Antiviral activity of novel oseltamivir derivatives against some influenza virus strains

Janusz Kocik¹, Marcin Kołodziej²*, Justyna Joniec³, Magdalena Kwiatek² and Michal Bartoszcze²

¹Military Institute of Hygiene and Epidemiology, Warsaw, Poland; ²Biological Threats Identification and Countermeasure Center of MIHE, Pulawy, Poland

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

Received: 26 August, 2014; revised: 27 August, 2014; accepted: 27 August, 2014; available on-line: 12 September, 2014

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). Development 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

ABBREVIATIONS: DMEM, Dulbecco modified Eagle's minimal essential medium; DMF, dimethylformamide; EC₅₀, half maximal effective concentration; FBS, fetal bovine serum; Hepes, 4-(2-hydroxethyl)-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; PRL, 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.
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) 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.

**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 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.

**Figure 1. Oseltamivir and its analogs examined in this study.**
Table 1. Cytotoxicity and antiviral activity of the compounds against H1N1 and H3N2, at infection dose of 1000 TCID<sub>50</sub>.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (mg/ml)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>TI</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (mg/ml)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS-11</td>
<td>0.562</td>
<td>32.01</td>
<td>0.32</td>
<td>n/d</td>
<td>3.2</td>
<td>0.32</td>
<td>4</td>
<td>141</td>
</tr>
<tr>
<td>OS-20</td>
<td>0.501</td>
<td>–</td>
<td>3.22</td>
<td>–</td>
<td>3.22</td>
<td>3.22</td>
<td>7</td>
<td>72</td>
</tr>
<tr>
<td>OS-23</td>
<td>0.562</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>OS-25</td>
<td>0.199</td>
<td>–</td>
<td>–</td>
<td>n/d</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>n/d</td>
</tr>
<tr>
<td>OS-27</td>
<td>0.178</td>
<td>–</td>
<td>0.33</td>
<td>–</td>
<td>–</td>
<td>33.51</td>
<td>0.33</td>
<td>10</td>
</tr>
<tr>
<td>OS-29</td>
<td>0.178</td>
<td>–</td>
<td>33.5</td>
<td>–</td>
<td>–</td>
<td>33.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>OS-35</td>
<td>0.158</td>
<td>35.17</td>
<td>3.52</td>
<td>n/d</td>
<td>3.52</td>
<td>0.33</td>
<td>3</td>
<td>53</td>
</tr>
<tr>
<td>OS-64</td>
<td>0.063</td>
<td>44.8</td>
<td>–</td>
<td>n/d</td>
<td>–</td>
<td>44.8</td>
<td>–</td>
<td>n/d</td>
</tr>
<tr>
<td>OS-ELTAMIVIR PHOSPHATE</td>
<td>0.562</td>
<td>24.37</td>
<td>2.44</td>
<td>n/d</td>
<td>2.44</td>
<td>2.44</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>OS-ELTAMIVIR CARBOXYLATE</td>
<td>0.501</td>
<td>n/t</td>
<td>n/t</td>
<td>n/t</td>
<td>n/t</td>
<td>n/t</td>
<td>n/t</td>
<td>n/t</td>
</tr>
</tbody>
</table>

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 TCID<sub>50</sub>/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 — EC<sub>50</sub> not determined in the range of tested doses, beyond scale; n/t — not tested, lack of data.

Table 2. Cytotoxicity and antiviral activity of the compounds against H1N1 and H3N2 at infection dose of 100 TCID<sub>50</sub>.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (mg/ml)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>TI</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (mg/ml)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS-11</td>
<td>0.562</td>
<td>3.3</td>
<td>0.03</td>
<td>2</td>
<td>281</td>
<td>3.3</td>
<td>0.03</td>
<td>0.6</td>
</tr>
<tr>
<td>OS-20</td>
<td>0.501</td>
<td>3.22</td>
<td>3.22</td>
<td>3</td>
<td>167</td>
<td>3.22</td>
<td>0.32</td>
<td>1</td>
</tr>
<tr>
<td>OS-23</td>
<td>0.562</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>OS-25</td>
<td>0.199</td>
<td>32</td>
<td>32</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>OS-27</td>
<td>0.178</td>
<td>3.35</td>
<td>0.33</td>
<td>1</td>
<td>178</td>
<td>3.35</td>
<td>0.03</td>
<td>3</td>
</tr>
<tr>
<td>OS-29</td>
<td>0.178</td>
<td>3.35</td>
<td>3.35</td>
<td>n/d</td>
<td>–</td>
<td>3.35</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>OS-35</td>
<td>0.158</td>
<td>3.52</td>
<td>0.33</td>
<td>5</td>
<td>32</td>
<td>0.35</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>OS-64</td>
<td>0.063</td>
<td>4.48</td>
<td>–</td>
<td>5</td>
<td>13</td>
<td>4.48</td>
<td>–</td>
<td>10</td>
</tr>
<tr>
<td>OS-ELTAMIVIR PHOSPHATE</td>
<td>0.562</td>
<td>2.43</td>
<td>0.24</td>
<td>n/d</td>
<td>n/d</td>
<td>2.43</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>OS-ELTAMIVIR CARBOXYLATE</td>
<td>0.501</td>
<td>0.0004</td>
<td>0.004</td>
<td>12 nM</td>
<td>141666</td>
<td>0.004</td>
<td>0.004</td>
<td>35 nM</td>
</tr>
</tbody>
</table>

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 TCID<sub>50</sub>/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 — EC<sub>50</sub> not determined in the range of tested doses, beyond scale.
23 and OS-25 did not show any activity. At the dose of 1000 TCID<sub>50</sub>, 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; Fukushima 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. EC<sub>50</sub> 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 administered 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 (Machała & Brydak, 2006).

**Acknowledgements**

Studies were conducted under the Ministry of Science and Higher Education project No. 00001909, Activity of non-nucleoside compounds against influenza virus strains.

**REFERENCES**


These references are cited in the document for supporting information on influenza virus and its antiviral treatments.