NOTE *Virology*

In Vitro Efficacies of Oseltamivir Carboxylate and Zanamivir against Equine Influenza A Viruses

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ABSTRACT. To investigate the possibilities of two NA inhibitors [oseltamivir carboxylate (OC) and zanamivir (ZA)] as the clinical agents for equine nifluenza A virus (EIV) infection, we examined the efficacies of these inhibitors against twelve EIVs *in vitro*. OC and ZA inhibited NA activities of all EIVs with 50% inhibitory concentrations with ranging from 0.017 to 0.130 and from 0.010 to 0.074 μM, respectively. OC and ZA inhibited plaque-forming of all EIVs in MDCK cells with 50% effective concentrations with ranging from 0.015 to 0.097 and from 0.016 to 0.089 μM, respectively, except for one strain (13.328 μM and 6.729 μM). These results suggest that these inhibitors are effective against most EIVs and might be useful for treatment of EI in horses.

KEY WORDS: equine, equine influenza A virus, neuraminidase inhibitor.

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Equine influenza A virus (EIV), a member of *Orthomyxoviridae*, is considered to be one of the most important pathogens of horses. EIV causes a severe respiratory infection characterized by a harsh cough, nasal discharge and pyrexia [13]. There are 2 subtypes of EIV: A/Equi 1/H7N7, first isolated in 1956, and A/Equi 2/H3N8, first isolated in 1963[13]. However, H7N7 has not been isolated since 1979 [13]. In contrast, H3N8 continues to circulate among horse populations worldwide with evolving into two genetically and antigenetically distinct "American" and "Eurasian" lineages after 1989 [7, 8, 10].

Influenza viruses have two major surface glycoproteins, viral hemaglutinin (HA) and neuraminidase (NA). The function of HA is to recognize and bind to the neuraminic acid-containing receptors, and the NA cleaves terminal neuraminic acid residues from adjacent oligosaccharide chains [6, 14]. In absence of functional NA, virus release is inhibited; virions are formed but remain attached to the cell surface and to each other, forming large aggregates on the surfaces of infected cells [6, 14].

Recently, NA inhibitors, oseltamivir phosphate (OP) and zanamivir (ZA) were introduced into human medicine worldwide [6]. OP is a bioavailable prodrug. After oral administration of OP, it is hydrolyzed to its active metabolite [oseltamivir carboxylate (OC)] by hepatic esterases [6]. ZA is applied topically to respiratory tract as an inhaled preparation because of its low oral bioavailability [6]. These compounds are the analogs of neuraminic acid and block the active site of influenza virus NA [6, 14]. It has been reported that the NA possesses an enzyme active site whose amino acid sequence was conserved among most NA subtypes, including EIVs [2, 11]. Moreover, it has been demonstrated that these compounds are effective against all nine NA subtypes of avian influenza A virus *in vitro* [4]. Therefore, we speculated that these compounds might have inhibitory efficacies to EIVs, and be valuable for treatment of horses infected with EIV. To confirm our speculation, it is necessary to observe the inhibitory efficacies of these compounds *in vivo* using horses. But, since the horse experiments need enough fund and labor, it is difficult to test many strains *in vivo*. In this study, therefore, we evaluated inhibitory efficacies of OC and ZA for several EIV strains *in vitro* ahead of *in vivo* studies.

OC was gained by hydrolysis of OP obtained from Tamiflu® capsules 75 (Chugai Pharmaceutical, Japan) by the modification of lithium hydroxide/tetrahydrofran method as follows [3]. The contents (net 16.2 g) removed from Tamiflu® capsules 75 were soaked in 50% methanol and sonicated. After filtration, filtrate was evaporated and lyophilized. In consequence, 10.0 g of OP was gained. 1.0 g of OP solved in 70 m*l* of methanol/tetrahydrofran (1:3 v/v) was incubated with aqueous lithium hydroxide solution (25.6 mmol/17.6 m*l*) at 50°C for 4 hr. The mixture was neutralized by the addition of 10% hydrochloric acid. The neutralized hydrolyzate was purified by HPLC. The fractions of OC were evaporated and lyophilized. The gained OC was characterized by the ^{1}H-nuclear magnetic resonance spectrometry and liquid chromatography/mass data. Relenza® (GlaxoSmithKline, Japan) powder was used as ZA in this study.

Twelve EIVs containing one H7N7 and eleven H3N8 strains were used in this study (Table 1). All viruses were propagated in allantoic cavities of embryonated hen's eggs. To evaluate the inhibitory efficacies of OC and ZA against the NAs of EIVs, NA inhibition assays were performed by colorimetric method as previously described with some modifications [1]. Viruses were diluted to give a standard level of enzymatic activity from 0.4 to 0.8 optical density unit at 550 nm (OD_{550}) by the spectrophotometer. Tenfold dilutions of test compounds ranging from 200 to 0.0002 μM were incubated with equal volume of diluted viruses for 30 min at 37°C and then with fetuin overnight. The cleaved product of fetuin was reacted with thiobarbituric acid for 15 min at 100°C. The chromophore was extracted by 1-

Table 1. Profiles of equine influenza A viruses used in this study

Virus	Subtype	Country of Origin	Lineage[a)]
A/Equi 1/Newmarket/77	H7N7	United Kingdom	–
A/Equi 2/Miami/63	H3N8	United States	–[b)]
A/Equi 2/Cavrot/87	H3N8	Holland	–[b)]
A/Equi 2/Suffolk/89	H3N8	United Kingdom	Eurasian
A/Equi 2/Taby/91	H3N8	Sweden	Eurasian
A/Equi 2/Italy/5/91	H3N8	Italy	Eurasian
A/Equi 2/Hong Kong/92	H3N8	Hong Kong	Eurasian
A/Equi 2/Avesta/93	H3N8	Sweden	Eurasian
A/Equi 2/La Plata/93	H3N8	Argentine	American
A/Equi 2/Kentucky/94	H3N8	United States	American
A/Equi 2/La Plata/95	H3N8	Argentine	American
A/Equi 2/La Plata/96	H3N8	Argentine	American

a) The lineages are based on the previous reports [7, 8, 10]. b): These strains are isolated before evolving into the two lineages.

Table 2. 50% inhibitory concentration (IC_{50}) and 50% effective concentration (EC_{50}) of oseltamivir carboxylate and zanamivir for equine influenza A viruses

Virus	IC_{50} (μM)[a)]		EC_{50} (μM)[b)]	
	Oseltamivir carboxylate	Znamivir	Oseltamivir carboxylate	Znamivir
A/Equi 1/Newmarket/77	0.017 ± 0.012	0.071 ± 0.022	0.015 ± 0.002	0.016 ± 0.005
A/Equi 2/Miami/63	0.112 ± 0.056	0.074 ± 0.017	0.057 ± 0.017	0.073 ± 0.029
A/Equi 2/Cavrot/87	0.034 ± 0.017	0.048 ± 0.034	0.087 ± 0.029	0.030 ± 0.007
A/Equi 2/Suffolk/89	0.077 ± 0.031	0.069 ± 0.020	0.025 ± 0.005	0.020 ± 0.000
A/Equi 2/Taby/91	0.076 ± 0.020	0.048 ± 0.017	0.097 ± 0.026	0.076 ± 0.029
A/Equi 2/Italy/5/91	0.031 ± 0.002	0.058 ± 0.047	13.328 ± 2.568	6.729 ± 2.218
A/Equi 2/Hong Kong/92	0.130 ± 0.013	0.051 ± 0.022	ND[c)]	
A/Equi 2/Avesta/93	0.032 ± 0.012	0.039 ± 0.021	0.052 ± 0.024	0.089 ± 0.009
A/Equi 2/La Plata/93	0.031 ± 0.002	0.010 ± 0.002	0.073 ± 0.029	0.019 ± 0.007
A/Equi 2/Kentucky/94	0.047 ± 0.015	0.046 ± 0.001	ND[c)]	
A/Equi 2/La Plata/95	0.081 ± 0.030	0.049 ± 0.036	0.044 ± 0.001	0.057 ± 0.017
A/Equi 2/La Plata/96	0.084 ± 0.011	0.019 ± 0.006	0.051 ± 0.013	0.082 ± 0.022

a) and b) were measured by neuraminidase inhibition assay and plaque reduction assay, respectively. All values are represented as mean ± standard deviation of three independent determinations. c): Not done.

butanol. OD_{550} of the extracted phase was measured by the spectrophotometer. The percent inhibition of NA activity relative to controls (NA activity in the absence of test compound) was calculated for each concentration of the test compound. The concentrations of test compound which reduced OD_{550} by 50% (50% inhibitory concentration, IC_{50}) were determined by using the SigmaPlot 9.0 software (SYSTAT, CA, U.S.A.).

The NA activities of all EIVs showed the susceptibilities to OC and ZA with the IC_{50}s ranging from 0.017 to 0.130 μM and from 0.010 to 0.074 μM, respectively (Table 2). Ten-fold or more remarkable difference in IC_{50} values was not observed between the compounds to each strain and among EIV strains to each compound, suggesting that the NAs of EIVs would have almost similar susceptibilities to OC and ZA, regardless of subtypes and lineages. The efficacies of these compounds to several human and avian influenza A viruses measured by NA inhibition assay (IC_{50}) have been documented [4, 9]. According to the previous report presenting the IC_{50}s for several human influenza A viruses (H1N1 and H3N2) determined by fluorometric method, the IC_{50}s of OC and ZA were ranged from 0.0003 to 0.001 μM and from 0.0003 to 0.0046 μM, respectively [9]. In regard to avian influenza A viruses of all NA subtypes (N1 to N9), the ranges of the IC_{50} values of OC and ZA measured by fluorometric method were from 0.0019 to 0.0692 μM and from 0.0022 to 0.0301 μM, respectively [4]. Although the correlation between the IC_{50}s determined by fluorometric and colorimetric methods is not established, the IC_{50}s of both compounds for EIVs used in this study are consistently higher than those for human influenza A viruses, but were comparable to those for avian influenza A viruses. Therefore, the NAs of EIVs might be less susceptible to OC and ZA than those of human influenza A viruses (H1N1 and H3N2), while being susceptible comparably to those for avian influenza A viruses.

To evaluate the effective efficacies of OC and ZA against the replications of EIVs, plaque reduction assays (PRAs) were performed as described previously with some modifications [15]. Monolayers of MDCK cells in 6-well culture plate were inoculated with EIV diluted in Eagle's minimal essential medium (EMEM) containing 2.0 μg/m*l* of acety-

lated trypsin to give 50 to 100 plaques per well. After 1 hr adsorption at room temperature, cells were covered with the overlay-medium (0.9% agar-EMEM) containing each concentration of the test compound. The final concentrations of test compound in overlay ranged in fourfold dilutions from 100 to 0.0015 μM. The plates were placed in a 37°C CO_2 incubator for three to 4 days. The percent reduction of plaque formation relative to control (plaques formed in the absence of test compound) was calculated for each concentration of the test compound. The concentrations of test compound that reduced the number of plaques in the PRA by 50% (50% effective concentration, EC_{50}) were determined by using SigmaPlot 9.0 software.

The EC_{50}s of OC and ZA for the EIVs examined in this study are presented in Table 2. Since A/Equi 2/Hong Kong/92 and A/Equi 2/Kentucky/94 did not form plaque by our method, these viruses were not examined in this assay. Except for A/Equi 2/Italy/5/91 (Italy91), the EC_{50}s of OC and ZA for the EIVs examined were ranged from 0.015 to 0.097 μM and from 0.016 to 0.089 μM, respectively. The EC_{50}s of OC and ZA for Italy91 were 13.328 μM and 6.729 μM, respectively, which were at least 75 times higher than those for the other viruses. No remarkable difference in EC_{50} values was observed between the compounds to each strain including Italy91 and among the EIVs except Italy91 to each compound. These data suggested that the majority of EIVs would have almost similar susceptibilities to OC and ZA in plaque formation. But, Italy91 seems to be less susceptible to OC and ZA than the other EIVs. As to human influenza A viruses (H1N1 and H3N2), it was reported that the ranges of EC_{50}s of OC and ZA measured by PRA were from 0.0006 to 0.094 μM and from 0.019 to 0.241 μM, respectively [9]. Since the EC_{50}s of OC and ZA for EIVs except Italy91 in this study were comparable to those for human influenza A viruses, it is suggested that the inhibitory efficacies of both compounds for the replication of EIVs and human influenza A viruses in MDCK cells would be approximately similar. On the other hand, the EC_{50}s of OC and ZA measured by enzyme linked immunosorbent assay (ELISA) using MDCK cells for avian influenza A viruses including all NA subtypes (N1 to N9), are ranged from 1.0 to 42.0 μM and from 4.0 to 58.3 μM, respectively [4]. Since the EC_{50}s of both compounds for avian influenza A viruses were 10 times higher or more than those for EIVs except Italy91, these compounds might be more efficacious to inhibit the replications of EIVs than that of avian influenza A viruses although the correlation between the EC_{50}s determined by PRA and ELISA has not been established.

Interestingly, Italy91 showed the unique relationship between the IC_{50} and EC_{50} in this study. Although the IC_{50}s of OC and ZA for Italy91 were almost similar to those for the other EIVs examined in this study, the EC_{50}s of both compounds for the virus were at least 75 times higher than those for the other EIVs. This phenomenon was observed in some human influenza A viruses [5, 6, 12, 15]. Studies on human strains with reduced susceptibility to ZA in PRA showed that the resistance could be associated with mutations in the HA gene, and that a weaker affinity of the HA for the cellular receptors by HA mutations would allow release of virus from infected cells even in the presence of a NA inhibitor [5, 6, 12]. To clarify the reason for the phenomenon observed in Italy91, further investigation on the HA gene of Italy91 would be necessary. However, since the human strains with reduced susceptibility to ZA in cell culture were shown to be sensitive to ZA *in viv*o [12,15], it is difficult to conclude that Italy91 is less susceptible to OC or ZA *in vivo* than the other EIVs from this limited study.

In summary, since our data suggest that OC and ZA are the broad spectrum inhibitors of the NA activities and viral replications of EIVs *in vitro*, OP and ZA might have the potentiality of clinical efficacies for EIV infection. However, since ZA has to be topically delivered to equine respiratory tract, veterinarians would be required to synchronize the period of administration with the horse's inspiration. This would limit the usefulness of ZA for veterinarians. Therefore, further studies to clarify the efficacy of orally administered OP in experimentally infected horses with EIVs are on the way.

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