



Susceptibility of antiviral drugs against 2009 influenza A (H1N1) virus

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ABSTRACT

The recent outbreak of the novel strain of influenza A (H1N1) virus has raised a global concern of the future risk of a pandemic. To understand at the molecular level how this new H1N1 virus can be inhibited by the current anti-influenza drugs and which of these drugs it is likely to already be resistant to, homology modeling and MD simulations have been applied on the H1N1 neuraminidase complexed with oseltamivir, and the M2-channel with adamantanes bound. The H1N1 virus was predicted to be susceptible to oseltamivir, with all important interactions with the binding residues being well conserved. In contrast, adamantanes are not predicted to be able to inhibit the M2 function and have completely lost their binding with the M2 residues. This is mainly due to the fact that the M2 transmembrane of the new H1N1 strain contains the S31N mutation which is known to confer resistance to adamantanes.

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Introduction

Since March 2009, the outbreak of a new strain of influenza A (H1N1) virus infection in humans has raised increasing concerns of the risk of a global flu epidemic. Many countries around the world, especially The United States of America and Mexico, have formally reported symptomatic human infections of this new A (H1N1) virus [1]. The most effective antiviral agent, oseltamivir (OTV), is recommended by The World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) for treatment of the infected patients, whilst the new A (H1N1) strain confers resistance to amantadine (AMT) and rimantadine (RMT). To overcome this or any other future pandemic, this urgent event needs a detailed understanding at the molecular level of any inhibitory machinery of such commercially available and well stocked front line drugs.

The new A (H1N1) virus contains the combination of gene segments of swine, avian and human influenza viruses. Based on genetic characterization, the hemagglutinin (HA) gene is similar to that of the swine influenza virus currently circulating amongst USA pigs, whilst the neuraminidase (NA) and matrix protein (M)

genes are similar to those of swine influenza viruses isolated from Europe. This unique genetic combination has not previously been detected elsewhere, and none of the patients have had direct contact with pigs leading to the possibility of increased human-to-human transmission of this new influenza virus [2]. In late April 2009, WHO has raised the pandemic alert for influenza A (H1N1) to phase 5, which is when the spread of disease between humans is occurring in more than one country [3].

Due to antigenic differences amongst influenza A strains, the current seasonal influenza vaccines cannot provide protection against this new strain of A (H1N1) influenza virus. Up to date, there are two classes of anti-influenza agents: (i) NA inhibitors, oseltamivir and zanamivir, protecting the release and spread of progeny virions; (ii) adamantane derivatives, amantadine and rimantadine, preventing the proton transfer in the M2 ion-channel [4]. The A (H1N1) viruses isolated from patients in USA and Mexico are sensitive to NA inhibitors but show resistance to adamantane derivatives [5].

To gain the fundamental knowledge on the structure and the drug–target interactions of the new strain of influenza A (H1N1) virus, homology modeling and molecular dynamics (MD) simulations were carried out on the three inhibitor–enzyme complexes: OTV-NA, AMT-M2 and RMT-M2. The present study is an extension from, and is compared to, our previous works on avian influenza H5N1 virus which were focused to understand the structural

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properties, intermolecular interactions and predictive inhibitory potencies of both wild- and mutant-type viruses at the NA and M2 targets [6–10].

Materials and methods

Models of the 2009 H1N1 influenza neuraminidase and M2-channel. The initial structures of the new strain of A (H1N1) influenza NA (N1/09H1N1) and M2-channel (M2/09H1N1) were modeled based on the available 3D structures and the genomic sequence data of virions recently isolated from infected patients in southern California, A/California/04/2009 (H1N1). Here, the crystal structure of avian influenza N1 (N1/04H5N1) with oseltamivir bound, (PDB entry code: 2HU4) [11], and the solid state NMR structure of the transmembrane segment of a homotetrameric M2 channel (2H95.PDB) [12], were used as the templates. The sequence alignment was performed using the homology modeling module implemented in the Discovery Studio 2.0 software. Relative to the amino acid sequences of the templates, the N1/09H1N1 and M2/09H1N1 proteins had 92% and 84% identity, respectively. To prepare the OTV-N1/09H1N1 complex, superimposition of OTV-N1/04H5N1 with N1/09H1N1 was performed and the N1/04H5N1 atomic coordinates were then deleted. For the AMT-M2/09H1N1 and RMT-M2/09H1N1 structures, the inhibitor was docked into the tetrameric M2 channel using the Autodock 3.0 program [13]. All built models were further refined by energy minimization and consequently MD simulations were performed for 20 ns on the NA complex, and for 8 ns on the M2 systems. All calculations were set up according to our previous studies on avian influenza NA [14] and M2-channel [9,10] (see detailed methodology in Supplementary material).

Linear interaction energy method. The linear interaction energy (LIE) method was used to calculate the binding free energies (ΔG_{bind}) of the oseltamivir bound to the NA enzyme. This method is a linear response semiempirical technique used to evaluate the free energy changes from the simulations of two states: (i) the solvated ligand (free state), and (ii) the ligand bound to the solvated protein (bound state). Based on the LIE method, the total binding free energy was contributed from van der Waals (U^{vdw}) and electrostatic interaction energies (U^{elec}) using the equation given below:

$$\Delta G_{\text{bind}} = \alpha(\langle U^{\text{vdw}} \rangle_{\text{bound}} - \langle U^{\text{vdw}} \rangle_{\text{free}}) + \beta(\langle U^{\text{elec}} \rangle_{\text{bound}} - \langle U^{\text{elec}} \rangle_{\text{free}}) + \gamma \quad (1)$$

where α and β are empirical scaling coefficients for the van der Waals and electrostatic interaction energies, respectively, and γ is

Table 1

Binding free energies (ΔG_{bind}) of oseltamivir and its functional groups to the 2009 H1N1 influenza neuraminidase (N1/09H1N1) based on the MD/LIE approach. Means and standard deviations are derived from four separate 5 ns simulations. The ΔG_{bind} for the OTV-N1/04H5N1 complex and the experimental values are also given for comparison.

System	ΔG_{bind} [kcal·mol ⁻¹]				
	Oseltamivir	-COO ⁻	-NH ₃ ⁺	-NHAc	-OCH ₂ Et ₂
N1/09H1N1	-12.8 ± 0.9	-5.2 ± 0.4	-2.6 ± 0.5	-2.9 ± 0.2	-4.6 ± 0.9
N1/04H5N1	-11.4 ± 0.4	-4.1 ± 0.3	-2.8 ± 0.1	-2.6 ± 0.1	-4.5 ± 0.4
Experimental	(-11.8)–(-13.1)	-	-	-	-

a constant. Here, Essex's coefficients ($\alpha = 0.472$, $\beta = 0.122$ and $\gamma = 2.603$), efficiently derived from a statistical analysis of the inhibitor sets binding to the NA enzyme [15], were used to fit the LIE equation. This set of coefficients has previously been found to be the most predictive model for the NA system amongst the many developed models, with or without the addition of the hydration term.

Results and discussion

All calculations are modeled and discussed in comparison to our previous studies on the avian H5N1 influenza NA (N1/04H5N1) [6–8,14] and M2-channel (M2/04H5N1) [9,10]. Relative to N1/04H5N1, all residues in the N1/09H1N1 binding pocket are conserved except for one, the tyrosine (Y374) being replaced by asparagine (N374) (Fig. 1A). This is not the case for the M2-channel in which amongst the 19 key residues three were changed, that is the I28 and N31 at the extracellular site and T43 close to the gating tryptophan (W41) of M2/09H1N1, were changed from V28, S31 and L43 of the M2/04H5N1, respectively (Fig. 1B).

Efficiency of oseltamivir against influenza A (H1N1) neuraminidase

To estimate the binding free energies (ΔG_{bind}) of oseltamivir and its four sidechains against the 2009 A (H1N1) influenza NA, 20 ns of MD simulations of OTV-N1/09H1N1 modeled system were carried out using the LIE method with Essex coefficients (Eq. (1)). Note that the energy of each functional group was evaluated from the fitted LIE equation without the addition of the γ constant. The results are summarized in Table 1, where the corresponding values for avian H5N1 influenza NA complexed with oseltamivir [14], and the experimental energies converted from the IC₅₀ and K_i values [16–19], are also given for comparison.

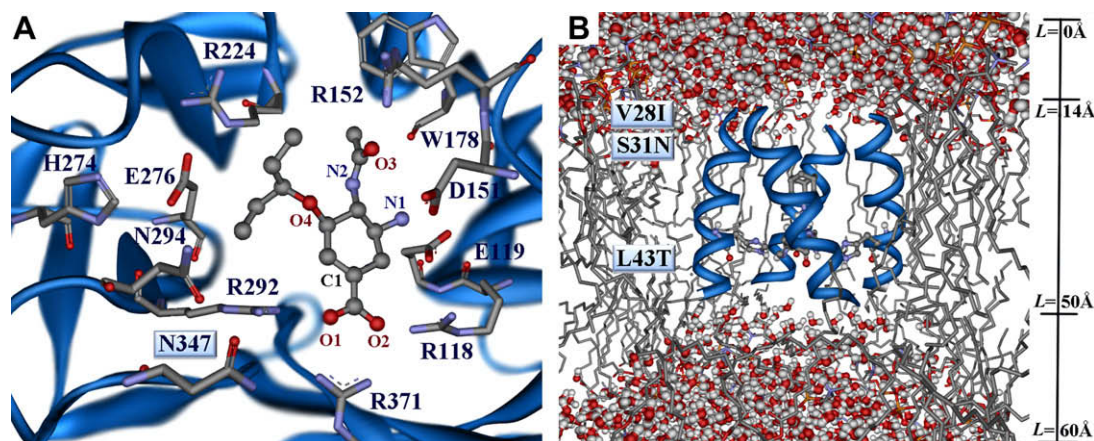


Fig. 1. Modeled structures of 2009 H1N1 influenza A virus: (A) oseltamivir bound to the binding site of neuraminidase, and (B) the adamantane inhibitor docked into the M2-channel. The residues which differ from those of avian H5N1 influenza are highlighted and some atoms are labeled for simplicity in the investigations and discussions.

The observed binding affinity of oseltamivir to the N1/09H1N1, at $-12.8 \text{ kcal} \cdot \text{mol}^{-1}$, is considerably higher than that of its binding to N1/04H5N1, by $1.4 \text{ kcal} \cdot \text{mol}^{-1}$, and falls within the range of experimental energies determined for the other N1 strains (-11.8 to $-13.1 \text{ kcal} \cdot \text{mol}^{-1}$) [16–19]. The contribution from each side chain of oseltamivir to the absolute binding free energy is in the following order: $-\text{COO}^- > -\text{OCHET}_2 > -\text{NHAc} \sim -\text{NH}_3^+$ with the corresponding values of -5.2 , -4.6 , -2.9 and $-2.6 \text{ kcal} \cdot \text{mol}^{-1}$, respectively (Table 1). These predicted energies were not notably different from those of the avian N1 system, except for the $-\text{COO}^-$ group of N1/09H1N1 which shows an increase in the energetic contribution of $1.1 \text{ kcal} \cdot \text{mol}^{-1}$. The calculated binding affinities lead us to conclude that the new A (H1N1) influenza virus is more sensitive to oseltamivir than the avian H5N1 influenza.

To examine the efficiency of oseltamivir binding to the new A (H1N1) influenza NA protein, the percentage and number of hydrogen bond (H-bond) interactions between oseltamivir and the NA binding residues were determined using the following criteria: (i) the distance between proton donor (D) and acceptor (A) atoms $\leq 3.5 \text{ \AA}$ and (ii) the D–H...A angle $\geq 120^\circ$. The results are shown in Fig. 2A (where description is given in Table S1, Supplementary materials) in comparison to the H5N1 influenza [14]. Distributions of the H-bond distance for the 118 and 347 residues are plotted in Fig. 2B.

In comparison between the two complexes, dramatic changes were found at the $-\text{COO}^-$ group of oseltamivir where a strong H-bond with R118 in the N1/09H1N1 system with 84% occupation (Fig. 2A), and the preferential O2–NH2 (R118) distance of 2.9 \AA , was newly formed (Fig. 2B, grey line). This is different for the N1/04H5N1 in which this distance takes place at $\sim 4.2 \text{ \AA}$ (Fig. 2B, black line). The loss of the H-bond with N347 in the N1/09H1N1 is due to its smaller side chain relative to that of Y347 for the N1/04H5N1 (Fig. 2A). This notion is supported by the distribution plots of the H-bond distances (Fig. 2C), in which the maxima of the two peaks were found at 2.8 and 5.3 \AA for N1/09H1N1 and N1/04H5N1, respectively. Note that the significance of Y347 was reported in a previous study on oseltamivir-resistance in the N1/04H5N1 isolate as being due to the N294S mutation, which was found to increase the O1(OTV)–OH(374) H-bond distance by $\sim 0.5 \text{ \AA}$, i.e., the occupation percentage of this H-bond was relatively decreased [14].

Interestingly, the residues within a spherical radius of 5 \AA around the oseltamivir of the new strain are completely identical to those of group-2 NA subtype N9 as shown in Fig. 3. In addition, oseltamivir was designed to fit to the cavity of this NA group. Superimposition of the snapshots of both the OTV–N1/04H5N1 and the OTV–N1/09H1N1 systems with the crystal structure of oseltamivir bound to N9 (2QWK.PDB) are consistent with the above statement. The guanidinium group of R118 in the N1/09H1N1 and N9 structures approaches significantly closer to stabi-

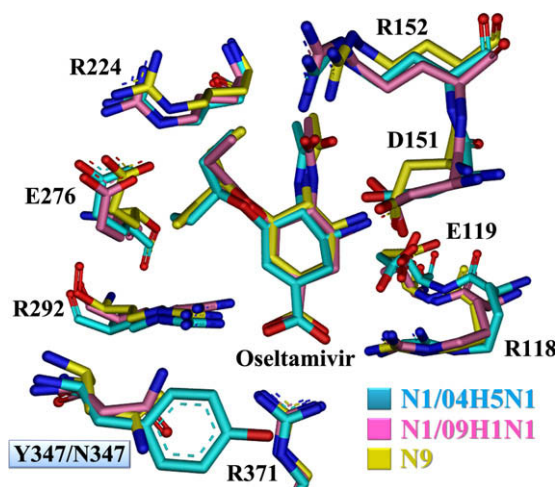


Fig. 3. Superimposition between the MD snapshots of OTV–N1/04H5N1 (cyan) and OTV–N1/09H1N1 (pink), and the crystal structure of OTV–N9 (yellow), where the closed view of oseltamivir in the binding pocket was shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lize the $-\text{COO}^-$ group of oseltamivir than that observed in the N1/04H5N1 complex. In contrast, the Y347 side chain of the N1/04H5N1 was, as expected, shown to locate much closer to oseltamivir than the other two systems.

2009 H1N1 influenza M2-protein resistance to adamantane derivatives

The susceptibility of the M2-channel of the 2009 H1N1 influenza (M2/09H1N1) to both amantadine and rimantadine is displayed in terms of water density along the channel (Fig. 1B). The results for both closed (0H) and open (3H) states, in comparison to those of the avian influenza H5N1 strain (M2/04H5N1) [9], are given in Fig. 4. To monitor how deep the amantadine and rimantadine molecules can penetrate into the M2-channel, the inhibitor distributions were also plotted in Fig. 4, in which the C^α positions of the four A30 and H37 residues were averaged and depicted as the reference. Note that in changing from the M2 channel of avian H5N1 to the new A (H1N1) influenza, three residues (V28, S31 and L43) were, respectively, replaced by I28, N31 and T43 (Fig. 1B). Amongst those changes, S31N is the most common mutation in the M2 protein which is known to confer adamantane-resistance [20–22] and, thus, the M2/09H1N1 resistance to both amantadine and rimantadine would be expected.

Considering all the inhibitor distributions (filled area), amantadine (dark grey) was found to locate deeper into the channel than

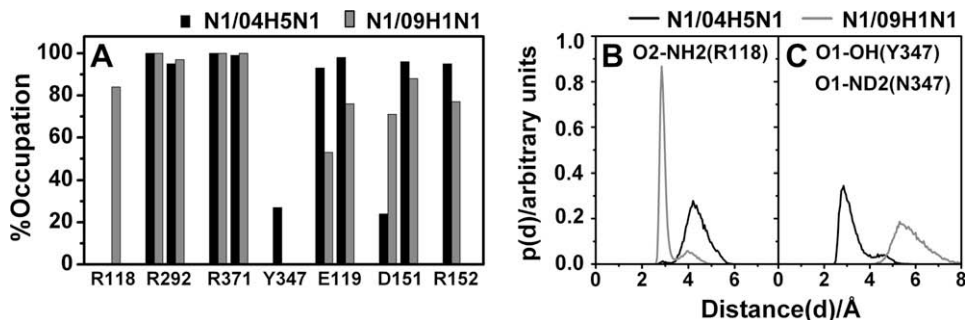


Fig. 2. (A) Percentage occupation of hydrogen bonding of oseltamivir (A) and its binding residues (see Fig. 1A for labels). The distribution plots of the H-bond distances for the NA residues: (B) R118 and (C) Y347/N347.

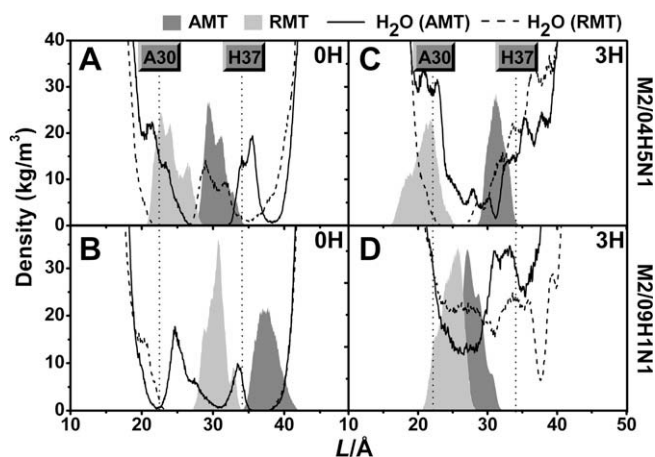


Fig. 4. Water density along the channel as a function of distance L starting from the extracellular site (see Fig. 1B for definition) for the new A (H1N1) M2 strain complexed with two inhibitors (amantadine (AMT) and rimantadine (RMT)) in the closed (OH) and open (3H) states. AMT and RMT distributions (filled area) were shaded by dark and light grey, whereas the vertical dashed lines represent the average C^α positions of the four A30 and H37 residues.

rimantadine (light grey). In the closed state (OH), zero-water density was observed for both M2 strains with the inhibitor bound (Fig. 4A and B), indicating that water cannot transport throughout the OH channel. In addition, zero water density is not detected before the filled area in all the examined systems. This, as expected, reveals that in the M2 channel, water cannot move across inhibitor in the closed state.

Dramatic changes were found for the open state (3H), where only rimantadine can inhibit water transportation through the M2/04H5N1 (Fig. 4C, dashed line). For the AMT-M2/04H5N1 complex, an almost zero-water density was slightly observed at $L \sim 32$ Å, which is in contrast to what is observed in the new A (H1N1) M2 strain where both inhibitors totally lose their functions (Fig. 4D). In addition, the detected density along the M2/09H1N1 channel is greater than those of the singlet S31N and A30T mutations for the AMT-M2/04H5N1, in which the experimental resistance degree for these two mutations are 267- and 3300-fold, respectively [10]. Therefore, the only conclusion based on this data is that the M2/09H1N1 resistance to amantadine is likely to be higher than what was previously observed.

As the inhibitory potency depends on the drug-target interaction, the H-bond between the ammonium group of the inhibitor and the viral M2 residues was measured, and the data are summarized in Table 2. As can be seen, there are two binding regions of inhibitors to the channel: (i) the extracellular site at residues L26, A30 and S31; and (ii) the H37 selective filter. Due to the bulky hydrophobic unit, the two adamantanes were found to interact weakly with the M2/04H5N1 channel in both states, whilst they completely lost their binding in the 3H state of the new M2 strain. Disappearance of H-bonding of the inhibitor to the M2 channel is consistent with the observed loss of adamantane susceptibility in blocking the M2/09H1N1 channel that contains the S31N-drug resistance as well as the replacement of the other two residues at the positions 28 and 43.

In conclusion, the evidence above suggests that both adamantanes will have lost their inhibitory activities towards the M2-channel of the 2009 influenza A (H1N1) virus, mainly due to this virus containing the S31N mutation which is found, in general, to be the main source of adamantane drug resistance against all influenza subtypes [20–22]. This mutation was proposed to indirectly increase the M2-protein mobility [23], and thus the drugs were unable to bind the M2 channel and to block the proton transport in this new A (H1N1) influenza.

Table 2

H-bond occupation between the residues of the M2-channel and the two drugs, amantadine and rimantadine, where I–IV refers to the M2 homotetrameric bundles. The results are given for both closed (OH) and open (3H) states of the M2-channel of the A (H5N1) and the new A (H1N1) influenza viruses.

System	% H-bond			
	AMT-M2/ 04H5N1	RMT-M2/ 04H5N1	AMT-M2/ 09H1N1	RMT-M2/ 09H1N1
<i>(i) OH state</i>				
A30-III	–	12	–	–
H37-I	–	–	13	22
H37-II	–	–	–	21
H37-III	56	–	–	–
H37-IV	29	–	–	–
<i>(ii) 3H state</i>				
L26-II	–	9	–	–
S31-II	20	–	–	–
S31-IV	–	10	–	–

Conclusions

In the present study, homology modeling and MD simulations were applied on the commercially available drugs bound to the NA and M2-channel of the new influenza A (H1N1) virus. Based on the MD/LIE method, the predicted binding affinity of oseltamivir towards the new A (H1N1) influenza isolate was considerably higher than the avian H5N1 strain. Except for the absence of a weak H-bond with residue 347, all interactions of OTV-N1/09H1N1 complex were considerably conserved. Interestingly, oseltamivir was well oriented in the binding pocket and its $-\text{COO}^-$ group interacted strongly with the arginine triad, similar to that found in the crystal structure of N9. For M2/09H1N1 channels with two adamantanes bound, water transport explicitly passed throughout the channel of the 3H state, representing the activated channel at a low pH. Either amantadine or rimantadine have totally lost the H-bond interactions with the M2 residues in this state, which results from the M2 transmembrane domain containing the S31N mutation as well as other two residues, I28 at the extracellular site and T43 close to the W41 gating residue, which differs from the avian H5N1. Overall, the simulated results have clearly explained at a molecular level how anti-influenza drugs can either potently inhibit (oseltamivir) or not (amantadine and rimantadine) the new A (H1N1) influenza virus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.066.

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