avian N9, human A(H7N9), and group N2 wild-type reference viruses (18).

Sequence database screening. Screening of all available 751 avian IAV N9 sequences in the NCBI Influenza Virus Resource database showed that none had threonine (T) and that all had isoleucine (I) and at amino acid residue 222.

DISCUSSION

In this OC exposure study, in which mallards were infected with an LPAI A(H7N9) that naturally circulates in wild ducks in Europe, the NA I222T substitution was detected after 2 days of 2.5 $\mu\text{g/liter}$ OC exposure in the first mallard generation passage and rapidly became the dominant genotype (Fig. 1; see also Fig. S2 in the supplemental material). Although there were no unexposed control mallards and infection and transmission were inhibited when drug pressure was increased, there are compelling reasons to believe that the OC exposure generated the change in the viral genotype. Drug-induced selection of the NA I222T substitution is supported by the absence of circulating avian N9 virus with the 222T genotype (based upon IAV sequence database screening), by previously observed NA I222R and NA I222K variants in A(H7N9) virus following oseltamivir treatment of humans (46), and by *in vivo* induction of NA I222T in A(H3N2) by low-dose OC exposure of infected mice (47).

TABLE 1 Neuraminidase inhibition by oseltamivir carboxylate and zanamivir

Virus	IC_{50} (nM) ^a	
	Oseltamivir carboxylate	Zanamivir
A/1621(H7N9) ^b ($n = 1$)	0.45 \pm 0.048	0.58 \pm 0.010
NA I222 ($n = 3$) ^c	0.51 \pm 0.12	0.50 \pm 0.24
NA I222T ($n = 10$) ^d	4.1 \pm 0.87	1.2 \pm 0.41

^a Drug concentrations that reduced viral NA activity by 50% (IC_{50}) in a fluorescence-based assay with the MUNANA substrate. Values are means \pm SD from 1, 3, and 10 duplicate sample analyses.

^b Virus used for inoculation of mallards.

^c Virus isolated from fecal samples during the experiment, with wild-type isoleucine (I) at NA residue 222.

^d Virus isolated from fecal samples during the experiment, with a threonine (T) substitution at NA residue 222.

At OC exposure levels of 2.5 and 7.2 $\mu\text{g}/\text{liter}$, the NA I222T mutant virus replicated, was excreted, and was transmitted similarly to wild type viruses measured in previous studies in this mallard model (31, 32, 39). However, when the OC exposure level was increased to 24 $\mu\text{g}/\text{liter}$ (84 nM), no viral shedding was detected, consistent with an OC concentration well above the median IC_{50} of 4.1 nM of the mutant. Interestingly, the 7.2 $\mu\text{g}/\text{liter}$ (25 nM) OC exposure was not sufficient to prevent replication and transmission of virus to two naive duck generations, which demonstrates that OC concentrations in environmental water cannot be directly translated to an antiviral effect at the site of infection in the mallard intestinal tract. As the IAV infection in waterfowl is intestinal and virus primarily invades apical epithelial cells (39), the virus is directly exposed to the active drug without a need for systemic uptake, which may increase drug exposure compared to that for oral prodrug administration and the deposition of active OC in airway tissue required in other species.

Our experiments did not indicate reduced fitness of the NA I222T mutant as excretion and transmission of virus were similar to previous observations with wild-type viruses (31, 32, 39), nor did propagation of the fecal samples without drug exposure in embryonated chicken eggs select for wild-type virus over I222T variants (including those with a mixed genotype). However, these measures are only proxies for viral fitness. Further studies with propagation of mutated virus in mallards without drug pressure and competitive infection studies with simultaneous passage of wild-type virus and mutated virus in unexposed hosts might better conclude if fitness of the mutant is indeed retained.

The active substrate binding site of NA is composed of 9 amino acids, supported by 10 framework residues, including I222 (48). NA I222T variants are described in human seasonal influenza B (49, 50), A(H3N2) (51), and A(H1N1)/pdm2009 (52) viruses, as well as in A(H5N1) virus (53), where the substitution was demonstrated to change the binding kinetics (loss of slow OC binding) (54). NA I222T and several other amino acid substitutions at the 222 NA residue generate reduced sensitivity to NAIs, either as independent resistance substitutions (55) or, perhaps of more concern, by enhancing resistance induced by H274Y in N1 virus (56, 57) or by E119V in N2 virus (58). Several substitutions also seem to restore reduced fitness: I222T/V/R combined with H274Y in N1 virus (56, 57) and I222V combined with E119V in N2 virus (59). Sequential evolution of permissive amino acids in a viral population over time, including at the NA 222 position, appears to facilitate acquisition and harboring of new resistance mutations, illustrated by the OC-resistant human seasonal A(H1N1)/H274Y virus that circulated in 2007 to 2009 (60).

Changes in inhibition by NAIs caused by NA I222T in our study, 8-fold and 2.4-fold increases in IC_{50} s by OC and ZA, respectively (Table 1), are similar to previously described levels for the substitution in other viral subtypes (49, 50, 52, 54, 61, 62). The level of change in IC_{50} does not meet the criteria for reduced inhibition according to the WHO phenotype criteria for resistance surveillance (>10-fold increase in IC_{50} compared to that for reference strains) (63). However, the results demonstrate that an OC-exposed avian N9 virus, in its natural host, can acquire a mutation that influences NAI sensitivity and that can modulate NAI-resistant viruses (53, 56). Environmental OC concentrations of 0.86 $\mu\text{g}/\text{liter}$ detected to date (25) are below the experimental concentrations at which resistance has evolved in IAVs in mallards, both in previous studies (31–33) and in this one. However,

the concentrations are on the same order of magnitude, environmental levels may occasionally be higher than thus far detected, particularly during an epidemic or pandemic peak, and different avian IAVs acquire resistance mutations at different exposure levels. Additionally, in the present study, as 2.5 $\mu\text{g}/\text{liter}$ was the lowest level of OC exposure, we cannot exclude the possibility that the I222T substitution could have been acquired at a lower OC concentration. Taking our results together, we believe there are good reasons not to ignore environmental OC as a risk factor for resistance development in avian IAVs of wild birds. Indeed, there are no indications that NAI resistance *per se* contributes to viral crossing of the avian to human species barriers. Rather, the reason for concern regarding NAI resistance in wild bird IAVs is the great genetic variability, which provides potential for a genetic context allowing emergence and persistence of resistance mutations, possibly also through a species-crossing evolutionary process, should such occur, to a novel human-pathogenic virus. Compared to NAI resistance evolving in treated humans, where the clinical outcome of individuals may be affected, a novel inherently resistant human IAV would change a pandemic scenario entirely and disarm preparedness plans.

In conclusion, the acquisition of I222T in a European wild duck LPAI A(H7N9) virus demonstrates that N9 viruses can harbor I222T substitutions and confirms the levels of reduced NAI sensitivity in other NA subtypes. Retained transmission and replication of the I222T mutant virus in our mallard model indicates that it might be sustained in wild migratory waterfowl. Although the LPAI A(H7N9) we used in our study is not related to the Chinese A(H7N9) outbreak strains, our findings demonstrate that a mutation which influences NAI sensitivity and can compensate for reduced fitness caused by other resistance mutations can occur among wild bird hosts in an IAV subtype that is potentially human pathogenic. To better understand the emergence and persistence of resistant IAVs, it is important to further study possible transmission of avian IAVs containing resistance-related mutations from wild birds to poultry, humans, and other mammals. As resistance mutation(s) in an A(H7N9) virus or in another novel human-pathogenic IAV with pandemic potential poses a public health threat, our results stress the need for prudent antiviral use and better sewage treatment as preventive measures, as well as resistance surveillance of IAVs in wild birds.

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