Novel oral anti-influenza drug candidate AV5080

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Objectives: Development of a novel drug candidate with improved potency against influenza virus neuraminidase compared with currently available therapeutics, and high activity against oseltamivir-resistant viruses.

Methods: A number of synthetic compounds were evaluated for antiviral properties in vitro and in vivo. Three-dimensional molecular docking, assisted by a pharmacophore model, was applied to classify compounds within the series by their inhibitory potency. Compound stability in blood and in animal models was determined. Pharmacokinetic studies in dogs and rats after oral or intravenous administration were performed.

Results: A novel highly potent drug candidate [(3R,4R,5S)-5-[(diaminomethylene)amino]-3-(1-ethylpropoxy)-4-[(fluoroacetyl)amino]cyclohex-1-ene-1-carboxylic acid; AV5080] was synthesized and tested. AV5080 exhibited high activity against influenza virus neuraminidase in vitro, with IC50 values of 0.03 nM and 0.07 nM against the neuraminidase of A/Duck/Minnesota/1525/1981/H5N1 and A/Perth/265/2009/H1N1 (wild-type), respectively. Notably, AV5080 was highly active against oseltamivir-resistant influenza viruses.

Conclusions: Based on the results presented in this study, AV5080 is a promising novel oral drug candidate for the treatment of influenza, including oseltamivir-resistant types. Further pre-clinical development of AV5080 is warranted.

Keywords: antiviral, neuraminidase inhibitors, pharmacokinetics

Introduction

Influenza is among the most current medical and socio-economic issues. According to the WHO, during influenza and related epidemics, up to 5 million people become ill, of whom up to 500,000 die annually.1,2 The current concept of the epidemic process, caused by the circulation of influenza A/H3N2, A/H1N1 and B, is mostly due to the variability of influenza virus surface proteins—haemagglutinin and neuraminidase (NA). The segmented structure of the genome may contribute to influenza A virus reassortants in humans, mammals and birds, which could lead to a new pandemic strain.1 This has been attributed to the infection of humans and animals with avian influenza A/H5N1, A/H7N7, A/H7N3 and A/H9N2 in countries of South and South East Asia (and recently in Europe), accompanied by high mortality that has become more frequent in recent years.2,3 In addition to vaccines, which are the primary means of prevention of influenza infection, small-molecule compounds are promising therapeutics against both viral types (A and B). The first generation of synthetic compounds against influenza were ion-channel blockers targeted against the M2 protein of influenza A, and include amantadine and rimantadine.4,5 However, the practical clinical application of these drugs is strictly limited by the lack of efficacy against influenza B, various side effects and the rapid emergence of drug resistance.

Second-generation drugs are NA inhibitors, and are also effective against both influenza A and B. They prevent the release of new virus particles from infected cells and their distribution throughout the body. This class of drugs includes oral medications oseltamivir carboxylate and phosphate (Tamiflu, available in the form of capsules),2,3,6,7 oseltamivir as well as zanamivir (available in the form an aerosol spray) and peramivir (available as an intravenous formulation). Favipiravir (T-705; available as tablets), an RNA-directed RNA polymerase (NS5B) inhibitor that has been filed for approval in Japan for the oral treatment of influenza A (including avian and H1N1 infections) and for the treatment of influenza B infection, was used as a cross-target control. T-705 has also been reported to be an NA inhibitor [50% inhibitory concentration (IC50) = 1.4 ± 0.8 nM, A/Victoria/3/1975, H3N2,
chemiluminescence assay. However, no relevant activity has been found against A/NWS/1933/H1N1 and A/Duck/Minnesota/1525/1981/H5N1; no inhibition at concentration $\leq 100$ mM.

Severe pandemics accelerate the development and release of novel NA inhibitors. In particular, the intravenous drug peramivir was designed. This compound is currently available in Japan as Rapiacta, in South Korea as Peramiflu and in China. It should be noted that peramivir is distributed as an intravenous (iv) formulation for treating swine flu. This drug is now being developed in a Phase III clinical trial in the USA. In 2009, the FDA approved the use of iv peramivir for the emergency treatment of H1N1 influenza. Compound 1 (Figure 1), a close structural analogue of zanamivir, has recently been reported by Kim et al. Nanomolar activity comparable to zanamivir was shown for this compound against many strains of influenza. In the B/Perth D197E and G70C H1N9 strains, the activity of compound 1 was 32-fold and 40-fold higher, respectively, than that observed for zanamivir. Like zanamivir, the compound showed a relatively high efficiency during iv and intranasal treatment of mice infected with influenza A (A/Hong Kong/1/1968/H3N2).

Compound 1 is a new type of irreversible inhibitor. Upon binding to the NA active site, it forms a covalent bond with amino acid Y406 via the nucleophilic substitution of the fluorine atom located in position 2 of the tetrahydropyran ring by the OH group of Y406 (Figure 1).

However, scepticism towards such compounds has recently been expressed owing to the suicide inhibition that in many cases is accompanied by various side effects. It is now well documented that the use of compounds that form covalent bonds with biomolecules in clinical practice can cause serious damage. Among these agents, compounds containing reactive groups, e.g. $\beta$-lactams and $\beta$-lactones, aziridines, epoxides, halogenethylamines, Michael acceptors (e.g. $\alpha,\beta$-unsaturated carbonyl compounds: 1,4-Michael addition), aldehydes, acid anhydrides and acyl halides, should be used cautiously. With regard to compound 1, the fluorine atom can be readily substituted by O-, N- and S-nucleophiles, e.g. by cysteine or lysine in proteins. Non-selective or poorly selective covalent binding of such agents to biomolecules can lead to serious damage to cells and tissues or to the activation of the innate immune system in response to the formation of modified proteins, which are considered as foreign, followed by degradation in lysosomes. In addition, many irreversible inhibitors have mutagenic and genotoxic effects.

In this paper we describe a series of novel NA inhibitors (compounds 2–10) (Figure 2). A medium-sized combinatorial library of (3R,4R,5S)-3-(1-ethyl-propoxy)-4-acylamino-5-guanidino-cyclohex-1-enecarboxylic acids has recently been synthesized in our laboratory. Among these compounds, AV5080 was the most potent inhibitor of the NA activity of influenza A/Duck/Minnesota/1525/1981/H5N1, influenza A/California/07/2009/H1N1 and influenza B/Florida/4/2006/H1N1 under the same assay conditions. The Hit-compound (AV5080) showed an IC$_{50}$ value of 0.03–0.61 nM.
Materials and methods

Cells and viruses

Madin Darby canine kidney (MDCK) cells were grown in minimal essential medium, supplemented with 10% fetal calf serum, 5 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin sulphate and 100 μg/mL kanamycin sulphate, in a humidified atmosphere of 5% CO₂. The influenza virus strains A/California/07/2009/H1N1, B/Florida/4/2006/H1N1 and A/Duck/Minnesota/1525/1981/H5N1 were obtained from the WHO and Utah State University (Logan, UT, USA). Mouse-adapted A/Aichi/2/1968/H3N2, A/Vladivostok/1609/H1N1 (oseltamivir resistant), A/Perth/16/2009/H3N2 (rimantadine resistant), A/Chicken/Rostov-on-Don/35/2007/H5N1 and B/Brisbane/60/2008 were obtained from the Ivanovsky Institute of Virology (Moscow, Russia). A/Perth/265/2009/H1N1 (wild-type (wt)), A/Perth/265/2009/H1N1/H275Y (oseltamivir resistant), B/Perth/211/2001 (wt) and B/Perth/211/2001/D179E (oseltamivir resistant) were obtained from the WHO Collaborating Centre for Research and Reference on Influenza (Australia).

NA

Purified N1 crystals from A/Puerto Rico/8/1934/H1N1 viruses were obtained from ATCC (Manassas, VA, USA).

Animals

Pathogen-free female albino mice and BALB/C mice (weight 12–14 g) were obtained from the nursery ‘Andreevka’ (Moscow reg., Russia). The animals were kept in separate climate-controlled rooms (temperature 18–26 °C, relative humidity 30%–70%, 8–10 volumes of room air per hour, and 12 h day/night cycle). Animal maintenance and care were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals.18 The animals were fed with briquetted feed in accordance with the approved standards. Marking of animals in groups was performed by colouring the body surface with dye (eosin). The experiments were carried out in winter and spring. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of the Chemical Diversity Research Institute, according to the Guide for the Care and Use of Laboratory Animals.

Chemistry

General and analytical chemistry, as well as synthesis protocols, are presented in the Supplementary methods (available as Supplementary data at JAC Online). Oseltamivir carboxylate and oseltamivir phosphate were prepared as described previously.19 AV5080S: (3R,4R,5S)-5-[(4-Azetidinylmethyl)amino]-3-(1-ethylpropoxy)-4-6-fluorophenyl)amino)-cyclohex-1-ene-1-carboxylic acid mesylate was synthesized starting from commercially available ethyl (3R,4R,5S)-4-amino-5-[(tert-butoxycarbonyl)amino]-3-(1-ethylpropoxy) cyclohex-1-ene-1-carboxylate20,21 according to the synthetic procedure shown in Scheme S1 (available as Supplementary data at JAC Online).

Molecular docking and pharmacophore modelling

Molecular docking and pharmacophore modelling were performed using Molecular Operating Environment (MOE) software (www.chemcomp.com) and crystallographic data for proteins isolated from influenza-type viruses that were available in the Protein Data Bank (PDB) [Research Collaboratory for Structural Bioinformatics (www.rcsb.org)]. For the in silico modelling we used crystallographic data reported for H5N1 influenza NA in complex with peramivir (PDB code: 2HU4 (H233Y) and 2HT8). The binding mode for oseltamivir was compared with that observed for peramivir, using ICM-Pro Software [Molsoft (www.molsoft.com)].

NA assay

The activity of the compounds against NA was determined using two screening approaches: (i) a chemiluminescence assay and (ii) a fluorometric assay. Each experiment with either method was performed at least three times.

(i) Inhibition of viral NA by the test compounds was evaluated using the NA-Star influenza NA Inhibitor Resistance Detection Kit (Applied Biosystems, Foster City, CA, USA) in 96-well solid white microplates, following the manufacturer’s instructions as previously reported.22 Samples were serially diluted 2-fold, then each concentration was mixed with virus in duplicate test wells and incubated for 10 min at 37 °C, followed by incubation with the chemiluminescent substrate for 30 min at 37 °C. Chemiluminescence was read using a Centro LB 960 luminometer (Berthold Technologies, Oak Ridge, TN, USA) following addition of the NA-Star accelerator solution. IC₅₀ values for viral NA activity were then determined by plotting chemiluminescence values versus test compound concentrations. Each test compound was evaluated in three independent experiments.

(ii) The fluorometric assay was performed using a WHO protocol.23 2’-[(4-Methylumbelliferyl)-α-N-acetylneuraminic acid sodium salt (MUNANA, Sigma, cat. No. M8639), at a final concentration of 0.1 mM, was used as fluorescent substrate. Diluted allantoic virus with 800–1200 fluorescence units of MUNANA was mixed with the test compound (0.01 mM–10 K mM) in 33 mM 2-[N-morpholino]ethanesulphonic acid (pH 6.5) containing 4 mM CaCl₂, and incubated for 30 min at 37 °C. After the substrate was added, the samples were incubated at 37 °C for 1 h, then the reaction was stopped by adding NaOH (0.14 M) in ethanol (83%). Fluorescence was measured at the excitation wavelength of 360 nm and the emission wavelength of 448 nm. The corresponding IC₅₀ values were calculated.

Antiviral activity and cytotoxicity of the synthesized compounds in MDCK cells infected with A/California/07/2009/H1N1 and A/Duck/Minnesota/1525/1981/H5N1

AV5080S was diluted to 20 or 50 mg/mL in DMSO, then test samples were prepared by half-log dilution (eight steps) in minimal essential medium, supplemented with 10% fetal calf serum, 5 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin sulphate and 100 μg/mL kanamycin sulphate, in a humidified atmosphere of 5% CO₂. The influenza virus strains A/California/07/2009/H1N1, B/Florida/4/2006/H1N1 and A/Duck/Minnesota/1525/1981/H5N1 were obtained from the WHO and Utah State University (Logan, UT, USA). Mouse-adapted A/Aichi/2/1968/H3N2, A/Vladivostok/1609/H1N1 (oseltamivir resistant), A/Perth/16/2009/H3N2 (rimantadine resistant), A/Chicken/Rostov-on-Don/35/2007/H5N1 and B/Brisbane/60/2008 were obtained from the Ivanovsky Institute of Virology (Moscow, Russia). A/Perth/265/2009/H1N1 (wild-type (wt)), A/Perth/265/2009/H1N1/H275Y (oseltamivir resistant), B/Perth/211/2001 (wt) and B/Perth/211/2001/D179E (oseltamivir resistant) were obtained from the WHO Collaborating Centre for Research and Reference on Influenza (Australia).

<table>
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<th>ID</th>
<th>IC₅₀: influenza A/Duck/ Minnesota/1525/1981/H5N1 (chemiluminescence assay)</th>
<th>SD</th>
<th>E_score</th>
<th>E_place</th>
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<tbody>
<tr>
<td>AV5080</td>
<td>0.03</td>
<td>0.00</td>
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<td>0.01</td>
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<td>0.01</td>
<td>−15.49</td>
<td>−122.485</td>
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<td>0.03</td>
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<tr>
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<td>0.01</td>
<td>−16.30</td>
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<tr>
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<td>7.41</td>
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<td>−132.87</td>
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<td>AV5070</td>
<td>12.57</td>
<td>3.18</td>
<td>−13.93</td>
<td>−133.076</td>
</tr>
</tbody>
</table>

*Score from the rescoring stage(s)/docking stage.

Score from the placement stage/pharmacophore stage.
MDCK cells. Three wells of each sample were infected with the test virus using a low multiplicity of infection (≤0.01; 50% cell culture infectious dose per cell). Two control wells were not infected with the virus. Oseltamivir carboxylate, zanamivir and favipiravir were tested in parallel as controls. After 2.5–4 days, when the cytopathic effect had reached its maximum in the untreated virus control wells, plates were stained with neutral red dye for ≏2 h, then supernatant dye was removed from the wells and the incorporated dye was extracted from the cells in 50:50 Sorensen citrate buffer:ethanol. The absorbance was determined using a spectrophotometer. Absorbance values were converted to percentage of cell controls and normalized to the virus control. The concentration of the test compound required to inhibit the cytopathic effect by 50% (EC50) was determined by regression analysis. Compound cytotoxic concentration 50% (CC50), which would cause 50% reduction in cell viability in the absence of the virus, was calculated in the same way. Then the selective index (SI), defined as the CC50 divided by EC50, was calculated. The statistical analysis was performed using GraphPad Prism v. 5.0 [GraphPad Software, Inc. (www.graphpad.com)].

Results and discussion

Three-dimensional (3D) molecular docking and pharmacophore modelling

The active binding site of NA was constructed based on the reported peramivir and oseltamivir binding modes. The model was successfully validated using oseltamivir, zanamivir, peramivir and their direct analogues with reported NA activity against A/Duck/Minnesota/1525/1981/HSN1. The results (Table 1 and Figure 3) were compared with the reported X-ray structures.
The predicted active conformations of the reference compounds correlated well with the available crystallographic data. For the molecular docking we used the 3D pharmacophore integrated approach, available in MOE. In brief, the first iteration was the 3D template construction (pharmacophore hypothesis) within the binding site of NA. We used the active conformation of peramivir, reported by Russell et al., as a 3D template. As shown in Figure 3, a pharmacophore with seven binding points (three H-bond acceptors, two H-bond donors and two hydrophobic areas) was initially constructed. The sphere diameter corresponded to the manually assigned tolerance. After the pharmacophore was sited, molecular docking was performed for all the compounds listed in Table 1. As shown in Figure 4, the predicted active conformations of the tested molecules correlated well with the reported crystallographic analyses. The developed 3D model demonstrated high predictive validity and effectively classified compounds by target activity within the scope of the title scaffold. For each compound, at least five different conformations were generated in MOE. During modelling we thoroughly analysed all scores that were generated in MOE. From these values, we selected the best scores for E_score2 and E_place for the conformation that is most closely related to the template supramolecular interface (Table 1). Generally, an E_score2 of $-15$ or lower is considered good, depending on the receptor. However, no reliable correlation was observed among F-substituted compounds, thus, based on the calculated score values, these compounds should be ranked by the predicted activity as follows: AV5080 (E_score2 = $-18.86$) > AV5068 (E_score2 = $-14.00$) > AV5070 (E_score2 = $-13.93$), while the experimental IC50 values were 0.03, 48.67 and 12.57 nM, respectively. It should be noted that for other viruses included in Table 1 a similar correlation was also observed, resulting in an unexpected inhibition behaviour, where the mono-F derivative was the most active, the di-F analogue demonstrated the weakest inhibition, and the tri-F compound showed moderate activity. There is no direct correlation with the estimated polarity, dipole moment, electronegativity or steric properties within this sub-series. Therefore, we may relate this to the kinetic and dynamic features of these groups under the experimental conditions, particularly associated with water re-solvation energy.

A convenient prediction model was then developed using the classical linear regression approach and two calculated scores: E_score2 and E_place. The developed model is depicted in Figure 5. As shown in Figure 5, good ‘prediction’ results were achieved for the molecules tested. Thus, the square of the Pearson correlation coefficient ($R^2$) for the A/Duck/Minnesota/1525/1981/H5N1 line is relatively high—0.89, while the analogue value for the ‘leave-one-out’ (LOO) procedure cross-validation set ($Q^2$) is slightly less—0.8. In statistics, the following three cross-validation methods are often used to examine a model for its effectiveness in practical application: independent dataset test, sub-sampling or K-fold cross-over test, and jackknife test. However, of the three test methods, the jackknife test is deemed the least arbitrary that can always yield a unique result for a given benchmark dataset, as elaborated by Chou29 and demonstrated by Li et al. in their equations 28–30. Accordingly, the jackknife test has been increasingly and widely used by investigators to examine the quality of various models or predictors. That is why we adopted the jackknife or LOO test to validate our findings in this study.

### Solubility

The solubility of AV5080 in urea-bisulphite solution (pH = 2.0, pH = 4.0 and pH = 7.0) with 2% DMSO was 208.6 μM, 228.4 μM and 211.6 μM, respectively. Thus, AV5080 demonstrated reasonably good solubility under these experimental conditions.

### In vitro stability in buffers, plasma and microsomes

The in vitro stability of AV5080 was evaluated as described previously. AV5080 was incubated in universal buffers (1 μM) at pH = 2, 4 or 7.4 (Table 2 and Figure 6) and in human, dog and rat plasma (Table 3 and Figure 6) in duplicate at 37°C with gentle shaking. Aliquots (60 μL) were collected at pre-determined timepoints (0, 1, 4 and 24 h), followed by extraction with acetonitrile (180 μL) containing 50 ng/mL of the internal standard tolbutamide. Samples were incubated at 20°C for 15 min and then centrifuged at 2000 g for 10 min. Supernatants were analysed by liquid chromatography–tandem mass spectrometry. Acetonitrile, DMSO,

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**Table 2. Stability of AV5080 in urea-bisulphite solution**

<table>
<thead>
<tr>
<th></th>
<th>pH=2</th>
<th>pH=4</th>
<th>pH=7.4</th>
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<tbody>
<tr>
<td></td>
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<td>4 h</td>
<td>24 h</td>
</tr>
<tr>
<td>AV5080 content, %</td>
<td>100</td>
<td>106±5.8</td>
<td>106±6.8</td>
</tr>
</tbody>
</table>

[Figure 4; the data from the X-ray diffraction analysis for AV5080 is available in Figure S1 (available as Supplementary data at JAC Online)]. The predicted active conformations of the reference compounds correlated well with the available crystallographic data. For the molecular docking we used the 3D pharmacophore integrated approach, available in MOE. In brief, the first iteration was the 3D template construction (pharmacophore hypothesis) within the binding site of NA. We used the active conformation of peramivir, reported by Russell et al., as a 3D template. As shown in Figure 3, a pharmacophore with seven binding points (three H-bond acceptors, two H-bond donors and two hydrophobic areas) was initially constructed. The sphere diameter corresponded to the manually assigned tolerance. After the pharmacophore was sited, molecular docking was performed for all the compounds listed in Table 1. As shown in Figure 4, the predicted active conformations of the tested molecules correlated well with the reported crystallographic analyses. The developed 3D model demonstrated high predictive validity and effectively classified compounds by target activity within the scope of the title scaffold. For each compound, at least five different conformations were generated in MOE. During modelling we thoroughly analysed all scores that were generated in MOE. From these values, we selected the best scores for E_score2 and E_place for the conformation that is most closely related to the template supramolecular interface (Table 1). Generally, an E_score2 of $-15$ or lower is considered good, depending on the receptor. However, no reliable correlation was observed among F-substituted compounds, thus, based on the calculated score values, these compounds should be ranked by the predicted activity as follows: AV5080 (E_score2 = $-18.86$) > AV5068 (E_score2 = $-14.00$) > AV5070 (E_score2 = $-13.93$), while the experimental IC50 values were 0.03, 48.67 and 12.57 nM, respectively. It should be noted that for other viruses included in Table 1 a similar correlation was also observed, resulting in an unexpected inhibition behaviour, where the mono-F derivative was the most active, the di-F analogue demonstrated the weakest inhibition, and the tri-F compound showed moderate activity. There is no direct correlation with the estimated polarity, dipole moment, electronegativity or steric properties within this sub-series. Therefore, we may relate this to the kinetic and dynamic features of these groups under the experimental conditions, particularly associated with water re-solvation energy.

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formic acid and water were of HPLC grade (Panreac). Tolbutamide and verapamil were obtained from Sigma. Pooled human plasma in sodium citrate, Sprague Dawley rat plasma and canine plasma (mixed breed) in Na₂EDTA were obtained from Innovative PlON-Inc. (USA) and used at the desired pH. Universal buffer was obtained from Research (Novi, MI, USA). Tolbutamide was extracted with ice-cold acetonitrile containing 50 ng/mL of the internal standard tolbutamide. Precipitated samples were incubated at 20°C for 15 min and then centrifuged at 2000 g for 10 min. The supernatant was assayed by liquid chromatography–tandem mass spectrometry. The pharmacokinetic parameters of AV5080 are summarized in Table S1 (available as Supplementary data at JAC Online). The related pharmacokinetic curves are presented in Figure 7. As shown in Table S1, the bioavailability of AV5080 in dogs is relatively low (6.3%) and it is 1.6-fold higher than in mice and ~3 times higher than in rats.

NA inhibition

Anti-NA activity of the test compounds was determined using the chemiluminescence NA assay, described earlier. The calculated IC₅₀ values are presented in Table 6, with oseltamivir carboxylate, zanamivir and peramivir as controls. These results indicate that all compounds inhibited NA, albeit with different potency. AV5068 and AV5070 were significantly less active against virus strains A/California/07/2009/H1N1, A/Duck/Minnesota/1525/1981/H5N1 and B/Florida/4/2006/H1N1 than peramivir. Note that AV5080 was ~3 times more potent against H5N1 of influenza A/Duck/Minnesota/1525/1981/H5N1 than oseltamivir. AV5080 was more active than its alkyynl-AV5063, difluoro-AV5068 and trifluoro-AV5070 analogues, as well as oseltamivir carboxylate and zanamivir. It should be noted that 5-guanidine derivatives (W=C(NH)NH₂; Figure 2) were significantly more active than 5-(4,5-dihydro-1H-imidaz-2-yl) derivatives (W=4,5-dihydro-1H-imidaz-2-yl; Figure 2). Based on the 3D molecular docking study, the binding of cyclic derivatives is less effective due to steric hindrance. Moreover, the calculated lipophilicity of AV5082 (logP=1.14) and AV5085 (logP=1.06) is markedly higher than that for AV5080 (logP=0.06) and AV5063 (logP=0.39).

In the fluorometric assay, AV5080 showed picomolar activity (IC₅₀<1 nM) against the NA of A/California/07/2009/H1N1, A/Aichi/2/1969/H3N2, A/Chicken/Rostov-on-Don/35/2007/
H5N1, A/Perth/16/2009/H3N2, A/Perth/265/2009/H1N1 and A/Perth/261/2009/H1N1/H275Y, and nanomolar activity (IC50 = 1.27–6.65 nM) against the NA of A/Vladivostok/16/2009/H1N1, B/Brisbane/60/2008, B/Perth/211/2001 and B/Perth/211/2001/197E (Table 7). It should be noted that the NA inhibitory activity of AV5080 was generally higher than that of both oseltamivir carboxylate and zanamivir. Interestingly, for the oseltamivir-resistant virus A/Vladivostok/16/2009/H1N1, the inhibition potency of AV5080 (IC50 = 4.45 nM, IC90 = 70.36 nM) was much lower than that of zanamivir (IC50 = 0.61 nM, IC90 = 7.69 nM). At the same time, AV5080 was considerably more active than oseltamivir against A/Vladivostok/16/2009/H1N1 (oseltamivir resistant) and ~2-fold more active against A/Perth/16/2009/H3N2 (rimantadine resistant). The results obtained with the two assays were in agreement. For example, the IC50 values for AV5080 were 0.06 nM and 0.19 nM, as determined using the chemiluminescence and fluorometric assays, respectively.

**In vitro activity of AV5080 in the MDCK cell line**

The results of the antiviral activity (EC50) and cytotoxicity (CC50) determination for AV5080, zanamivir, oseltamivir carboxylate and T-705 in MDCK cells infected with A/California/07/2009/H1N1 or A/Duck/Minnesota/1525/1981/H5N1 are presented in Table 8. AV5080 was most active against strain A/California/07/2009/H1N1, as well as against the highly pathogenic A/Duck/Minnesota/1525/1981/H5N1 strain, as compared with oseltamivir carboxylate, zanamivir and favipiravir (T-705). The activity of AV5080 against A/California/07/2009/H1N1 was 13 and 20 times higher than that for oseltamivir carboxylate and zanamivir, respectively. Similarly, AV5080 was 24 and 16 times more active against A/Duck/Minnesota/1525/1981/H5N1 than oseltamivir carboxylate and zanamivir, respectively. It should also be noted that under the experimental conditions, the activity of AV5080 was more than three orders of magnitude higher than that of favipiravir. No cytotoxicity was observed for AV5080 in these studies.
Table 7. NA inhibition activity of AV5080, oseltamivir carboxylate and zanamivir against a panel of influenza strains

<table>
<thead>
<tr>
<th>Virus</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ± SD, nM (number of measurements)</th>
<th>IC&lt;sub&gt;90&lt;/sub&gt; ± SD, nM (number of measurements)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AV5080</td>
<td>oseltamivir carboxylate</td>
</tr>
<tr>
<td>A/California/07/2009/H1N1</td>
<td>0.19 ± 0.08 (6)</td>
<td>0.76 ± 0.43 (6)</td>
</tr>
<tr>
<td>A/Aichi/2/1969/H3N2</td>
<td>0.69 ± 0.19 (6)</td>
<td>0.43 ± 0.24 (6)</td>
</tr>
<tr>
<td>A/Vladivostok/16/2009/H1N1 (oseltamivir resistant)</td>
<td>4.45 ± 0.85 (7)</td>
<td>844.4 ± 110.0 (7)</td>
</tr>
<tr>
<td>A/Perth/16/2009/H3N2 (rimantadine resistant)</td>
<td>0.46 ± 0.018 (2)</td>
<td>0.92 ± 0.01 (2)</td>
</tr>
<tr>
<td>A/Chicken/Rostov-on-Don/35/2007/H5N1</td>
<td>0.20 ± 0.00 (2)</td>
<td>1.78 ± 0.09 (2)</td>
</tr>
<tr>
<td>B/Brisbane/60/2008</td>
<td>1.27 ± 0.14 (2)</td>
<td>25.42 ± 0.51 (2)</td>
</tr>
<tr>
<td>A/Perth/265/2009/H1N1 (wt)</td>
<td>0.07 ± 0.05 (4)</td>
<td>1.002 ± 0.068 (4)</td>
</tr>
<tr>
<td>A/Perth/265/2009/H1N1/H275Y (oseltamivir resistant)</td>
<td>1.39 ± 0.38 (4)</td>
<td>359.9 ± 87.2 (4)</td>
</tr>
<tr>
<td>B/Perth/211/2001 (wt)</td>
<td>2.08 ± 0.71 (4)</td>
<td>39.24 ± 2.436 (4)</td>
</tr>
<tr>
<td>B/Perth/211/2001/D179E</td>
<td>6.65 ± 0.90 (4)</td>
<td>230.3 ± 62.6 (4)</td>
</tr>
</tbody>
</table>

Table 8. Antiviral activity and cytotoxicity of the selected compounds in MDCK cells infected with A/California/07/2009/H1N1 or A/Duck/Minnesota/1525/1981/H5N1 strains

<table>
<thead>
<tr>
<th>Compound</th>
<th>A/California/07/2009/H1N1</th>
<th>A/Duck/Minnesota/1525/1981/H5N1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;, nM</td>
<td>CC&lt;sub&gt;50&lt;/sub&gt;, μM</td>
</tr>
<tr>
<td>AV5080</td>
<td>0.75</td>
<td>&gt;690                     &gt;920000</td>
</tr>
<tr>
<td>Zanamivir</td>
<td>15.95 (21.2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;30                      &gt;1881</td>
</tr>
<tr>
<td>Oseltamivir carboxylate</td>
<td>9.85 (13)</td>
<td>&gt;35                      &gt;3553</td>
</tr>
<tr>
<td>T-705</td>
<td>1530 (2040)</td>
<td>&gt;796                     &gt;520</td>
</tr>
</tbody>
</table>

<sup>a</sup>Coeff.: EC<sub>50</sub> of reference compound/EC<sub>50</sub> of AV5080.
In vivo activity of AV5080 in the influenza A mouse model: treatment and prophylaxis

The results of AV5080 evaluation in mice challenged with a lethal dose of A/Aichi/2/1969/H3N2 or A/Puerto Rico/8/1934/H1N1 (mouse pneumonia model) are presented in Figure 8 and Figure S2 (available as Supplementary data at JAC Online). A relatively high mortality rate (82%–100%) and loss in body weight (10%–21%) were observed after viral infection in the absence of treatment. AV5080 and oseltamivir phosphate were administered intragastrically 2 h before and 6 h after viral inoculation, then twice a day for 5 days. Animals that showed signs of severe disease and weight loss of 25% were humanely killed and regarded as having died. As shown in Figure 8, the survival rate (%) was higher for oseltamivir phosphate than for AV5080 across almost all doses administered. For example, at 5 mg/kg per day the survival rate was 50% for oseltamivir phosphate and 20% for AV5080, while at 40 mg/kg per day the survival rates were 80% and 90%, respectively. Therefore, the intragastric route of administration was not considered optimal for AV5080 in this mouse model.

The results of oral administration of AV5080 in mice infected with A/Aichi/2/1969/H3N2, are presented in Table 9. The compound was highly effective against viral infection at all doses studied. For example, administration at 25 mg/kg per day significantly increased survival rate (up to 90%–100%) and time to death compared with untreated control mice. The survival rate increase was dose dependent, and for the 25–50 mg/kg per day dose range the efficacy of AV5080 and oseltamivir phosphate was very similar. During the oral treatment of mice infected with A/Puerto Rico/8/1934/H1N1 with 10 mg/kg per day AV5080, the survival rate reached 66%, and was comparable to that observed for oseltamivir phosphate (Table 9).

A significant gain in body weight was observed in mice infected with A/Puerto Rico/8/1934/H1N1 after the oral administration of

![Figure 8. Survival rate in mice infected with A/Puerto Rico/8/1934/H1N1 after the intragastric administration of 5–50 mg/kg per day AV5080. OsP, oseltamivir phosphate.](image-url)

![Table 9. Antiviral efficacy of AV5080 and oseltamivir phosphate after oral administration in mice infected with influenza strain A/Aichi/2/1969/H3N2 or A/Puerto Rico/8/1934/H1N1](table-url)
AV5080 at $\geq 10$ mg/kg per day (Figure S2). For instance, starting from day 8 after the first administration of AV5080 at the dose of 5 mg/kg per day, mouse body weight continuously increased up to $\approx 50\%$ on day 14.

3D molecular docking of the tested molecules and oseltamivir carboxylate, zanamivir and peramivir revealed similar binding modes and supramolecular interfaces for these compounds within the active binding site of NA from influenza A/Duck/Minnesota/1525/1981(H5N1). Thus, the highest scores were calculated for AV5080, AV5063, oseltamivir, zanamivir and peramivir. The lowest score was observed for cyclic guanidine analogues AV5082 and AV5085. It should be noted that the docking scores showed excellent correlation with the observed biological activities except for the two outliers described earlier. Two scores that were output from MOE were used for the development of the linear regression model with a relatively high $R^2$ value of 0.89 (LOO). On the basis of the study by Kim et al., we have also suggested covalent bonding with S179 for our F-substituted analogues, as the distance between S179 and the carbon of the AV5080 2-fluorine acetylamino group is 2.8 Å. However, our biological data clearly indicate no covalent bonding with NA based on the recorded enzymatic kinetics. In addition, we clearly showed that AV5080 did not react with S179 affected by glutathione, arginine, lysine and phenol in water solutions (pH=7.4–8, temperature=40°C).

Conclusions

In conclusion, AV5080 is a new oseltamivir analogue with improved antiviral activity against a number of influenza strains, including oseltamivir-resistant strains. AV5080 demonstrated good physico-chemical and metabolic properties, as well as a favourable pharmacokinetic profile in animals. Further pre-clinical development of this drug candidate for the treatment of influenza is warranted.

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Transparency declarations

A. V. I. is chief scientific officer and founder of ChemDiv, managing member of ASAIV LLC and author of the patent application cited in reference 17. Y. A. I. and V. V. B. are employees of ChemDiv. All other authors: none to declare.

Supplementary data

Supplementary methods, Scheme S1, Figure S1, Table S1 and Figure S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


