

Antiviral activity of novel oseltamivir derivatives against some influenza virus strains

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The aim of this study was to investigate the *in vitro* cytotoxicity of oseltamivir derivatives and determine their activity against A/H1N1/PR/8/34 and A/H3N2/Hong-Kong/8/68 — strains of influenza virus. Antiviral activity of these compounds was determined by using two methods. MTT staining was used to assess the viability of MDCK cells infected with influenza viruses and treated with various concentrations of drugs. In parallel, the effect of drugs on viral replication was assessed using the hemagglutination test. The most toxic compounds were: OS-64, OS-35, OS-29, OS-27 and OS-25, whereas OS-11, OS-20 and OS-23 were the least toxic ones. Statistically significant antiviral effect at a higher virus dose was shown by compounds: OS-11, OS-20, OS-27, OS-35, and OS-64. H3N2 virus was sensitive to 10-times lower concentrations of OS-11 and OS-35 than H1N1. At a lower infection dose, the antiviral activity was observed for OS-11, OS 27, OS-35 and OS-20. OS-64 turned out to be effective only at a high concentration. OS-23 showed no antiviral effect.

Key words: influenza virus, neuraminidase inhibitors, oseltamivir, antiviral activity, cytotoxicity

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INTRODUCTION

According to WHO, every year 5–10% of adults and 20–30% of children fall ill with flu, 3–5 million patients require hospitalization, and 250000–500000 die (<http://www.who.int/mediacentre/factsheets/fs211/en/>). The large variability of the virus makes the disease difficult to control and eradicate. Frequent point mutations (antigenic drift) and the exchanges of viral RNA segments between strains (antigenic shift) favour the emergence of new variants, potentially threatening to people. For example, the avian influenza H5N1 virus, which has been detected in humans since 1997, displayed an extremely high virulence (http://www.who.int/mediacentre/factsheets/avian_influenza/en/). Then, in 2009, a new pandemic variant H1N1 managed to spread across almost the entire globe in just a few weeks. The primary way to protect people against the disease is vaccination, but the variability of the virus impedes development of a vaccine with long-lasting protection and makes annual changing of vaccine composition necessary (Barnett *et al.*, 2000; Berhane *et al.*, 2012; Mehrbod *et al.*, 2009). De-

velopment of a vaccine for a new virus strain takes time, so the key to minimizing effects of a potential pandemic may be antiviral drugs (Hedlund *et al.*, 2010; Kwiatek *et al.*, 2009). The available anti-flu drugs are divided into two groups: M2 ion channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (zanamivir, oseltamivir, registered in Japan laninamivir and still tested peramivir). These substances are not free of drawbacks: they provoke side effects or their therapeutic effectiveness is undermined (Ng *et al.*, 2010). Importantly, the emergence of drug-resistant strains is frequently reported (Saito *et al.*, 2003; Sheu *et al.*, 2008; Nguyen *et al.*, 2010; Ghedin *et al.*, 2012; Ng *et al.*, 2010).

It has been shown that seasonal H1N1 strain and H3N2 strain currently circulating in the population are mostly resistant to oseltamivir (Sheu *et al.*, 2008; Okomo-Adhiambo *et al.*, 2010; Hurt *et al.*, 2009a). Among the pandemic H1N1 (2009) isolates there were also identified those having a mutation H274Y in the neuraminidase gene, conditioning resistance to this drug (Payungporn *et al.*, 2011; Okomo-Adhiambo *et al.*, 2010; Hurt *et al.*, 2009b; Meijer *et al.*, 2012). Reports of oseltamivir-resistant H5N1 virus isolates (de Jong *et al.*, 2005; Earhart *et al.*, 2009; Triana-Baltzer *et al.*, 2009; Hayden *et al.*, 2005) are particularly disturbing. These facts encourage searching for new compounds of similar molecular structure, which would be active against influenza strains currently circulating in the population and the newly emerging ones (Hurt *et al.*, 2009a; Ghedin *et al.*, 2012). The extension of the pool of available antiviral drugs seems to be a priority in controlling influenza infections (Hayden, 2009).

AIM

The aim of this study was to evaluate the *in vitro* cytotoxicity and antiviral activity of eight compounds derived from oseltamivir against two strains of type A influenza

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Abbreviations: DMEM, Dulbecco modified Eagle's minimal essential medium; DMF, dimethylformamide; EC₅₀, half maximal effective concentration; FBS, fetal bovine serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC₅₀, half maximal inhibitory concentration; MIHE, Military Institute of Hygiene and Epidemiology, Warsaw, Poland; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PRI, Pharmaceutical Research Institute, Warsaw, Poland; SDS, sodium dodecyl sulfate; TCID₅₀, 50% tissue culture infective dose; TI, therapeutic index; TPCK-treated trypsin, trypsin modified by L-(tosylamido-2-phenyl) ethyl chloromethyl ketone.

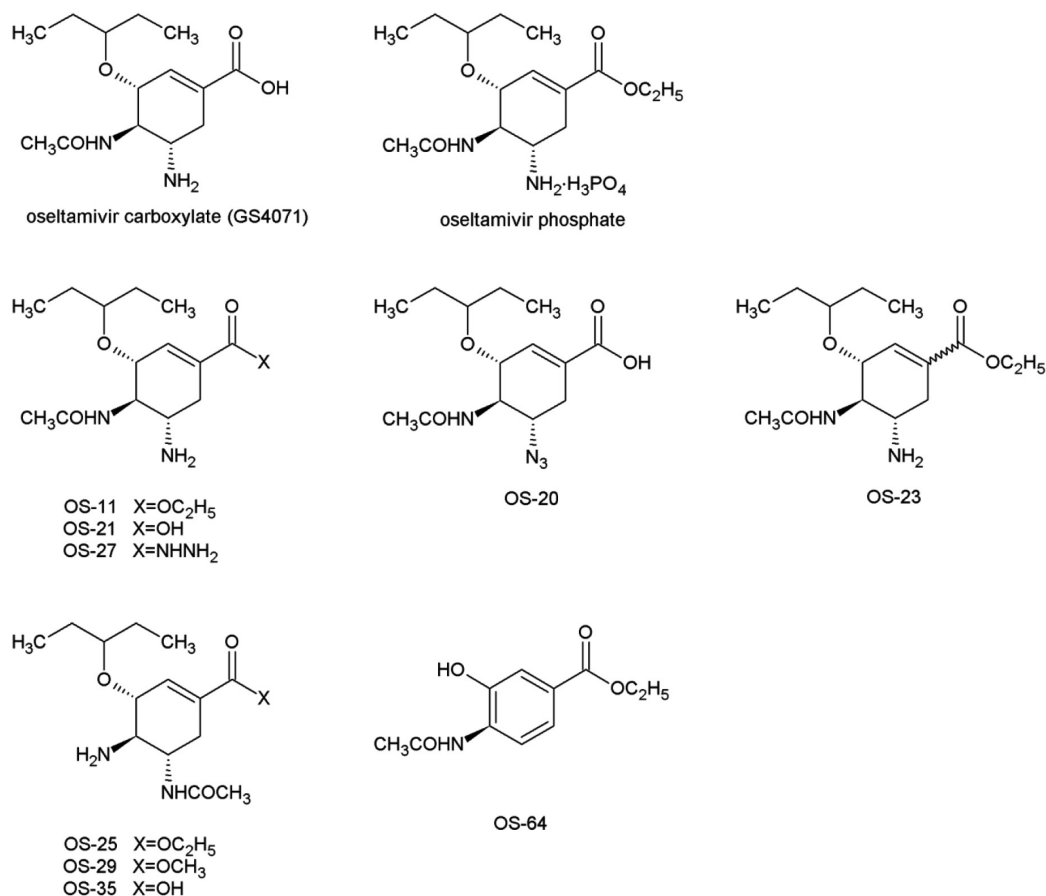


Figure 1. Oseltamivir and its analogs examined in this study.

virus, for selection of the original substances that would be effective and have an acceptable toxicity profile.

MATERIALS AND METHODS

Compounds. This study involved eight analogs of oseltamivir (Fig. 1), which were synthesized in the Pharmaceutical Research Institute (Warsaw). Oseltamivir carboxylate and phosphate were also obtained from PRI.

Cells. Madin-Darby Canine Kidney cells (obtained from the National Influenza Center of the National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland) were cultured in DMEM (Sigma-Aldrich) supplemented with 7.5% FBS (Sigma-Aldrich), 10 U/ml penicillin and 10 µg/ml streptomycin (Sigma-Aldrich) in culture flasks (Nunc) and 96-well plates (TPP Techno Plastic Products) at 37°C, under 5% CO₂. Passaging was carried out by treating cell culture with trypsin (Sigma-Aldrich) every 3 days.

Viruses. Activity of the compounds was tested against two strains of influenza virus type A: A/H1N1/PuertoRico/8/34 (kindly provided by NIPH-NIH) and A/H3N2/HongKong/8/68 (purchased at ATCC, Manassas, VA, USA). Viruses were propagated in MDCK culture in DMEM containing antibiotics, 1% HEPES (Sigma-Aldrich) and 2 µg/ml TPCK-treated trypsin (Sigma-Aldrich) at 35°C, under 5% CO₂. After 3–4 days supernatant was collected, aliquoted and stored at –72°C. Titration was performed by infecting 1-day MDCK culture on a 96-well plate with 10-fold dilutions of the supernatant. After 4 days of incubation at 35°C, under

5% CO₂ the medium was removed from the wells and cell staining was performed using the MTT (according Levi *et al.*, 1995, Shi *et al.*, 2007 with modifications). 25 µl of 2.5 mg/ml solution MTT solution (Sigma-Aldrich) were added to wells. After 2 h incubation at 35°C, 100 µl of lysis buffer: 13.5 g/100 ml SDS (POCH), 45% (v/v) DMF (Sigma-Aldrich) was added and plates were incubated overnight at 35°C. At test termination, absorbance was measured at λ = 560 nm using Ultramark plate reader (Bio-Rad) and a TCID₅₀ values were determined.

Cytotoxicity of the compounds: The weighted portions of the test compounds were dissolved in DMSO (Sigma-Aldrich) and their 10-fold dilutions (10 µg/ml – 100 pg/ml) were prepared in DMEM with FBS, providing 0.05% concentration of DMSO in each of them. 100 µl of the compound solutions were added to a 1-day MDCK culture on a 96-well plate. The culture was incubated for 4 days at 37°C, under 5% CO₂. Then, supernatant was removed from the wells and MTT staining was performed. Each solution was examined at least in triplicate. After absorbance measurement, the concentration causing 50% decrease in cell viability (IC₅₀) was determined for each compound.

Impact of the compounds on survival rate of infected cells: 1-day MDCK cultures on 96-well plates were infected with 50 µl of influenza virus at the concentration of 1000 and 100 TCID₅₀/100 µl/4 days in DMEM supplemented with antibiotics, HEPES, and TPCK-treated trypsin. The solutions of the compounds (100 pg/ml – 10 µg/ml), in identical medium but with addition of DMSO, were also added to wells. Plates were incubated for 4 days at

Table 1. Cytotoxicity and antiviral activity of the compounds against H1N1 and H3N2, at infection dose of 1000 TCID₅₀.

Compound	IC ₅₀ (mg/ml)	H1N1				H3N2			
		A	B	EC ₅₀	TI	A	B	EC ₅₀	TI
		(μM)	(μM)	(μg/ml)		(μM)	(μM)	(μg/ml)	
OS-11	0.562	32.01	0.32	n/d	n/d	3.2	0.32	4	141
OS-20	0.501	–	3.22	–	–	3.22	3.22	7	72
OS-23	0.562	–	–	–	–	–	–	–	–
OS-25	0.199	–	–	–	–	–	–	–	–
OS-27	0.178	–	0.33	–	–	33.51	0.33	10	18
OS-29	0.178	–	33.5	–	–	–	33.5	–	–
OS-35	0.158	35.17	3.52	n/d	n/d	3.52	0.03	3	53
OS-64	0.063	44.8	–	n/d	n/d	44.8	–	n/d	n/d
OSELTAMIVIR PHOSPHATE	0.562	24.37	2.44	n/d	n/d	2.44	2.44	n/d	n/d
OSELTAMIVIR CARBOXYLATE	0.501	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t

MDCK cells were infected with influenza virus and treated with various drug doses. A — the lowest tested drug concentration giving a statistically significant protective effect for MDCK cells infected with a dose of 1000 TCID₅₀/100 ml/4 days in cell viability assay with MTT staining; B — the lowest tested drug concentration causing statistically significant reduction in hemagglutination titer of the virus; n/d — EC₅₀ not determined in the range of tested doses, beyond scale; n/t — not tested, lack of data.

35°C, under 5% CO₂, and then the MTT staining was performed. The assay was repeated in 3 independent experiments, each solution was examined in triplicate. After absorbance measurement, the lowest compound concentration that affect the survival rate of infected cells and concentration that helps 50% cells to survive (EC₅₀) were determined. The therapeutic indices (TI), i.e. IC₅₀/EC₅₀ were also determined if possible.

Impact of the compounds on virus hemagglutination titer. Medium from the wells was subjected to hemagglutination test. The 2-fold solutions of medium in PBS were prepared in a 96-well plate with V-shaped bottoms and 50 μl of turkey erythrocytes suspended in PBS were added. After 0.5 h incubation at room temperature, hemagglutination titer was evaluated visually.

Statistical analysis. The results were treated using a one-parameter analysis of variance ANOVA. Normality

of data distribution was analysed with Shapiro-Wilk test, equality of variances was tested and a non-conservative *post-hoc* test was performed. The results were statistically significant if $p \leq 0.01$.

RESULTS

Cytotoxicity and antiviral activity are shown in Table 1 and Table 2.

Cytotoxicity studies on MDCK cells showed that OS-64, OS-35, OS-29, OS-27 and OS-25 were the most toxic compounds, whereas OS-11, OS-20 and OS-23 had the lowest toxicity that was similar to oseltamivir phosphate and carboxylate. For a higher virus dose (Table 1), antiviral activity was observed for OS-11, OS-27 and OS-35. OS-64 was active only at high concentrations, OS-

Table 2. Cytotoxicity and antiviral activity of the compounds against H1N1 and H3N2 at infection dose of 100 TCID₅₀.

Compound	IC ₅₀ (mg/ml)	H1N1				H3N2			
		A	B	EC ₅₀	TI	A	B	EC ₅₀	TI
		(μM)	(μM)	(μg/ml)		(μM)	(μM)	(μg/ml)	
OS-11	0.562	3.3	0.03	2	281	3.3	0.03	0.6	937
OS-20	0.501	3.22	3.22	3	167	3.22	0.32	1	501
OS-23	0.562	–	–	–	–	–	–	–	–
OS-25	0.199	32	32	n/d	n/d	–	–	–	–
OS-27	0.178	3.35	0.33	1	178	3.35	0.03	3	59
OS-29	0.178	–	33.51	–	–	33.51	33.51	n/d	n/d
OS-35	0.158	3.52	0.35	5	32	0.35	0.04	0.06	2633
OS-64	0.063	4.48	–	5	13	4.48	–	10	6
OSELTAMIVIR PHOSPHATE	0.562	2.43	0.24	n/d	n/d	2.43	0.02	3	187
OSELTAMIVIR CARBOXYLATE	0.501	0.0004	0.0004	12 nM	141666	0.004	0.004	35 nM	48571

MDCK cells were infected with influenza virus and treated with various drug doses. A — the lowest tested drug concentration giving a statistically significant protective effect for MDCK cells infected with a dose of 100 TCID₅₀/100 ml/4 days in cells viability assay with MTT staining; B — the lowest tested drug concentration causing statistically significant reduction in hemagglutination titer of the virus; n/d — EC₅₀ not determined in the range of tested doses, beyond scale.

23 and OS-25 did not show any activity. At the dose of 1000 TCID₅₀ the antiviral effect was dependent on the virus strain. H3N2 strain was sensitive to ten times lower concentrations of the compounds than H1N1 strain, especially for OS-11, OS-35 and oseltamivir phosphate. At a lower infection dose (Table 2), the highest activity was observed for OS-11, OS 27, OS-35 and OS-20. OS-64 showed activity only at high concentrations. OS-23 showed no activity. It has been noticed that virus strains differed partially in sensitivity to various compounds. In case of H3N2, OS-11, OS-20 and OS-35 reached values of TI higher than oseltamivir phosphate.

DISCUSSION

In studies on antiviral drugs, two main approaches can be applied. The first one is an attempt to computerized design of molecules that should have high specificity and high efficiency against influenza viruses (Rungrotmongkol *et al.*, 2009; Hussain Basha & Prasad, 2012; Wang *et al.*, 2010; Durrant & McCammon, 2010; Park & Jo, 2010; Li *et al.*, 2009; Mitrasinovic, 2009). However, this approach has limitations related to the difficulties in synthesizing newly designed molecules, their potential toxicity and lack of knowledge about potential metabolic transformations in the organism. Another approach is a screening test that is carried out empirically to select compounds with the strongest antiviral properties. In our case, it was a reasonable strategy because we tested derivatives of oseltamivir, which is a known antiviral drug. To assess both cytotoxicity and antiviral activity, we used MTT staining, a method which is widely exploited in such studies (Levi *et al.*, 1995; Shi *et al.*, 2007). In addition, to confirm the antiviral activity in the infected cell culture, the virus titer in the culture medium was examined. It was justified by the fact that neuraminidase inhibitors are compounds able to fit in and block the active site of neuraminidase, an enzyme that is crucial for release of progeny virions from the host cell membrane. A disturbance in neuraminidase functioning may reduce the spread of the virus in a tissue and reduce the virus titer. In our studies on cytotoxicity, two compounds turned out to have a similar effect to oseltamivir phosphate, the remaining were several times more toxic. Still, when an effective antiviral demonstrates a disruptive effect on the cells, its toxicity may be reduced through chemical modifications. In our studies, the antiviral activity was noticed for three compounds, and it was evident that the infection dose and virus strains used influence the results. At a higher virus dose, antiviral activity was less manifested, and also has been affected by the virus strain. The H1N1 strain was less sensitive to the action of the compounds than the H3N2. Such a variable sensitivity among influenza virus strains was also observed by other authors (Govorkova *et al.*, 2004; Smee *et al.*, 2009; Smee *et al.*, 2009). At a lower virus dose these differences disappeared. Analysis of the therapeutic index values revealed that three compounds had therapeutic activity higher than oseltamivir phosphate and two had a lower TI value. In light of these results, closer attention should be paid to the three selected derivatives of oseltamivir, OS-11, OS-27 and OS-35, which may raise hopes for the future. It must be underscored that, despite the emergence of strains resistant to known neuraminidase inhibitors, these drugs (Govorkova *et al.*, 2001; Yamanaka *et al.*, 2005; Vavricka *et al.*, 2011; Kubo *et al.*, 2010; Gubareva *et al.*, 2001; Smee & Sidwell, 2002), their combinations, and combinations with substances of other kinds (Govorkova *et al.*, 2004; Nguyen *et al.*, 2010;

Smee *et al.*, 2009; Fukushi *et al.*, 2012; Galabov *et al.*, 2006; Smee *et al.*, 2010) still have the greatest therapeutic potential. For these reasons, it seems advisable to search for compounds with similar structures that may be helpful in cases of infection with strains resistant to the currently used neuraminidase inhibitors, especially since the synthesis of oseltamivir depends on the supply of raw materials (Satoh *et al.*, 2009; Nie & Shi, 2009).

Our results indicate that the novel synthesized compounds used manifested their activity at a higher dose than oseltamivir carboxylate, tested by the same methods. EC₅₀ of oseltamivir carboxylate in our study was comparable with the results of other authors (Nguyen *et al.*, 2009; Smee *et al.*, 2009; Smee *et al.*, 2010). The orally administrated oseltamivir analogs may be metabolized (oseltamivir phosphate is metabolized in the liver to the active form of carboxylate (Davies, 2010)) and can exhibit antiviral activity *in vivo*, which justifies further research on compounds from this group. Furthermore, in contrast to known blockers of the M2 ion channel, neuraminidase inhibitors have the advantage of acting against both influenza virus type A and B (Machala & Brydak, 2006).

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