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Synthesis and in vitro study of novel neuraminidase inhibitors against avian influenza virus

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ABSTRACT

Evidences of oseltamivir resistant influenza patients raised the need of novel neuraminidase inhibitors. In this study, five oseltamivir analogs **PMC-31**–**PMC-36**, synthesised according to the outcomes of a rational design analysis aimed to investigate the effects of substitution at the 5-amino and 4-amido groups of oseltamivir on its antiviral activity, were screened for their inhibition against neuraminidase N1 and N3. The enzymes used as models were from the avian influenza A H7N1 and H7N3 viruses. The neuraminidase inhibition assay was carried out by using recombinant species obtained from a baculovirus expression system and the fluorogenic substrate MUNANA. The assay was validated by using oseltamivir carboxylate as a reference inhibitor. Among the tested compounds, **PMC-36** showed the highest inhibition on N1 with an IC_{50} of 14.6 ± 3.0 nM (oseltamivir 25 ± 4 nM), while **PMC-35** showed a significant inhibitory effect on N3 with an IC_{50} of 0.1 ± 0.03 nM (oseltamivir 0.2 ± 0.02 nM). The analysis of the inhibitory properties of this panel of compounds allowed a preliminary assessment of a structure–activity relationship for the modification of the 4-amido and 5-amino groups of oseltamivir carboxylate. The substitution of the acetamido group in the oseltamivir structure with a 2-butenylamido moiety reduced the observed activity, while the introduction of a propenylamido group was well tolerated. Substitution of the free 5-amino group of oseltamivir carboxylate with an azide, decreased the activity against both N1 and N3. When these structural changes were both introduced, a dramatic reduction of activity was observed for both N1 and N3. The alkylation of the free 5-amino group in oseltamivir carboxylate introducing an isopropyl group seemed to increase the inhibitory effect for both N1 and N3 neuraminidases, displaying a more pronounced effect against N1.

1. Introduction

The spreading of avian influenza in birds increased the probability of direct infection in humans. To date, person to person transmission has been very rare and associated with close human contact.[1] Nonetheless, the possibility of genetic reassortment of avian influenza virus in suitable 'mixing vessels' thus generating novel viral strains raised the concern about the next pandemic.

Currently, anti-influenza medications consist of two classes of drugs: M2 protein inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir).[2] M2 protein inhibitors are specifically active against influenza A; they interfere with the viral uncoating process through a direct interaction with the matrix (M2) protein, which functions as a channel for hydrogen ions.[3] Neuraminidase inhibitors prevent the removal of the sialic acid (*N*-acetylneuraminic acid) residue from the glycopeptide

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receptor by the viral neuraminidase, which would otherwise allow the virus particles to be released from the infected cell thus spreading to neighboring cells.[4] As a class, neuraminidase inhibitors are effective against all neuraminidase subtypes and, therefore, against all strains of influenza. This is a key point in epidemic and pandemic preparedness and an important advantage over the M2 protein inhibitors which are effective only against sensitive strains of influenza A.[2] Among all antiviral drugs, oseltamivir is the recommended antiviral to treat patients infected with avian influenza (H5N1) including chemoprophylaxis in high-risk populations.[5]

Alarming oseltamivir resistances, such as the one associated to the N294S mutation of the neuraminidase detected in an influenza A (H5N1) infected patient in Egypt[6] or the H274Y mutation in influenza A (H5N1) reported in Vietnamese patients during treatment,[7,8] are important examples stressing the present need of novel putative neuraminidase inhibitors.

The available X-ray crystallographic data suggest that the strategy of designing neuraminidase inhibitors binding to the highly conserved region of the neuraminidase, achieves inhibitors effective against all influenza neuraminidase subtypes, N1–N9 and influenza B viruses.[9–13] However, X-ray crystallographic studies of neuraminidase from type A influenza viruses, also revealed that the neuraminidase structures can be divided in two different classes, known as group-1 (comprising N1, N4, N5, N8) and group-2 neuraminidases (comprising N2, N3, N6, N7, N9), according to distinctive structural features near the oseltamivir binding site. In fact, the major difference between the two groups is the presence of a large cavity, known as the '150-cavity', adjacent to the active site in group-1 but not present in group-2 neuraminidases.[14] Since currently available neuraminidase inhibitors were targeted against the structures of group-2 neuraminidases, the discovery of the '150-cavity' in group-1 neuraminidases prompted the design the novel neuraminidase inhibitors in order to improve the efficiency of current antiviral treatments. In fact, many studies have been focused on using computational drug design for modifying the structure of oseltamivir.[10–15] However, these data are limited only to in silico screening. In this study, we report a preliminary in vitro assessment of the neuraminidase inhibition activity of five novel putative inhibitors synthesised so far that contain extensions of the 4-amido and 5-amino groups intended to fill the 150-cavity of the group-1 neuraminidases. These analogs were proposed by computer-assisted combinatorial design of more potent N1 inhibitors[17] taking advantage of the aforementioned recent X-ray crystallographic studies of neuraminidase N1 co-crystallized with oseltamivir[14] and structure-based in silico screening of a virtual library of oseltamivir analogs. The compounds studied in this work do not contain structural modifications of the 3-ether group and thus are not expected to display inhibitory activity against the oseltamivir-resistant N294S and H247Y variants of N1. The mutated residues are located near the 3-ether group and thus will most probably not be significantly affected by the 4-amido and 5-amino groups extensions. The neuraminidases used in this study were expressed in baculovirus and found on the viral membrane capsule, the sequences were taken from the genome of influenza A H7N1 and H7N3 viruses.[21,22] Our study has identified oseltamivir analogs with elevated binding affinity to N1 and N3 neuraminidase subtypes.

2. Materials and methods

2.1. Oseltamivir and its analogs

Oseltamivir carboxylate was purchased from Toronto Research Chemical Inc. The compound was dissolved in distilled water obtaining a stock solution with concentration 17.58 mM. The stock solution was aliquoted and stored at −20 °C until further use.

Synthetic oseltamivir analogs were dissolved in water in order to obtain a stock solution of the compounds. All the stock solutions were aliquoted and stored at −20 °C until further use. For **PMC-35** (**5**) the use of 10% DMSO in water was necessary in order to obtain a clear solution.

2.2. Reagents

2′-(4-Methylumbelliferyl)-α-D-acetyl neuraminic acid (MUNANA) was purchased from Gold-Biotechnology Inc., 4-methylumbelliferone (4-MU) was purchased from Alfa Aesar GmbH & Co. KG. 2-*N*-Morpholino-ethanesulfonic acid (MES) and Glycine were purchased from Sigma, $CaCl_2$ was purchased from Sigma–Aldrich.

2.3. Neuraminidase

Lyophilized aliquots of baculovirus supernatant expressing recombinant Neuraminidase (N1) and (N3) were provided by OFFLU Network on Avian Influenza. The lyophilized material was dissolved in double distilled water. These solutions were aliquoted and stored at −80 °C until further use. The enzymatic activity of the supernatant was assessed as previously described.[18,19]

2.4. Neuraminidase inhibition assay

2.4.1. Analysis of inhibition against N1

In a microtitre plate, 90 μl of 33 mM MES buffer pH 6.5 containing 4 mM $CaCl_2$ were dispensed in a suitable number of wells. Since **PMC-35** was insoluble in 33 mM MES buffer pH 6.5, 10% DMSO was added in this case. Ten microliter of 1 mM oseltamivir carboxylate and its analogs were added in a first well, and the solution was thoroughly mixed obtaining a 0.1 mM inhibitor concentration. A 10 μl aliquot of this solution was transferred in a second well and thoroughly mixed, reaching after dilution an inhibitor concentration of 0.01 mM. The process was continued until a seventh, 0.1 nM inhibitor concentration was obtained, at the end of the dilution process. Each well contained a final volume of 90 μl. Twenty-five microliter of enzyme stock solution were added to each well, and the mixtures were incubated for 2 h at 37 °C; after the incubation time, 25 μl of a 20 μM MUNANA solution were added, with final concentrations of oseltamivir carboxylate or its analogs spanning the 0.064 nM–64.2 μM range. After incubating the plate for 5 h at 37 °C, 50 μl of stop solution (0.1 M glycine in 25% aqueous EtOH, pH 10.7) were added. The fluorescence of the solutions contained in each well was read with the Victor[3] multilabel counter. The 50% inhibitory concentration (IC_{50}) was determined from the dose–response curve using the GraphPad Prism 5 software (GraphPad, San Diego, CA). All measurements were replicated in 3–5 independent experiments.

2.4.2. Analysis of inhibition against N3

2.4.2.1. Procedure for PMC-34 (4), PMC-35 (5), PMC-36 (6) and oseltamivir free acid. Ninety microliter of 33 mM MES buffer pH 6.5 containing 4 mM $CaCl_2$ were dispensed in a suitable number of wells in a microtiter plate. Namely, 10 μl of 10 μM inhibitor solutions (**PMC-34**, **PMC-35** and commercial oseltamivir) were added in a first well and the mixture was thoroughly mixed obtaining a 1 μM inhibitor concentration. A 10 μl aliquot of this solution was transferred in a second well and thoroughly mixed obtaining a 0.1 μM inhibitor solution. The process was continued until a seventh 0.001 nM solution was obtained, at the end of the dilution process. Each well contained a final volume of 90 μl. Twenty-five microliter of enzyme stock solution were added to each well, and the mixtures were incubated for 2 h at 37 °C; after the incubation time, 25 μl of a 20 μM MUNANA solution were added, with final concentrations of oseltamivir carboxylate or its analogs spanning

Table 1
Kinetic parameters of N1 and N3

Recombinant protein	Neuraminidase activity (mU/mg)	$K_m \pm SD$[a] (μM)	$IC_{50} \pm SD$[a,b] (nM)
N1	0.04	400 ± 60	25 ± 4
N3	0.4	87 ± 4	0.2 ± 0.02

[a] K_m, IC_{50} values were obtained from three independent experiments.
[b] IC_{50} is a concentration of oseltamivir which inhibits by 50% the neuraminidase activity.

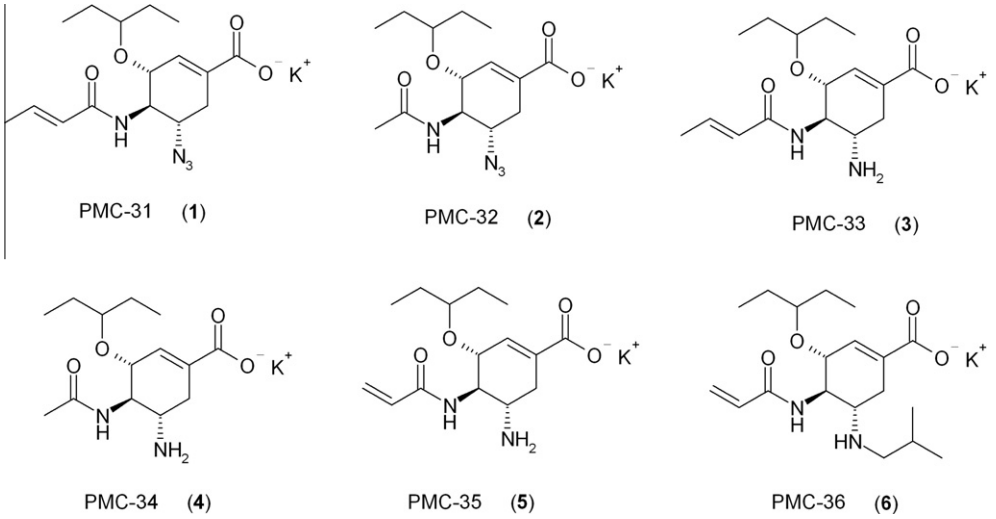


Figure 1. Structures of the compounds tested in the study.

the 0.00064–642 nM range. After incubating the plate for 5 h at 37 °C 50 μl of stop solution (0.1 M glycine in 25% aqueous EtOH, pH 10.7) were added. The fluorescence of the solutions contained in each well was read with the Victor[3] multilabel counter. The 50% inhibitory concentration (IC_{50}) was determined from dose–response curve using a GraphPad Prism 5 software (GraphPad, San Diego, CA). All measurements were replicated in 3–5 independent experiments.

2.4.2.2. Procedure for PMC-31 (1), PMC-32 (2), PMC-33 (3). For **PMC-31** (**1**), **PMC-32** (**2**), **PMC-33** (**3**), the same procedure described for the N1 study was used. The 50% inhibitory concentration (IC_{50}) was determined from dose–response curves using the GraphPad Prism 5 software (GraphPad, San Diego, CA). All measurements were replicated in 3–5 independent experiments.

3. Results and discussion

3.1. Characterization of recombinant neuraminidase

Both N1 and N3 containing supernatants were examined to assess their enzymatic activity as previously described.[23,24] The concentration of active enzyme in both the supernatants was determined by incubating each preparation with excess substrate and monitoring the extent of substrate cleavage over time. The amount of released product was monitored fluorimetrically, the concentration of 4-methylumbelliferone was determined by comparison with a calibration curve obtained by using a reference sample of 4-methylumbelliferone. This analysis revealed that the N3 containing supernatant displayed a neuraminidase activity 10 times higher than the N1 containing supernatant. The measured concentrations of active enzyme were 0.4 mU/mg in the case of N3 and 0.04 mU/mg in the case of N1 where one unit of enzyme activity was defined as the amount of active neuraminidase enzyme required to convert one micromole of substrate per minute at 37 °C. The enzymes were further characterized by determining their Michaelis–Menten constant against the substrate; the Michaelis constant of N1 was 400 ± 60 μM, while that of N3 was 87 ± 4 μM. The inhibitory activity of oseltamivir carboxylate against the two enzymes was assessed and used as reference to compare the different inhibitors synthesized. The IC_{50} of commercially available oseltamivir carboxylate on N3 and N1 were found to be 0.2 ± 0.02 nM and 25 ± 4 nM, respectively, the obtained data are reported in Table 1.

3.2. Synthesis of oseltamivir carboxylate (PMC 34) and of oseltamivir analogs PMC 31–33, PMC 35,36

The structures of oseltamivir carboxylate **PMC-34**/compound **4** and of five oseltamivir analogs **PMC 31–33**, and **PMC 35–36** (compounds **1–3**, **5** and **6**), prepared and biologically characterized in this study as potassium salts, are reported in Figure 1.

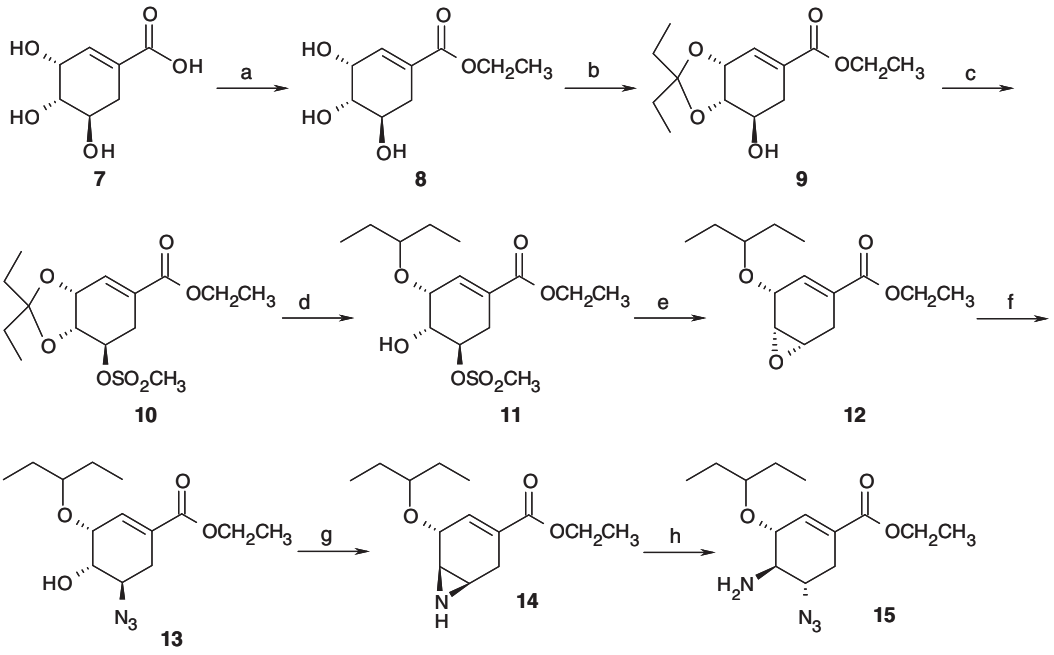
Their synthesis was achieved through the adaptation of some published experimental protocols,[14–26] and required at first the preparation of a common, key amino-azide intermediate **15** (Scheme 1).

Key intermediate **15** was acylated (**PMC 31** and **32**), acylated and reduced (**PMC-33–35**), or acylated, reduced and alkylated (**PMC-36**) to provide, respectively, the six carboxylate esters **16**, **17**, **19**, **21**, **24** and **26**. The six esters were hydrolyzed with KOH to give the six potassium carboxylates **PMC 31–36** (Scheme 2).

3.3. Neuraminidase Inhibition assay

3.3.1. Inhibitory activity of oseltamivir analogs on N1

All the inhibitors were tested at different concentrations, spanning the range 0.064 nM–64.2 μM. A dose–response curve was obtained for each compound. Fitting of the experimental data

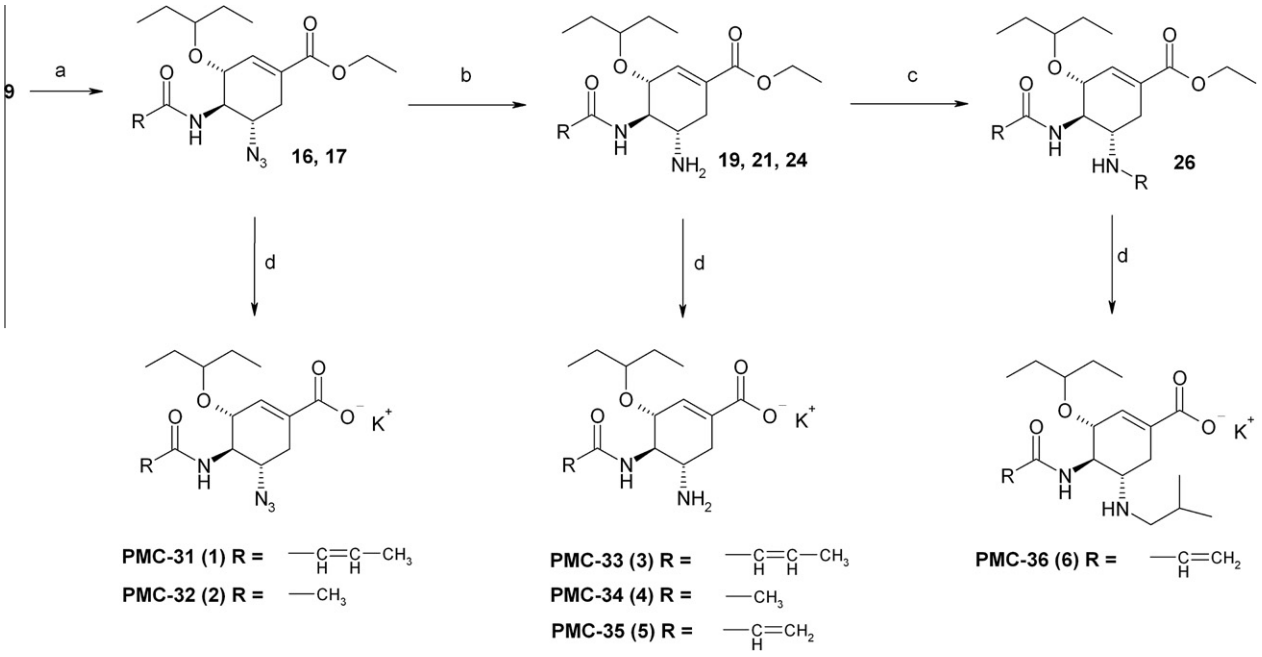


Scheme 1. Reagents and conditions: (a) Ethanol, $SOCl_2$, reflux 3 h; (b) 3-pentanone, *p*-TSA, toluene, 100 °C, MW, 15 min; (c) MsCl, TEA, DCM, rt, 2 h; (d) Et_3SiH, $TiCl_4$, DCM, −35 °C, 1 h; (e) $KHCO_3$, H_2O, EtOH, 60 °C, 1 h; (f) NaN_3, NH_4Cl, H_2O, EtOH, 68 °C, 14 h; (g) PMe_3, THF, CH_3CN, 25 °C, 10 min; (h) NaN_3, NH_4Cl, DMF, 70 °C, overnight.

allowed the determination of the IC_{50} for each compound; the experimental data are graphically reported in Figure 2.

The analysis of the experimental data indicated **PMC-36** (**6**) as the best inhibitor of N1 activity within the panel of tested compounds, displaying an IC_{50} of 14.6 ± 3.0 nM, while commercial oseltamivir displayed an IC_{50} of 24.9 ± 3.9 nM; a synthetically prepared sample of oseltamivir, **PMC-34** (**4**), was also included in this analysis and it displayed an IC_{50} of 39.3 ± 3.2 nM (Table 2). Compound **PMC-35** (**5**) displayed an IC_{50} of 31.8 ± 6.9 nM, while **PMC-32** (**2**) was less active with an IC_{50} of 84.4 ± 20 nM. Both **PMC-33** (**3**) and **PMC-31** (**1**) had a limited inhibitory activity with an IC_{50} of 138.9 ± 21 μM and 51.6 ± 3.0 μM, respectively (Table 2).

The variations in the measured inhibition for the different compounds revealed that an isobutyl group installed on the 5-amino group site to form a secondary amine is well tolerated and appears to increase the affinity for the enzyme, as shown in the case of **PMC-36** (**6**). This might be because the alkyl group affects the strengths of hydrogen bonds of the amine group with acidic residues of the N1 neuraminidase, or more probably due to favorable van der Waals contacts in the 150-loop cavity, which is located



Scheme 2. Reagents and conditions: (a) Acyl chloride, NMM, DCM, rt; (b) PMe_3, DCM, 1 M HCl, rt; (c) isobutyraldehyde, DCM, then $NaBH(OAc)_3$, 0 °C to rt; (d) 1 M aq KOH, dioxane, 4–23 h, rt.

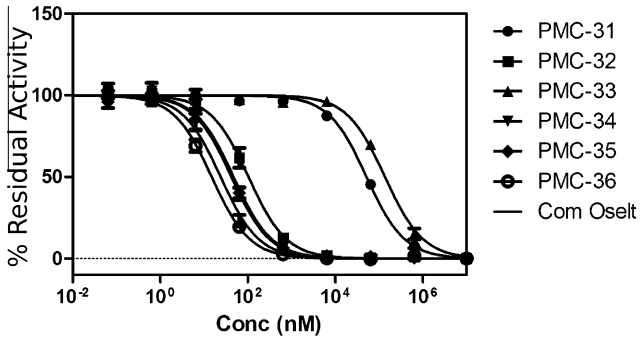


Figure 2. Titration curves of oseltamivir and its analogs against N1.

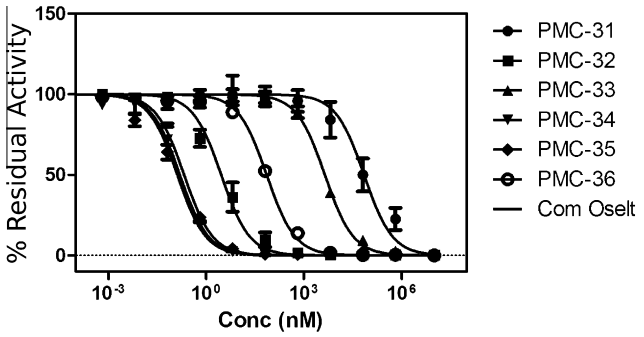


Figure 4. Titration curves of oseltamivir and its analogs against N3.

Table 2
The IC_{50} of oseltamivir analogs on N1 and N3

Compounds	IC_{50} ± SD on N1 (nM)	IC_{50} ± SD on N3 (nM)
PMC-31	51,576 ± 2904	99,295 ± 32,416
PMC-32	84.4 ± 20	2.2 ± 1.4
PMC-33	138,962 ± 21,589	4512 ± 1172
PMC-34 (oseltamivir)	39.3 ± 3.2	0.1 ± 0.08
PMC-35	31.8 ± 6.9	0.1 ± 0.03
PMC-36	14.6 ± 3.0	28.1 ± 9.7
Commercial oseltamivir	24.9 ± 3.9	0.2 ± 0.02
Oseltamivir carboxylate[a]	36.1[b] – 53.2[c]	2.9[d] – 3.3[e]

[a] Taken from Govorkova et al., 2001.[21]
[b] Influenza virus: A/teal/Hong Kong/W312/97 (H6N1).
[c] Influenza virus: A/duck/Alberta/35/76 (H1N1).
[d] Influenza virus: A/duck/Singapore/3/97 (H5N3).
[e] Influenza virus: A/duck/Germany/1215/73 (H2N3).

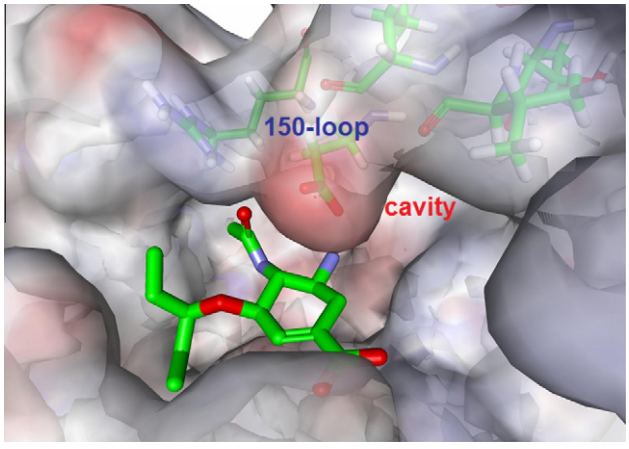


Figure 3. Oseltamivir binding site in the crystal structure of neuraminidase N1.[14] Partially transparent molecular surface (colored according to interpolated atomic charge) shows the 150-loop and the associated 150-cavity specific for the group-1 neuraminidases. Oseltamivir is shown in a stick representation, hydrogen atoms are omitted for better clarity. Coloring scheme: hydrogen: white, carbon: green, nitrogen: blue, oxygen: red).

in the vicinity of the amino and acetamido groups of bound oseltamivir (Fig. 3).[14]

Moreover, a small increase in size for the acyl group on the 4-amido group from the original acetyl group of oseltamivir also seems to enhance the inhibitory activity of the novel species, as observed in the case of **PMC-36** (**6**) and **PMC-35** (**5**). However, this position seems to be intolerant to the introduction of larger groups, since the presence of a 2-butenyl amide dramatically reduces the affinity of compound **PMC-33** (**3**) with respect to the oseltamivir benchmark. The IC_{50} of commercial oseltamivir on N1 did not perfectly match that of synthesized oseltamivir (**PMC-34**), but its variability is well within the acceptable limits. The titration curves and the IC_{50} values of all the tested compounds on N1 are reported in Figure 2 and Table 2, respectively.

3.3.2. Inhibitory activity of oseltamivir analogs on N3

For neuraminidase N3, all the inhibitors were tested at different concentrations either spanning the 0.64 pM–642.8 nM range (**PMC-34** (**4**); **PMC-35** (**5**), commercial oseltamivir), or the 0.064 nM–64.2 μM range (**PMC-31** (**1**), **PMC-32** (**2**), **PMC-33** (**3**); **PMC-36** (**6**)). A dose–response curve was obtained for each compound, and fitting of the experimental data allowed the determination of the IC_{50} of each putative inhibitor. The dose–response curves for each compound are reported in Figure 4.

Among the tested compounds **PMC-35** (**5**), **PMC-34** (**4**) and commercial oseltamivir showed a potent inhibitory activity on neuraminidase subtype 3 with IC_{50} of 0.1 ± 0.03, 0.1 ± 0.08 and 0.2 ± 0.02 nM, respectively. All the other compounds displayed a lower inhibitory potency, with IC_{50} values ranging from 2.2 ± 1.4 nM for **PMC-32** (**2**) to 99.3 ± 32.4 μM for **PMC-31** (**1**). The data for the whole set of tested compounds is reported in Table 2.

The differences in the observed inhibitory properties of the investigated species indicated that the primary 5-amino group is essential for inhibitory activity on neuraminidase subtype 3. Modification of the amine seems to decrease the activity, as shown for **PMC-32** (**2**) and **PMC-36** (**6**); this observation infers a different trend in comparison with N1, where **PMC-36** (**6**) outperformed **PMC-35** (**5**). Adding a small group on the 4-amido function as for **PMC-35** (**5**) does not affect the activity. In contrast, the presence of a bulky group on the amide as in **PMC-31** (**1**) and **PMC-33** (**3**) greatly decreases the ability to inhibit the N3 neuraminidase. The IC_{50} of commercial oseltamivir is similar to that of synthesized oseltamivir (**PMC-34**). Higher inhibitory potency of oseltamivir towards the group-2 neuraminidase N3 over the group-1 neuraminidase subtype N1 as reported also by other groups.[9]

4. Conclusion

The modification at both the 5-amino and 4-amido group of oseltamivir carboxylate is needed in order to make more efficient neuraminidase inhibitors based on this scaffold. In this paper, we reported a preliminary activity screening of novel putative neuraminidase inhibitors obtained by computer-assisted combinatorial design on the basis of the oseltamivir structure as well as the crystal structure of N1 neuraminidase. The collected data suggest that the introduction of small substituents on the 5-amino group and 4-acetamido group of oseltamivir increases the affinity of the new analogs against N1, but it seems to be rather ineffective with respect to N3. In addition, the introduction of large substituents on

the 4-amino group of oseltamivir carboxylate decreases the affinity of the compounds for N1 while this structural modification seems to be somewhat better tolerated by the N3. Although the investigated analogs present an activity similar to that of oseltamivir carboxylate, among the panel of analyzed compounds two different species, namely **PMC-36** (**6**) and **PMC-35** (**5**), displayed a promising activity as inhibitors of neuraminidase N1 and N3, respectively. In general, neuraminidase N3 was more sensitive to the structural modifications of the oseltamivir analogs, while neuraminidase N1 was less sensitive. This is most probably due to the presence of the cavity formed by the 150-loop in the group-1 neuraminidases located next to the 5-amino and 4-acetamido groups of bound oseltamivir, which can more readily accommodate the aliphatic extensions of these groups.

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Supplementary data

Supplementary data (synthetic procedures and spectral data of the described compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.026.

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