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ORIGINAL ARTICLE

Molecular prediction of oseltamivir efficiency against probable influenza A (H1N1-2009) mutants: molecular modeling approach

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Abstract To predict the susceptibility of the probable 2009 influenza A (H1N1-2009) mutant strains to oseltamivir, MD/LIE approach was applied to oseltamivir complexed with the most frequent drug-resistant strains of neuraminidase subtypes N1 and N2: two mutations on the framework residues (N294S and H274Y) and the two others on the direct-binding residues (E119V and R292K) of oseltamivir. Relative to those of the wild type (WT), loss of drug–target interaction energies, especially in terms of electrostatic contributions and hydrogen bonds were dominantly established in the E119V and R292K mutated systems. The inhibitory potencies of oseltamivir towards the WT and mutants were predicted according to the ordering of binding-free energies: WT (-12.3 kcal mol^{-1}) > N294S (-10.4 kcal mol^{-1}) > H274Y (-9.8 kcal mol^{-1}) > E119 V (-9.3 kcal mol^{-1}) > R292K (-7.7 kcal mol^{-1}), suggesting that the H1N1-2009 influenza with R292K substitution, perhaps, conferred a high level of oseltamivir resistance, while the other mutants revealed moderate resistance levels. This result calls for an urgent need to develop new potent anti-influenza agents against the next pandemic of potentially higher oseltamivir-resistant H1N1-2009 influenza.

Keywords 2009-H1N1 influenza A neuraminidase · Oseltamivir resistance · Mutations · Molecular dynamics simulations

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Introduction

The 2009 influenza A (H1N1) virus has rapidly spread across the world with an evidence of human to human transmission. The probable mutation in the neuraminidase (NA) genes could cause resistance to the available drugs, especially oseltamivir. A new drug-resistant strain probably leads to a large scale outbreak of novel pandemic flu and an increase the national and global public health concerns. Common mutations in N1 (a subtype in NA group 1) are detected at N294S and H274Y, while the E119V and R292K mutations are mostly found in the N2 and N9 subtypes (in NA group 2), with oseltamivir resistance levels relative to the wild type (WT) of 20–80, 700–1,700, 20–1,000 and 1,500–10,000-fold higher, respectively (Abed et al. 2008; Collins et al. 2008; Yen et al. 2005, 2007). To date, oseltamivir has been found to effectively inhibit this new virus [2009 A (H1N1)] due to the following reasons: the N1 of the new H1N1 influenza and N9 share an identical active site (Rungrotmongkol et al. 2009a, b), and oseltamivir was designed to fit well to the active site of the NA group 2. With an increase in medical use and stockpile of oseltamivir for the recent

outbreak, the question arises, and is the main goal of this study, can we predict the inhibitory activity of oseltamivir with respect to those frequent mutations that take place in the influenza A (H1N1-2009) strain, and thus the potential evolution and spread of resistant strains. The oseltamivir-resistant influenza NA mutants would perhaps serve as the emergence of a potential pandemic strain of the 2009-H1N1 virus.

Oseltamivir is an antiviral drug against NA that functions by preventing viral replication in the last step of the viral life cycle. It was found to directly interact with the catalytic residues of the NA active site, while the framework residues stabilized the enzyme structure (Fig. 1) (Ferraris and Lina 2008). Mutations at the conserved residues of NA appear to associate with oseltamivir resistance in a subtype specific manner. Thus, the mutated framework residues H274Y and N294S are regularly indentified in N1, while in the N2 and N9 sub-types, mutations on the binding residues (E119V and R292K) of oseltamivir were detected after treatment in infected patients with high oseltamivir resistance (Abed et al. 2008; Boivin and Goyette 2002; Collins et al. 2008; Mishin et al. 2005; Zürcher et al. 2006).

To provide information at the molecular level to aid the control and prevention of emerging potential pandemic strains of the 2009-H1N1 influenza, multi-molecular dynamics (MD) simulations in conjunction with the linear interaction energy (LIE) method have been performed on complexes of oseltamivir bound to each of the four most likely 2009-H1N1-mutated strains; that is, with the H274Y, N294S, E119V and R292K substitutions. The structural property, drug–target interaction and the binding affinity of oseltamivir against the mutated models are extensively discussed and compared with those recently published for the wild-type strain of the 2009-H1N1 influenza A virus (Rungrotmongkol et al. 2009a, b).

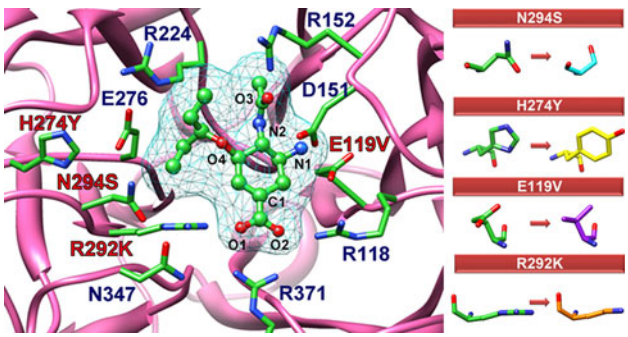

Fig. 1 Modeled structure of oseltamivir bound to the wild-type strain of 2009-H1N1 influenza neuraminidase. Among the labeled residues, four residues colored in *red* are singly mutated for investigation in this work: there are N294S, H274Y, E119V and R292K mutations. The selected atoms of oseltamivir are numbered for simplicity in the discussion

Materials and methods

The homology model of oseltamivir bound to the wild-type NA (OTV-WT) of the 2009-H1N1 virus (Rungrotmongkol et al. 2009a, b) was used as the initial structure for the modeling of the four single mutations: N294S, H274Y, E119V and R292K. To prepare each mutant, the specific residue was changed using the LEaP module of the AMBER 10 program package (Case et al. 2008), keeping the backbone and identical side chain atoms. All mutated NA strains with oseltamivir bound were then set-up and treated in accordance with the 20-ns MD simulations for the wild-type novel H1N1 influenza (Rungrotmongkol et al. 2009a, b), as follows.

Each simulated system was performed by MD simulations with spherical boundary condition under the surface constrained all atom solvent model (King and Warshel 1989) using the Q-program (Marelius et al. 1998), version 5. The atomic charges of oseltamivir were taken from our previous study (Malaisree et al. 2008). The AMBER force field (Case et al. 2008) was applied to the amino acid and inhibitor atoms. To set-up the environment for oseltamivir to be the most similar in all simulated systems and to take the conformational change of the oseltamivir into consideration, the C1 atom of oseltamivir was then chosen to be the center of simulation and the whole oseltamivir structure was thus considered as ligand. In the simulations, the system was capped by a 25 Å sphere of TIP3P water molecules centered on the C1 atom of oseltamivir (see Fig. 1 for atomic label). Atoms positioned further than 25 Å from the C1 center were taken into consideration as structural restrains. All acidic and basic side chains of residues lying within a 22 Å sphere were fully charged. In contrast, these ionizable residues positioning between 22 Å and 25 Å distances were neutralized, except for the pairs of charged residues with a probable formation of hydrogen-bonding interactions. The rest ionizable residues located outside a 25 Å sphere were considered as uncharged entities. Local reaction field approximation was employed for calculating the long-range electrostatic (ES) interactions, with a 10-Å cut-off radius for the non-bonded interactions. The SHAKE algorithm (Ryckaert et al. 1977) was applied to fix all bonds involving hydrogen atom. The NVT ensemble was performed, and a 2-fs time step was used. Initially, locations of the water molecules were simulated by MD simulations at 5 K, keeping all other atoms fixed to their initial positions, and the whole structure was then relaxed by four steps of simulations. Afterwards, the system was heated to 298 K over 300 ps, followed by equilibration phase. At last, the four equilibrated structures were randomly chosen for employing a production phase of 5-ns simulation.

To predict the binding-free energies (ΔG_{bind}) of oseltamivir towards the NA mutants, the LIE method (Åqvist

Table 1 Changes in the electrostatic and van der Waals interactions for the four substituent functional groups of oseltamivir in the bound states of the modeled H1N1 mutant relative to those of wild type

Functional group	$\langle \Delta\Delta U^{ES} \rangle_{bound}$ (kcal mol^{-1})			
	N294S	H274Y	E119V	R292K
(a) Electrostatic interactions				
–COO$^-$	8.3 ± 0.2	11.3 ± 0.1	5.4 ± 0.1	14.1 ± 0.2
–NH$_3^+$	−2.7 ± 0.4	−2.8 ± 0.5	11.1 ± 0.6	2.3 ± 1.2
–NHAc	3.0 ± 0.1	2.0 ± 0.1	6.0 ± 0.1	4.2 ± 0.2
–OCHEt$_2$	1.3 ± 0.6	0.6 ± 0.6	1.5 ± 0.9	4.3 ± 1.2
Oseltamivir	10.0 ± 0.6	11.1 ± 0.6	23.9 ± 0.9	24.9 ± 1.2
Functional group	$\langle \Delta\Delta U^{vdW} \rangle_{bound}$ (kcal mol^{-1})			
	N294S	H274Y	E119V	R292K
(b) Van der Waals interactions				
–COO$^-$	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.8 ± 0.3
–NH$_3^+$	0.6 ± 0.1	0.6 ± 0.0	−0.3 ± 0.1	0.8 ± 0.1
–NHAc	0.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.0	−0.7 ± 0.1
–OCHEt$_2$	1.4 ± 0.1	1.5 ± 0.0	0.1 ± 0.0	3.2 ± 0.2
Oseltamivir	2.5 ± 0.0	3.4 ± 0.0	1.2 ± 0.0	4.2 ± 0.2

Means and standard deviations are derived from four separate 5-ns simulations

et al. 1994; Hansson et al. 1998) was used. The total binding-free energy, which includes the van der Waals (vdW) (U^{vdW}) and the ES interaction energies (U^{ES}), of the two simulated states: (1) the solvated ligand (free state), and (2) the ligand bound to the solvated protein (bound state) were evaluated using the equation:

$$\Delta G_{bind} = \alpha\left(\langle U^{vdW}\rangle_{bound} - \langle U^{vdW}\rangle_{free}\right) + \beta\left(\langle U^{ES}\rangle_{bound} - \langle U^{ES}\rangle_{free}\right) + \gamma \quad (1)$$

where α and β are the empirical scaling coefficients for the vdW and ES interaction energies, respectively, and γ is a constant. Here, Wall's coefficients ($\alpha = 0.472$, $\beta = 0.122$ and $\gamma = 2.603$), which were efficiently derived from a statistical analysis of the inhibitor sets binding to the relevant NA enzyme (Wall et al. 1999) were chosen to fit the LIE equation due to the three following reasons. (1) Because the outbreak of the novel H1N1 pandemic flu was just arisen in April 2009, the experimental inhibitory activities required for the construction and validation the LIE model for the training set are not available. (2) This set of coefficients was successfully applied on the avian influenza A (H5N1) virus in prediction, the inhibitory activity of oseltamivir against both WT and mutant strains (Rungrotmongkol et al. 2009a, b). (3) The four single mutated strains of 2009-H1N1 neuraminidase virus in the present study were built by a specific mutation on the wild-type strain modeled from the crystal structure of the H5N1 neuraminidase with the sequence identity of 91%

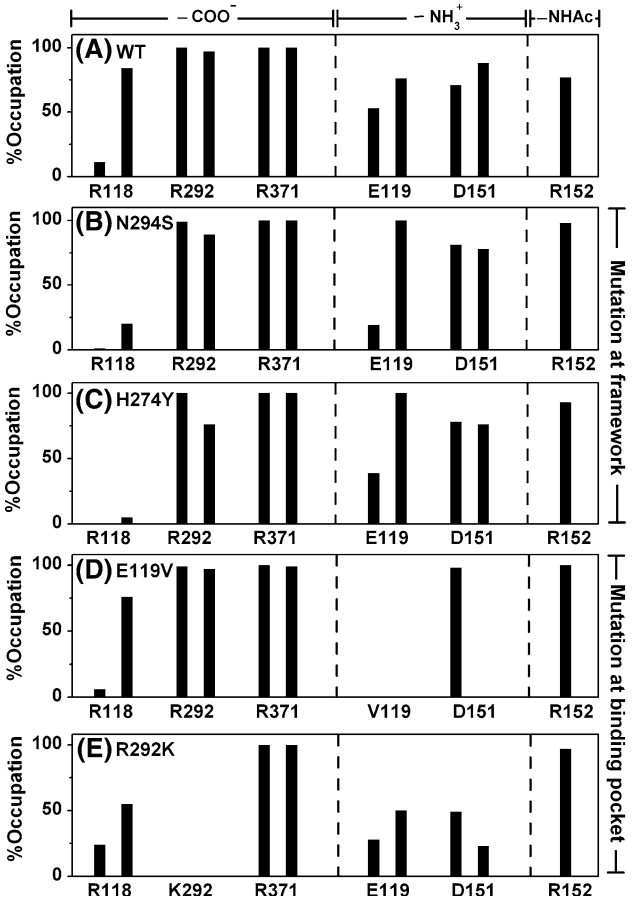

Fig. 2 Percentage occupation of H-bonds between the functional groups of oseltamivir and the NA residues (see Fig. 1 for residue positions) in the mutant models with the single substitution at two district regions: the framework residues closed to the hydrophobic pocket (N294S and H274Y), and the direct-binding residues (E119V and R292K)

(Rungrotmongkol et al. 2009a, b). Therefore, both WT and mutant NA strains of 2009-H1N1 are relatively similar to the H5N1 NA enzyme.

Results and discussion

Reduced oseltamivir binding to probable H1N1-2009 mutants

To examine oseltamivir susceptibility within the neuraminidase pocket of the 2009-H1N1 mutant models, intermolecular hydrogen-bond (H-bond), ES and vdW interactions between the oseltamivir's side chains and the NA residues were evaluated and compared with those of the WT (Rungrotmongkol et al. 2009a, b). The ES and vdW energetic differences were evaluated using Eqs. 2a and 2b, and are summarized in Table 1:

$$\langle \Delta\Delta U^{\mathrm{ES}} \rangle_{\mathrm{bound}} = \langle U^{\mathrm{ES}} \rangle_{\mathrm{bound}}[\mathrm{mutant}] - \langle U^{\mathrm{ES}} \rangle_{\mathrm{bound}}[\mathrm{wild\ type}]. \quad (2a)$$

$$\langle \Delta\Delta U^{\mathrm{vdW}} \rangle_{\mathrm{bound}} = \langle U^{\mathrm{vdW}} \rangle_{\mathrm{bound}}[\mathrm{mutant}] - \langle U^{\mathrm{vdW}} \rangle_{\mathrm{bound}}[\mathrm{wild\ type}]. \quad (2b)$$

The positive and negative values of the energy components indicate that the selected moiety of oseltamivir in the mutants decreases and increases its binding potency, relative to that of the WT, respectively. The H-bonds were calculated according to the two criteria that (1) the proton donor (D) and acceptor (A) distance is $\leq$3.5 Å and (2) the D–H..A angle is $\geq$120°. The results are shown in Fig. 2, whereas the descriptions are given in Table S1 of the supplementary materials. The schematic views of hydrogen bonds formed between oseltamivir and its binding residues extracted from the simulations were given in Fig. 3.

Lower oseltamivir binding-free energies to the probable H1N1-2009 mutants were observed in terms of the H-bonds, ΔU^{ES} and ΔU^{vdW} energies relative to the WT, depending on where the mutation is located. As expected, the ES effect of single mutation at the framework residues (H274Y and N294S) is drastically less than that at the direct-binding residues (E119V and R292K), which leads to the ΔU^{ES} reduction being in the range of c.a. 10–11 and 24–25 kcal mol^{-1}, respectively (Table 1a).

For the H1N1 WT, the strong oseltamivir–NA interactions were found via five, three and one H-bonds ($\geq$75%) with the –COO$^-$, –NH$_3^+$ and –NHAc moieties, respectively (Figs. 2a, 3a). In the two framework region mutations, it can be seen that the –COO$^-$ group of oseltamivir

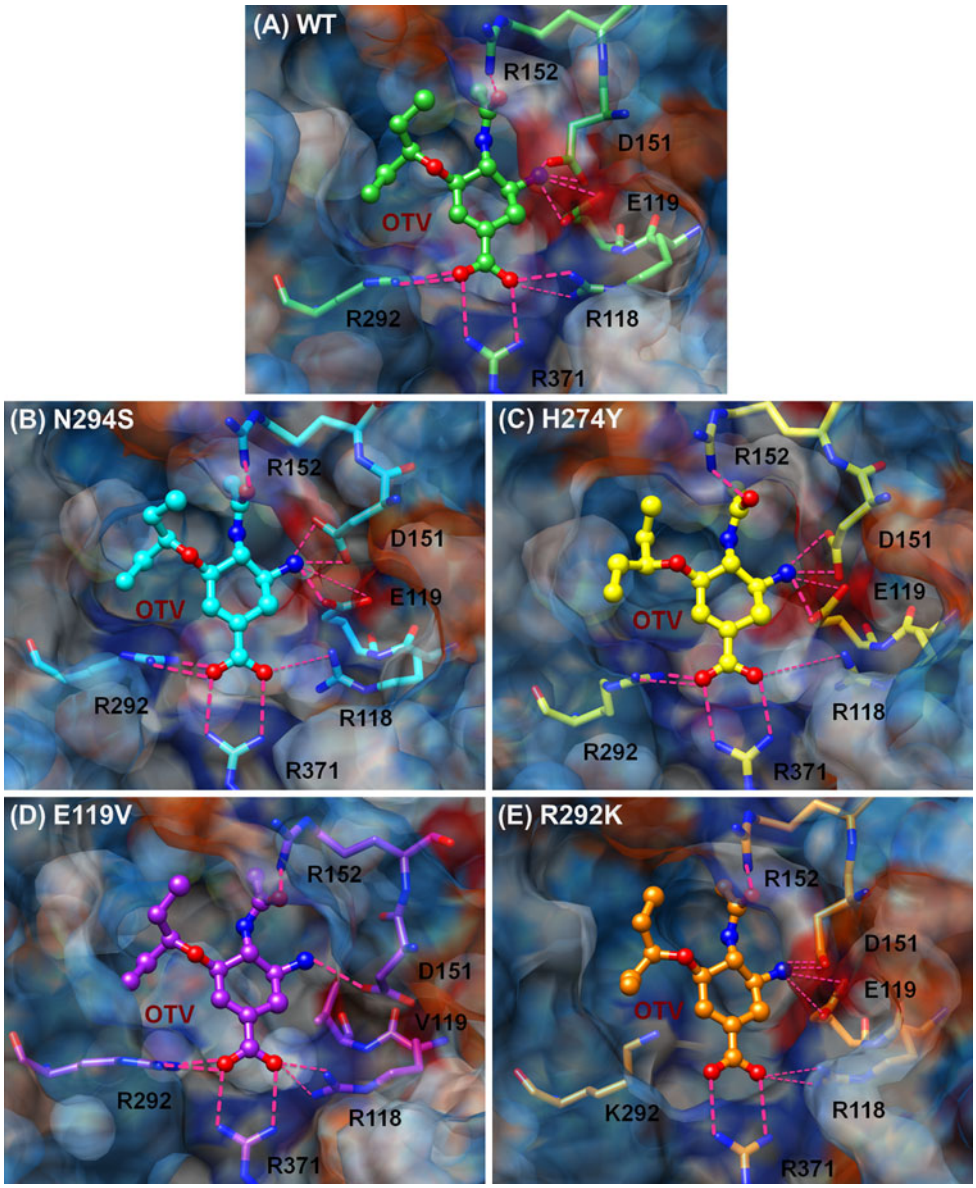

Fig. 3 Electrostatic potential of five different NA strains complexed with oseltamivir where negative regions are in *red* and positive regions are in *blue*: **a** wild type, **b** N294S, **c** H274Y, **d** E119V and **e** R292K. Closeup of oseltamivir, hydrogen bonds to its binding residues are represented by *red dashed line*

Table 2 MD/LIE binding-free energies (ΔG_{bind}) of oseltamivir towards the 2009-H1N1 influenza neuraminidases [A/California/04/2009(H1N1)] for the wild type (WT) and the probable single mutations: N294S, H274Y, E119V and R292K

NA strain	ΔG_{bind} (kcal mol^{-1})				
	WT	N294S	H274Y	E119V	R292K
Predictive					
A/California/04/2009(H1N1)	−12.8 ± 0.9	−10.4 ± 0.9	−9.8 ± 1.0	−9.3 ± 0.8	−7.7 ± 0.7
Experimental[a]					
A/WSN/33 (H1N1)[b]	−12.1	−9.3	−8.5	–	–
A/Puerto Rico/8/34 (H1N1)[c]	−11.4	−8.8	−8.1	–	–
A/Vietnam/1203/04 (H1N1)[c]	−13.0	−11.2	−8.6	–	–
A/Vietnam/1203/04 (H5N1)[d]	−13.1	−10.5	−9.8	–	–
A/Sydney/5/97 (H3N2)[b]	−12.8	−8.3	–	−8.6	−7.3
A/Wuhan/359/95 (H3N2)[e]	−12.4	–	–	−9.1	−6.2

Means and standard deviations are derived from four separate 5-ns simulations

The experimental ΔG_{bind} for different strains of N1 and N2, converted from the K_I inhibitory and IC_{50} values, are also given for comparison

[a] $\Delta G_{experiment}$ was calculated from the experimental data using the following references: (b) Abed et al. 2008, (c) Yen et al. 2007, (d) Collins et al. 2008 and (e) Yen et al. 2005

has almost lost the H-bonds with R118 (see Figs. 2b, 3b for N294S; Figs. 2c, 3c for H274Y) in correspondence with the ΔU^{ES}(–COO^-) reduction of 11.3 kcal mol^{-1} in H274Y and 8.3 kcal mol^{-1} in N294S (Table 1a). As expected (Table 1b), the decreased ΔU^{vdW} (oseltamivir) of 2.5 kcal mol^{-1} by N294S and of 3.4 kcal mol^{-1} by H274Y were mainly contributed from the loss of vdW interactions at the bulky $OCHEt_2$ group ($\sim$1.5 kcal mol^{-1}). Previous theoretical studies on the influenza NA mutants have already explained how the H274Y mutation confers oseltamivir resistance by a meaningful change of E276's sidechain conformation with a consequent effect upon the shape and size of the hydrophobic pocket for the –$OCHEt_2$ moiety (Malaisree et al. 2009; Rungrotmongkol et al. 2009a, b; Wang and Zheng 2009) while E276 in the N294S mutant acted as the center of H-bond network between R224 and S294 (Rungrotmongkol et al. 2009a, b) similar to that found in the crystal structure of the oseltamivir-resistant H5N1 N294S variant (Collins et al. 2008).

With a relatively high reduction in the ES contribution to oseltamivir in the mutations at the binding residues (E119V and R292K, Table 1a), the mutated residues V119 and K292 showed a complete loss of H-bond interactions with the –NH_3^+ and –COO^- moieties of oseltamivir (Figs. 2d, 3d for E119V; Figs. 2e, 3e for R292K), supported by an increase in the ΔU^{ES}(–NH_3^+) by 11.1 kcal mol^{-1} and in the ΔU^{ES}(–COO^-) by 14.1 kcal mol^{-1}. In addition, only one H-bond with D151 in the E119V mutant was maintained, while lower H-bond strengths in the R292K mutant were observed at R118, E119 and D151. Moreover, a reduced vdW interaction of 3.2 kcal mol^{-1} was found at the –$OCHEt_2$ group in the R292K mutant because the side chain of K292 (Fig. 3e) is smaller and shorter than that of R292 (Fig. 3a). The results of the R292K mutation were somewhat comparable to the computational study of the sialic acid analogs binding to the R292K mutated NA subtype N9 (Chachra and Rizzo 2008).

Prediction of inhibitory activity against the H1N1 mutated strains

Based on the MD/LIE approach, the binding affinities of oseltamivir towards different mutant models of the 2009-H1N1 influenza [A/California/04/2009(H1N1)], according to Eq. 1, were predicted and are summarized in Table 2. As expected, oseltamivir's binding-free energy against the WT is the most favorable one at −12.8 kcal mol^{-1}. Only moderate binding-free energy values were found for the N294S, H274Y and E119V mutated strains, in which the corresponding ΔG_{bind} of −10.4, −9.8 and −9.3 kcal mol^{-1}, respectively. The lowest favorable binding of oseltamivir is found in the R292K mutant with a predicted ΔG_{bind} of −7.7 kcal mol^{-1}. All the calculated binding-free energies were found to fall within the ranges of those experimentally determined for various WT and mutant strains of the other influenza N1 and N2 subtypes (Table 2) (Abed et al. 2008; Boivin and Goyette 2002; Collins et al. 2008; Mishin et al. 2005; Zürcher et al. 2006).

Taking all the above data into consideration, it seems likely that oseltamivir will be significantly less potent an inhibitor for all the modeled mutants of the 2009-H1N1 strains, with the ranked order of: R292K < E119V < H274Y < N294S.

Conclusions

In the present study, multi-MD simulations in conjunction with the LIE method was performed on oseltamivir–NA-bound complexes for the four probable NA mutants of influenza A (H1N1-2009): two mutations on the framework residues (N294S and H274Y) and the two others on the direct-binding residues (E119V and R292K) of oseltamivir. Reduction in the oseltamivir–enzyme interaction energies, particularly in the ES term, and in the hydrogen bonding were both observed in the two mutated systems with substitution on the direct-binding residues, E119V and R292K.

Based on the MD/LIE approach, the inhibitory potencies of oseltamivir towards the WT and mutants were predicted in accordance with their derived binding-free energies (ΔG_{bind}) with: WT (-12.3 kcal mol^{-1}) > N294S (-10.4 kcal mol^{-1}) > H274Y (-9.8 kcal mol^{-1}) > E119 V (-9.3 kcal mol^{-1}) > R292 K (-7.7 kcal mol^{-1}). This means that oseltamivir (which, to date, effectively inhibits the current H1N1-2009 wild-type strain) is less effective in protection and/or treatment of patients with these probable mutants, and especially with the R292K variant. Therefore, surveillance of any mutations in the influenza A (H1N1-2009) needs to be closely watched, and prompt action taken for preparation for the next pandemic of potentially higher oseltamivir-resistant H1N1 influenza strains. This calls for the urgent development of new potent anti-influenza agents against both native and mutants forms of H1N1-2009.

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