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Oseltamivir boosts 2009 H1N1 virus infectivity *in vitro*

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ABSTRACT

The neuraminidase inhibitor oseltamivir has been identified to have significant anti-influenza activity in clinical practice. However, its efficacy has not been verified in enough subtypes of influenza A virus, particularly, the current pandemic virus, H1N1. *In vitro*, using our influenza pseudotyped particle system, oseltamivir displayed significant inhibitory effects on viral NA activity and pp release. Conversely, a boosting effect on viral infection was observed, particularly with the 2009 H1N1 pp at oseltamivir concentrations above 0.025 μM. Further testing on two wild 2009 H1N1 virus strains, A/California/07/09 and A/Sichuan/1/09, as well as a seasonal flu virus, A/Baoan/51/2008, confirmed these findings.

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Introduction

The surface proteins of influenza viruses, HA and NA, play important roles in virulence, host specificity, and human immune response. Influenza A viruses are believed to enter host cells via HA binding to sialic acid receptors on the cell surface [1]. Human viruses known to be capable of tropism bind preferentially to sialic acid linked to galactose via α-2,6 linkages, while avian viruses of this type tend to bind to α-2,3 linkages [2,3]. In particular, the 2009 H1N1 virus prefers both α-2,6 and α-2,3 linked sialic acids [4]. NA is believed to remove sialic acid from sialyloligosaccharides to aid viral release and spread [1,5,6], NA inhibitor oseltamivir is designed to inhibit this function and has been lead to the expected effects with some influenza viruses [7–9].

While, as a NA inhibitor, oseltamivir could play contradictory roles in the influenza virus life cycle; that is, when NA is inhibited by oseltamivir, viral release is inhibited, but more receptors in target cells may be provided to the HA of the viruses already released. In this study, we evaluated the role of oseltamivir in both the early infection stage and release event using our newly established influenza virus pseudotyped particle system (pp) and wild virus strains. Our study advances our knowledge of oseltamivir and urges us to consider the possible consequences related to its effect on viral infection in clinical practice.

Materials and methods

Cells and NA inhibitor oseltamivir. MDCK cells, human alveolar epithelial type II cell line A549, and human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified essential medium (DMEM; Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum. Oseltamivir carboxylate [(3R,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid] was provided by Hoffmann-La Roche.

Establishment of influenza virus pp system and infection assays. cDNA fragments encoding the full-length HA and NA of 2009 H1N1, 1918H1N1, H5N1 strains were synthesized and inserted into the pVRC vector, as reported previously [10]. The pps were produced by transfecting 293T cells with four plasmids: a HA expression plasmid, NA expression plasmid, Gag-pol-encoding plasmid, and reporter plasmid. At 72 h post-transfection, pps were harvested from the supernatants of the transfected cells by filtration through a 45-μm Durapore PVDF membrane filter (Millipore, Cork, Ireland).

The infection assay was performed as reported previously [10]. Briefly, A549 cells ($5–10 \times 10^3$) were seeded in a 96-well plate 1 day prior to infection. The medium was replaced with 100 μL of 1:1 diluted pps, followed by 4 h inoculation, and then replaced with DMEM supplemented with 3% FBS. At 72 h post-infection, the number of GFP reporter-positive cells was calculated using FACS (BD FACSAria, BD Biosciences, Franklin Lakes, NJ). For the infection assay in the presence of oseltamivir, according to clinical dosage [7–9], 10-fold serial dilutions of oseltamivir in PBS were mixed 1:1 with pps (50 μL pp and 50 μL diluted oseltamivir) to final concentrations of 0.00025–2.5 μM. PBS was mixed 1:1 with pps as a control.

Abbreviations: HPAI A (H5N1), highly pathogenic avian influenza virus of type A; HA, hemagglutinin; NA, neuraminidase; pp, pseudotyped particle.

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Release assay of pps in the presence of oseltamivir. The effect of oseltamivir on the pp release process was assessed at 24 h after transfection. The culture medium for 293T cells was replaced with media containing serially diluted oseltamivir (final concentrations, 0.00025–2.5 μM; 0 μM as a control) and cultured for 48 h. The pps were harvested and subjected to the infection assay.

NA activity and hemagglutination assays. The NA activity of pps treated with oseltamivir was measured using the NA-Star Influenza Neuraminidase Inhibitor Resistance Detection kit (Applied Biosystems, Foster City, CA). All reagents were prepared according to the manufacturer's instructions. For each sample, 25 μL of pps was incubated 1:1 with diluted oseltamivir (same final concentrations as in the infection assay) at 37 °C for 1 h and then incubated with 10 μL of NA-Star substrate for 30 min at room temperature (RT). The samples were then analyzed using a luminometer (2103 Envision Multilabel Reader, PerkinElmer, Waltham, MA).

Hemagglutination assays were also performed. The virus samples were diluted serially in PBS (twofold from 1 to 1/1024) in 96-well plates with 50 μL per well. Next, 50 μL aliquots of 1% turkey red blood cells were added to each well and left to stand for 30 min; hemagglutination was then scored.

Preparation of viruses. All aspects of the study were performed in accordance with the national ethics regulations and approved by Ethics Committee of CDC, China. The 2009 H1N1 virus A/Sichun/1/09 was isolated from the first confirmed case in China, a 25-year-old male patient with uncomplicated, upper respiratory tract illness. The A/California/07/09 viral stock was provided by the US CDC. The two 2009 H1N1 viruses were compared to a representative seasonal flu virus, A/Baoan/51/2008, of similar antigenic characteristics to A/Brisbane/59/2007. The viruses were grown in chorioallantoic fluid of 10-day-old embryonated chicken eggs and purified by sucrose gradient centrifugation. Virus particles were prepared in PBS, aliquoted, and stored at −80 °C until use. The viral titer was determined by titration in MDCK cells and the tissue culture infectious dose affecting 50% of the cells (TCID50) was calculated using the Reed-Muench formula. All experiments with 2009 H1N1 were performed in approved enhanced biosafety level 3 (BSL-3) containment laboratories.

Cell-based viral entry assay in a single infection cycle. Confluent A549 cells (5×10^4) were infected with the viruses at a multiplicity of infection (MOI) of 0.5 in the infection medium containing 2 mg/L TPCK-trypsin and oseltamivir at final concentrations of 0–250 μM, and then incubated for 1 h at 35 °C. Cells were then washed and cultured in infection medium without TPCK-trypsin to avoid re-entry of virus. Mock-treated cells were treated similarly in parallel except that virus was not added. At 8 h point of infection (p.o.i), the cells were harvested and permeabilized. Viral infectivity was measured by anti-influenza A NP staining (1:400; US CDC) for 30 min at RT. Unbound antibody was removed by washing three times before the addition of goat anti-mouse IgG Fc-FITC (1:100) for 30 min at RT, followed by FACS detection.

Cell-based virus reduction assay in multiple infection cycles. Confluent A549 cells (3×10^4) were infected with the viruses at MOI of 0.01 for 1 h at 35 °C and then washed and overlaid with infection medium containing TPCK-trypsin and 10-fold serial dilutions of oseltamivir (0–250 μM). Virus replication was determined by measuring the HA activity after 72 h of incubation at 35 °C.

Results

Because oseltamivir is used widely as a NA inhibitor, it might impact pp infectivity by inhibiting NA activity. For this purpose, we established an influenza pseudotyped particle (pp) system carrying HA and NA only, with a GFP reporter gene [10], which can mimic the process of viral entry to allow us to clarify what happened in the presence of oseltamivir during viral infection. Serial dilutions of oseltamivir were applied to the pps infection assay (two H1N1 strains and a highly pathogenic avian influenza virus H5N1, named H1N1-2009pp, H1N1-1918pp, and H5N1pp, respectively). H1N1-2009pp, H1N1-1918pp, and H5N1pp displayed various infectivity profiles with increasing concentrations of oseltamivir. In H1N1-2009pp, infectivity jumped from 2.5 ± 0.8% at 0.0025 μM to 18.7 ± 6.5% at 0.025 μM and even 31.3 ± 12.5% at 0.25 μM, whereas H1N1-1918pp and H5N1pp displayed gradually enhanced infectivity with increasing oseltamivir (0–2.5 μM) (Fig. 1A). Our data indicated that oseltamivir boosted pp entry and supported our hypothesis. The differential enhancements among pps may be partially explained by their sialic acid binding preference of HAs and cleavage preference of NAs [1,4,11,12]. To confirm the inhibitory effect of oseltamivir on viral NA activity, all pps treated by serial oseltamivir concentrations were subjected to the NA activity assay in parallel. The 2009 H1N1pp, 1918 H1N1pp, and H5N1pp were all sensitive to oseltamivir, and their respective NA activity was significantly inhibited at 0.00025 μM and almost eliminated at 0.025 μM or higher; although the NA activity of H1N1-2009pp was almost 300-fold greater than that

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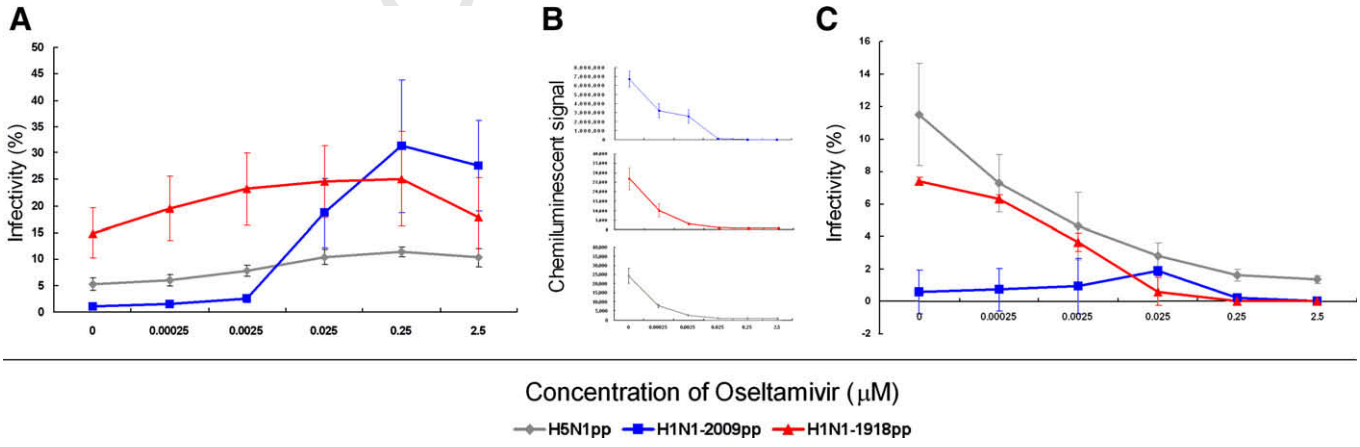

Fig. 1. Osetamivir boosts pp infection and suppresses pp release. (A) Infectivity of pps in the presence of serially diluted oseltamivir. Infectivity represents the pp infected cells. Gray represents H5N1pp; blue, H1N1-2009pp; red, H1N1-1918pp. (B) NA activity of pps treated with serially diluted oseltamivir. CS was used to quantify NA activity. Upper: H1N1-2009pp NA activity (blue); middle: H1N1-1918pp NA activity (red); bottom: H5N1pp NA activity (gray). (C) Osetamivir suppresses pp release. Infectivity was used to indicate the quantity of pps released from producer 293T cells treated with serially diluted oseltamivir. All data are presented as means ± SEM from three sets of experiments. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

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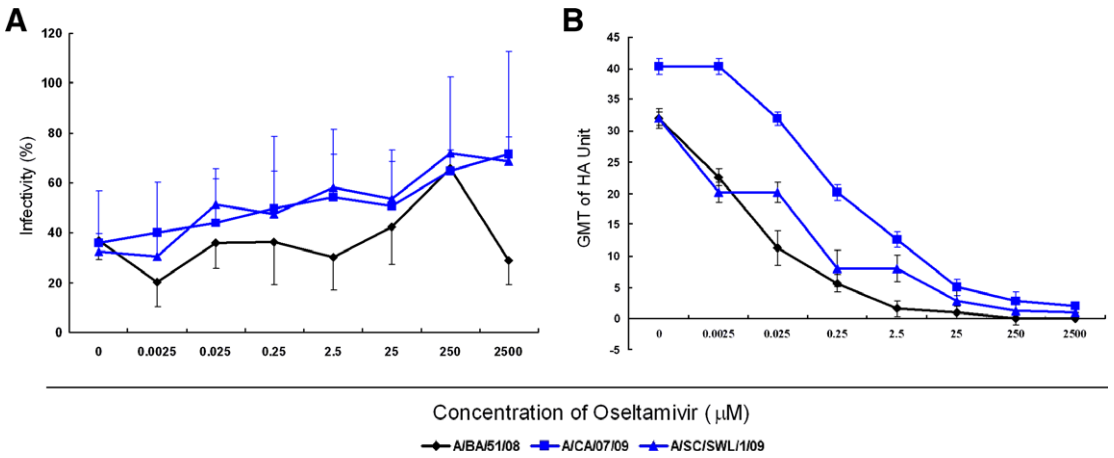

Fig. 2. Oseltamivir enhances viral infection and inhibits viral release. (A) Infectivity of wild influenza A viruses (MOI 0.5) in one single infection cycle in the presence of serially diluted oseltamivir. Blue represents the two strains of 2009 H1N1, gray represents a seasonal influenza A virus. The data are presented as means ± SEM from four sets of experiments. (B) Viral release inhibited by oseltamivir in multiple infection cycles. The data are presented as the geometric mean titers (GMT) ± SEM of viral titer tested by hemagglutination assay from three sets of experiments. HAU, hemagglutinin unit. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

of H1N1-1918pp and H5N1pp (Fig. 1B). Of H1N1-2009pp, although the NA activity was significantly inhibited, at 0.025 μM, its activity still remained at 86,106.6 ± 33,248.7 chemiluminescent signal (CS) which was much higher than that of 1918 H1N1pp (26,960.0 ± 5774.3CS) and H5N1pp (24,266.7 ± 4176.6CS) at absence of oseltamivir, these data could also explain the extraordinary enhancement of oseltamivir on H1N1-2009pp entry, which kept increasing to 0.25 μM oseltamivir.

Next, we confirmed the theoretical effect on viral release in our pp system. Serially diluted oseltamivir was applied in the medium of pp producer 293T cells, because the inhibition of pp release may be reflected by the infection assay. As depicted in Fig. 1C, the infectivity of H1N1-2009pp, H1N1-1918pp, and H5N1pp decreased significantly as the concentration of oseltamivir increased. The presence of 0.25 μM oseltamivir thoroughly inhibited the release of H1N1-2009pp and H1N1-1918pp. For 2009 H1N1pp, the infectivity increased slightly at oseltamivir concentrations from 0 to 0.025 μM, but remarkably, was eliminated at 0.25 μM; this may be due to remaining oseltamivir in the supernatant.

To investigate whether oseltamivir can interrupt viral early infection stages and to confirm the observations from the pp system, we mimicked one cycle of viral infection at MOI of 0.5 and any changes in infectivity were measured by viral NP protein expression at 8 h p.o.i. The wild viruses were pre-incubated with serially diluted oseltamivir, as in the pp assays, and inoculated to A549 cells for 1 h. The infectivity of all A/BA/51/08, A/SC/1/09 and A/CA/07/09 viruses displayed an increasing tendency with increasing concentrations of oseltamivir. Among these, the infectivity of two 2009 representative wild strains, A/SC/1/09 and A/CA/07/09, increased from 32.25 ± 7.65% and 35.97 ± 20.76% at 0 μM to 58.03 ± 13.43% and 54.30 ± 27.15%, respectively, at 2.5 μM and this tendency remained at oseltamivir concentrations as high as 2500 μM, indicating that oseltamivir also boosted the entry process of wild viruses, consistent with the observations in our in pp system (Fig. 2A).

To assess whether virus release could be inhibited by oseltamivir, a low virus-load infection in multiple infection cycles was performed and the hemagglutination assay was used to measure virus titers. Significant inhibition effects were observed in all virus strains with increasing concentrations of oseltamivir. Among these, the viral titer of two 2009 representative wild strains, A/SC/1/09 and A/CA/07/09, was reduced to around 2 haemagglutinin unit/50 μL at 25 μM, indicating the effect of oseltamivir on the current pandemic H1N1 virus (Fig. 2B).

Discussion

Accumulating clinical evidence indicates that some severely ill patients might even deteriorate if treated with oseltamivir [13,14] and drug-resistant variants were thought to be responsible for this [13]. In this study, none of the strains represented by pps and the wild viruses possessed H274Y or other mutations [15]. If we recheck current clinical cases, deterioration after oseltamivir treatment may possibly be partially due to this viral infection enhancement effect in individuals with high viral load, thus, it is urgent to evaluate this possibility and distinguish resistance from the enhancement effect of oseltamivir.

Our study advised us the anti-viral profile of oseltamivir with different subtypes of influenza viruses should be evaluated comprehensively prior to its administration to treat a certain virus strain and it is important to evaluate the anti-viral effect in all processes of the viral life cycle especially for drugs like NA inhibitors.

Conclusion

This study has advanced our knowledge of oseltamivir and urges consideration of the possible consequences related to its effect on virus infection during the use of oseltamivir in clinical practice.

Conflict of interest

The authors have no conflict of interests to declare.

Acknowledgments

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