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Molecular Basis of Drug Resistance in A/H1N1 Virus

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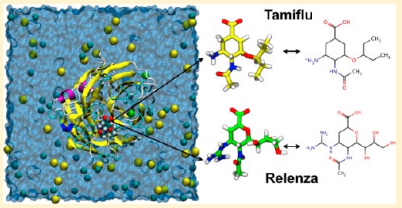
Supporting Information

ABSTRACT: New mutants of human influenza virus (A/H1N1) exhibit resistance to antiviral drugs. The mechanism whereby they develop insensitivity to these medications is, however, not yet completely understood. A crystallographic structure of A/H1N1 neuraminidase has been published recently. Using molecular dynamic simulations, it is now possible to characterize at the atomic level the mechanism that underlies the loss of binding affinity of the drugs. In this study, free-energy perturbation was used to evaluate the relative binding free energies of Tamiflu and Relenza with H274Y, N294S, and Y252H neuraminidase mutants. Our results demonstrate a remarkable correlation between theoretical and experimental data, which quantitatively confirms that the mutants are resistant to Tamiflu but are still strongly inhibited by Relenza. The simulations further reveal the key interactions that govern the affinity of the two drugs for each mutant. This information is envisioned to prove useful for the design of novel neuraminidase inhibitors and for the characterization of new potential mutants.



INTRODUCTION

Influenza viral infections affect all populations of the world and represent a leading cause of mortality in elderly and immune-compromised populations. The 2009 A/H1N1 pandemic clearly illustrated how drug resistant mutants can impact a population before a vaccine is available.[1] Understanding the mechanism of resistance of these mutants is likely essential to the development of potent and effective antiviral drugs and, therefore, of paramount importance for human health.

The influenza virus infects the epithelial cells of the respiratory tract through two glycoproteins, namely hemagglutinin and neuraminidase, located in the virus capsid. Specifically, neuraminidase interacts with sialic acid contained in this cell type, allowing attachment of the virus and the subsequent release of virion progeny.[2] Because the active site of neuraminidases is highly conserved, it has been employed as a target for the design of structure-based drugs. Two neuraminidase inhibitors are currently utilized to combat viral dissemination—namely, oseltamivir (Tamiflu), which binds the active site by mimicking sialic acid and zanamivir (Relenza), which is a sialic-acid derivative, wherein the 4-hydroxyl group is replaced by a guanidinium group.[3] The effectiveness of these antiviral drugs is, however, limited due to a high-mutation rate of the influenza virus.

During the 2009 pandemic of the influenza virus (A/H1N1), important mutations close to the active site were observed (H274Y, N294S, and Y252H), causing a dramatic loss of binding affinity of Tamiflu and diminishing the efficacy of the drug.[4] Similar mutations had already been reported for H5N1 neuraminidase,[5] which could be explained by the marked sequence identity of the active site of both neuraminidases. In a recent study by Collins et al.,[6] binding and inhibitory parameters were determined for Tamiflu and Relenza interacting with the wild type and three mutants of H5N1 neuraminidase: (1) the H274Y mutant observed in patients treated with Tamiflu,[7] showing a drug resistance 256-fold higher compared with the wild-type virus; (2) the N294S mutant isolated from patients treated with Tamiflu in viruses containing N1 or N2 neuraminidase,[8] exhibiting a drug resistance 81-fold higher than for the wild type; and finally (3) the Y252H mutant isolated from infected patients, has not been associated with clinical resistance, although experimentally showed to be inhibited by Tamiflu, but not Relenza.[9] The rapid emergence of drug resistance for both H5N1 avian flu and A/H1N1 influenza virus, therefore, constitutes the primary motivation for understanding its mechanism of action and should lead to the development of potent antiviral drugs that circumvent their resistance strategies.

Previous theoretical studies[10−13] have revealed the specific interactions for both wild-type and mutants H5N1 neuraminidase bound to Tamiflu and Relenza. However, the recent elucidation of the crystal structure of A/H1N1 neuraminidase[14]

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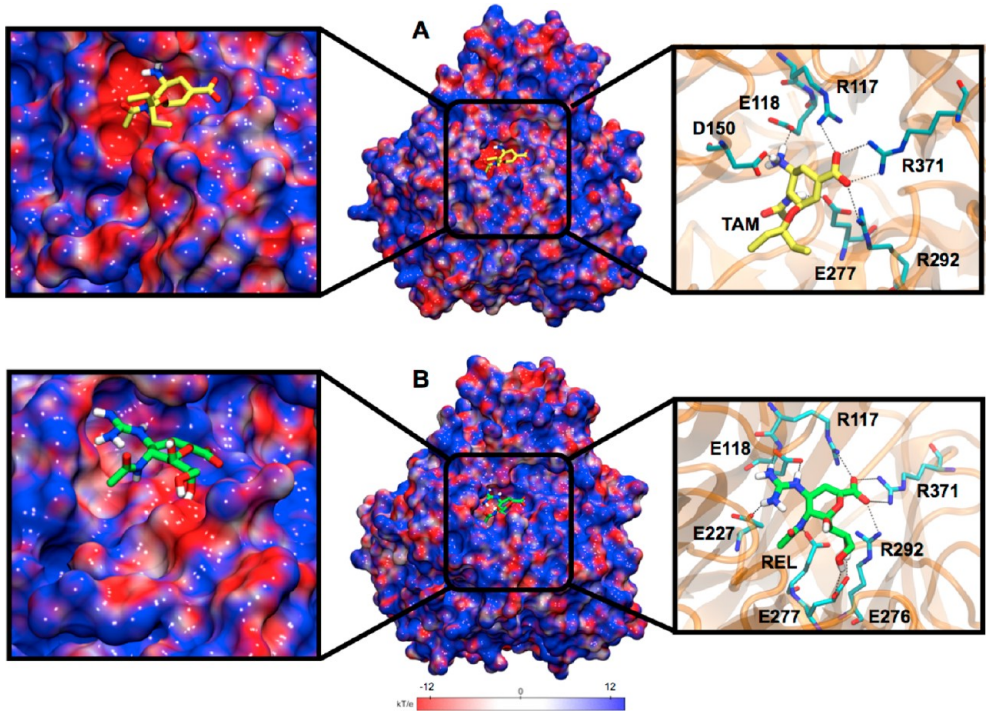

Figure 1. Electrostatic potential surface and hydrogen-bond network of the binding pocket of A/H1N1 neuraminidase in interaction with Tamiflu and Relenza. Complete map (center) and a close-up (left) of the binding pocket with Tamiflu (A, in yellow) and Relenza (B, in green) bound to A/H1N1 neuraminidase. Hydrogen bonds between residues in the binding pocket and each drug are also shown (right). These hydrogen bonds are generally the same between the drugs, although Relenza shows additional interactions with E227 and E276. The internal cavity exhibits a negative electrostatic potential (in red) and the region around the binding site possesses a positive electrostatic potential (in blue) that interacts with the $-NH_2$ and $-COO^-$ moieties of both drugs. The electrostatic potential surfaces were generated using the APBS software interfaced through VMD.

(PDB ID: 3NSS), provides a framework for capturing the mechanisms of drug binding aimed at this protein specifically. On the basis of this novel three-dimensional structure, we employed molecular dynamics simulations to analyze the binding of Tamiflu and Relenza to wild-type and mutant neuraminidases and to characterize the drug-resistance resulting from the H274Y, N294S, and Y252H mutations. At the energetic level, we performed a thermodynamic analysis of the association process using free-energy perturbation (FEP) calculations, with the objective of measuring the relative binding affinity of A/H1N1 neuraminidase mutants to selective inhibitors of the A/H1N1 virus.

RESULTS AND DISCUSSION

To evaluate the specific interactions of Tamiflu and Relenza with the wild-type A/H1N1 neuraminidase, structural analyses of equilibrated complexes were performed. The results show that the hydrogen-bond network is well conserved for both drugs, in agreement with previous studies.[15−17] The complex with Tamiflu exhibits seven hydrogen bonds on average, while that featuring Relenza is stabilized by ten hydrogen bonds (see Figure 1). Specifically, both neuraminidase inhibitors share the same binding pocket,[18,19] which includes hydrogen bonds to residues R117, E118, D150, E277, R292, and R371 of the neuraminidase; Relenza, however, forms additional hydrogen bonds with residues E227 and E276. Although the residues surrounding both drugs are highly conserved, the guanidinium and hydroxyl groups of Relenza allow a hydrogen-bond network to be formed, markedly more robust than that for Tamiflu.

It is notorious that in addition to the well-conserved hydrogen-bond network, the electrostatic environment within the active site contributes to drug-binding stability. In order to understand how the charge distribution of the amino acids surrounding the active site modulates the interaction of the drugs with the wild-type neuraminidase, the three-dimensional electrostatic potential of the equilibrated complexes was calculated and averaged for each trajectory. The resulting electrostatic surfaces, characterizing both complexes, are shown in Figure 1. In the wild-type neuraminidase:Tamiflu complex, we observed that the residues around the drug are oriented in such a way as to form an electronegative surface within the binding pocket, while the entrance to the active site has some electropositive and electronegative areas, in agreement with previous studies.[20,21] On the other hand, the binding pocket residues surrounding Relenza show less electronegative area than for Tamiflu and the entrance to the active site tends to be electronegative. Therefore, while the network of hydrogen bonds is larger for Relenza, the electrostatic potential is more favorable for Tamiflu, leading to similar affinities and, therefore, to similar inhibitory effects, in agreement with previous clinical studies.[22] The residues that contribute predominantly to the electrostatic potential of the binding pocket for both drugs are R117, E118, L133, D150, R151, W178, S179, I222, R224, E227, S246, E276, E277, R292, N294, R371, and Y406. The root mean-square deviation (RMSD) profiles for these amino acids as well as those that form the hydrogen-bond network remain unchanged over the 5 ns equilibrium MD trajectory, which implies that the protein:ligand complexes have reached a steady conformational state for subsequent binding free-energy calculations.

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Table 1. Overview of Free-Energy Changes Associated with H274Y, N294S, and Y252H Mutations

		ΔΔG, kcal/mol		error	
transformation	total simulation time, ns	experimental	BAR[a]	systematic	statistical
H274Y−Tamiflu	20.7	+3.29	+2.61	1.15	0.07
H274Y−Relenza	21.3	+0.37	+0.93	1.63	0.07
N294S−Tamiflu	20.0	+2.59	+3.25	1.24	0.17
N294S−Relenza	19.2	+1.16	+0.32	1.39	0.15
Y252H−Tamiflu	25.6	−1.35	−0.91	0.97	0.05
Y252H−Relenza	25.6	+0.10	+0.49	1.51	0.14

[a]BAR estimates were obtained by combining the forward and backward transformations. The total simulation time was ∼135 ns and required approximately 650 wall-clock hours on a cluster of 128 CPUs.

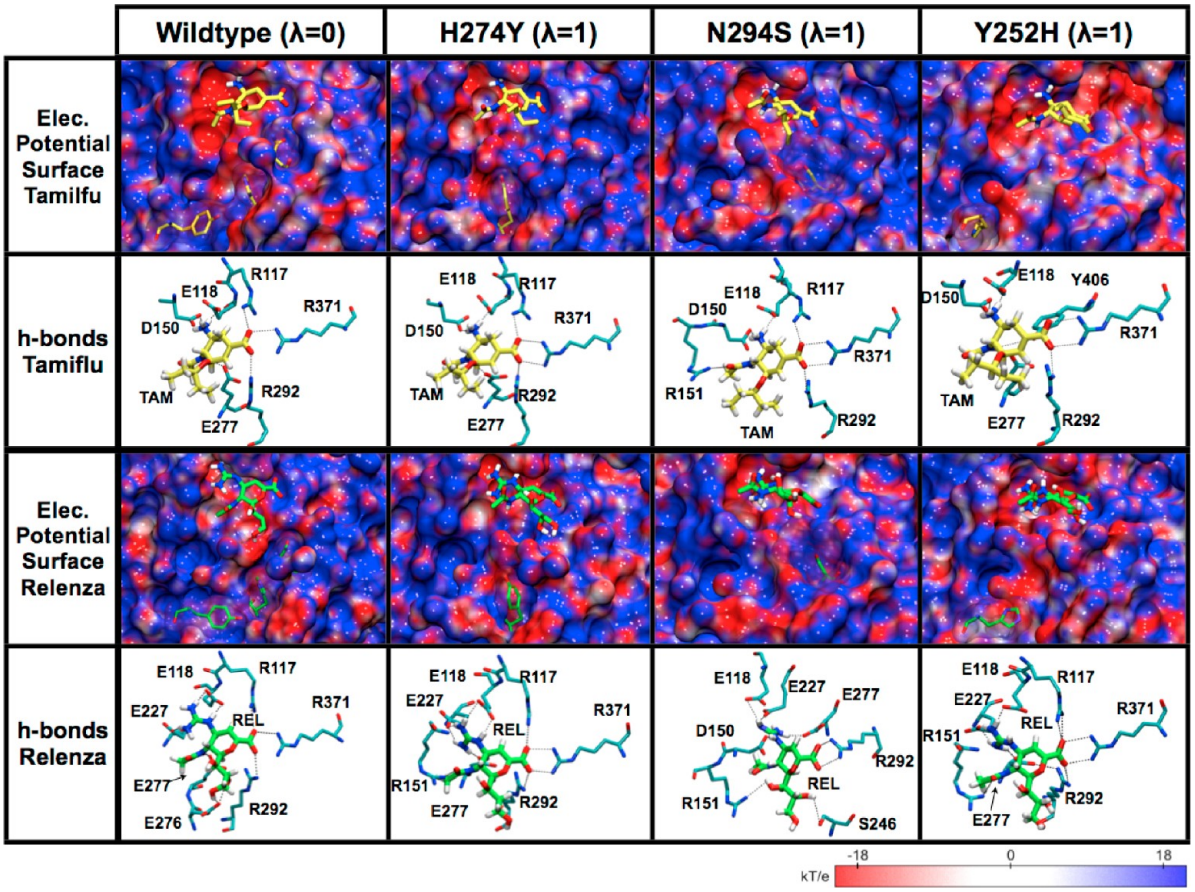

Figure 2. Images of the active site in the wild-type and mutant states of A/H1N1 neuraminidase in association with Tamiflu and Relenza. Average electrostatic potential surface and hydrogen-bond network for Tamiflu and Relenza bound to A/H1N1 neuraminidase for the wild type and mutants (H274Y, N294S, and Y252H). The values $\lambda = 0$ and 1 indicate that the wild-type and mutants serve as the reference and target states, respectively, for the FEP calculations. Despite the fact that the mutations do not involve residues in direct contact with the drugs, they cause significant changes in the environment that makes direct contact with Tamiflu and Relenza.

For each equilibrated system, we generated a hybrid mutant with the objective of evaluating the relative binding free-energy for the drug-resistant mutants of A/H1N1 neuraminidase (H274Y, N294S, and Y252H) associated with Tamiflu and Relenza. As previously mentioned, these mutants have already been explored experimentally in H5N1 with the same drugs. Owing to the high sequence identity between A/H1N1 and H5N1 neuraminidase (91.47%), these experimental data were used as a reference for the present study.[6] The relative binding affinity of Tamiflu and Relenza in complex with the wild-type and mutant A/H1N1 neuraminidases were separately calculated using free-energy perturbation (FEP)[23] following the thermodynamic cycle depicted in the Supporting Information (Figure S1). The free-energy changes corresponding to the "alchemical transformation"[24] of H274Y, N294S, and Y252H mutants are gathered in Table 1. The Bennett acceptance ratio (BAR)[25] net free-energy differences for Tamiflu and Relenza bound to the H274Y mutant are 2.6 ± 0.1 and 0.9 ± 0.1 kcal/mol, respectively. Similarly, for the N294S mutant, Tamiflu yields a BAR net free-energy change of 3.3 ± 0.2 kcal/mol while Relenza yields 0.3 ± 0.2 kcal/mol. Both mutants show an unfavorable energy in the presence of Tamiflu, suggesting a higher resistance to this drug and, hence, a better inhibitory capacity of Relenza in comparison with Tamiflu. On the other hand, the Y252H mutant leads to a BAR net free-energy change of −0.9 ± 0.1 kcal/mol for Tamiflu and +0.5 ± 0.1 kcal/mol for

Table 2. Summary of Structural Properties of Simulated Complexes[a]

complexes	residues that form hydrogen bonds											EP active site	EP entrance active site	infiltration water molecules
WT-TAM	R117	E118	D150					E277	R292	R371		E−	E−, E+	0
WT-REL	R117	E118			E227		E276	E277	R292	R371		E−	E−	0
H274Y-TAM	R117	E118	D150					E277	R292	R371		↓E−	↑E+	6
H274Y-REL	R117	E118		R151	E227			E277	R292	R371		↑E−	↑E+	2
N294S-TAM	R117	E118	D150	R151					R292	R371		↓E−	↑E+	9
N294S-REL		E118	D150	R151	E227	S246		E277	R292			↑E−	↑E+	3
Y252H-TAM		E118	D150					E277	R292	R371	Y406	↑E−	↑E−	0
Y252H-REL	R117	E118		R151	E227			E277	R292	R371		↑E−	↓E+	4

[a]Analysis includes residues that form hydrogen bonds and variations in the electrostatic potential of the active site and the entrance to the latter, as well as the number of water molecules surrounding the drugs. [b]The arrows indicate the increase (↑) or decrease (↓) of the electrostatic potential (EP) compared to wild-type complexes.

Relenza, thus, suggesting that Tamiflu remains an effective inhibitor for the Y252H mutant. Overall, the present set of results shows an excellent agreement (∼90%) with the experimental estimates (see Table 1), confirming the ability of the computational approach used here to predict relative binding affinities of drugs against a specific protein.

Considering that the FEP calculations confirm the difference of affinity of the drugs observed experimentally, a structural analysis of the mutant:drug complexes was performed to characterize the molecular interactions lost in the case of drug resistance. Hydrogen-bond and electrostatic-potential analyses for each complex in the initial state ($\lambda = 0$) and the final state ($\lambda = 1$) of the FEP calculations are shown in Figure 2. Although the H274Y, N294S, and Y252H mutations do not involve residues in direct contact with the drugs, important structural changes were observed. In comparison with the wild-type neuraminidase, the H274Y:Tamiflu complex conserves the hydrogen-bond network, albeit the local electrostatic surface potential differs, revealing a binding pocket less electronegative relative to the wild-type. At the same time, the region that includes the mutated (H274Y) residue is observed to be more electropositive, suggesting that the affinity of the drug is altered in part by a change in the electrostatic potential due to a spatial reorganization of residues within the neighborhood of H274Y. In the case of Relenza, a hydrogen bond formed with E276 in the wild-type complex is lost; however, a new hydrogen bond is formed with R151. In the H274Y:Relenza complex, the electrostatic potential, compared to wild-type complex, becomes slightly more negative in the binding pocket and is rather more positive in the region in contact with the hydroxyl groups of the drug, which likely explains in part the affinity of Relenza versus Tamiflu in presence of this mutant. Similarly, the N294S mutant interacting with either drug exhibits a similar hydrogen-bond network, although for Tamiflu the hydrogen bond with E277 is replaced by a new hydrogen bond with R151. For Relenza the interactions with R117, E276, and R371 are lost, while new hydrogen bonds with D150, R151, and S246 appear. It is likely, then, that the spatial reorganization of these residues contribute to the change of electrostatic potential within the binding pocket of the N294S:Tamiflu complex in comparison to the wild-type. Meanwhile, the electrostatic potential surface of the N294S:Relenza complex becomes more negative in this region, and as in the previous mutant, a new electropositive area is observed in the vicinity of hydroxyl groups of the drug. Therefore, although the H274Y and N294S mutations do not substantially alter the number of hydrogen bonds, they appear to cause reorganization of neighboring residues, leading to a significant change in the local electrostatic potential perceived by the drugs.

In the case of the Y252H mutant, Tamiflu features an additional hydrogen bond with Y406, while the interaction with R117, observed in the wild-type complex, is lost. For the same mutant, Relenza loses its interaction with E276 and forms a new hydrogen bond with R151. Just as in the case of the mutants described above, the Y252H mutant exhibits for both drugs an electronegative binding pocket; however, the potential at the entrance of the active site shows a more negative potential for Tamiflu as compared to Relenza. Moreover, this mutant, in contrast with H274Y and N294S, shows a different side-chain orientation for residue H252 for Tamiflu or Relenza, which may explain the drastic change in the electrostatic potential. We therefore find a similar picture for the third mutant as for the other two (see Table 2): variation in the hydrogen bonds between the mutants and the wild-type is moderate, to the extent that the difference in affinity between the mutants and the drugs is ascribed primarily to the change of the electrostatic potential induced by the residues located at the entrance of the active site (namely, within the neighborhood of H274Y, N294S, and Y252H). In addition, these structural analyses are consistent with the relative binding free energy calculated by FEP, that is, the mutations H274Y and N294S alter the electric potential of the binding pocket in a way that matches well with the charge distribution of Relenza, but not of Tamiflu. Specifically, the region of negative electrostatic potential of the binding pocket of both mutants coincides with the positively charged guanidinium group of Relenza, while the negatively charged hydroxyl groups of the drug coincide with the positive potential that form the residues surrounding them. In contrast, the remarkable negative binding pocket of the Y252H:Tamiflu complex yields a more favorable electrostatic interaction for Tamiflu than for Relenza, and Tamiflu, therefore, remains effective for the Y252H mutant, in agreement with the aforementioned computed relative binding free-energy.

The wide variation in electrostatic potential around the active site of neuraminidase in the Tamiflu and Relenza systems prompted an investigation of how subtle alterations in drug association can produce such profound differences in local binding potentials. One well-known phenomenon that can substantially alter the electrostatic interaction of a drug and a protein is the infiltration of water molecules, which induces a reorganization of the residues that surround the active site and, therefore, entails a spatial redistribution of the charges. To analyze this infiltration, we turned our attention to examining

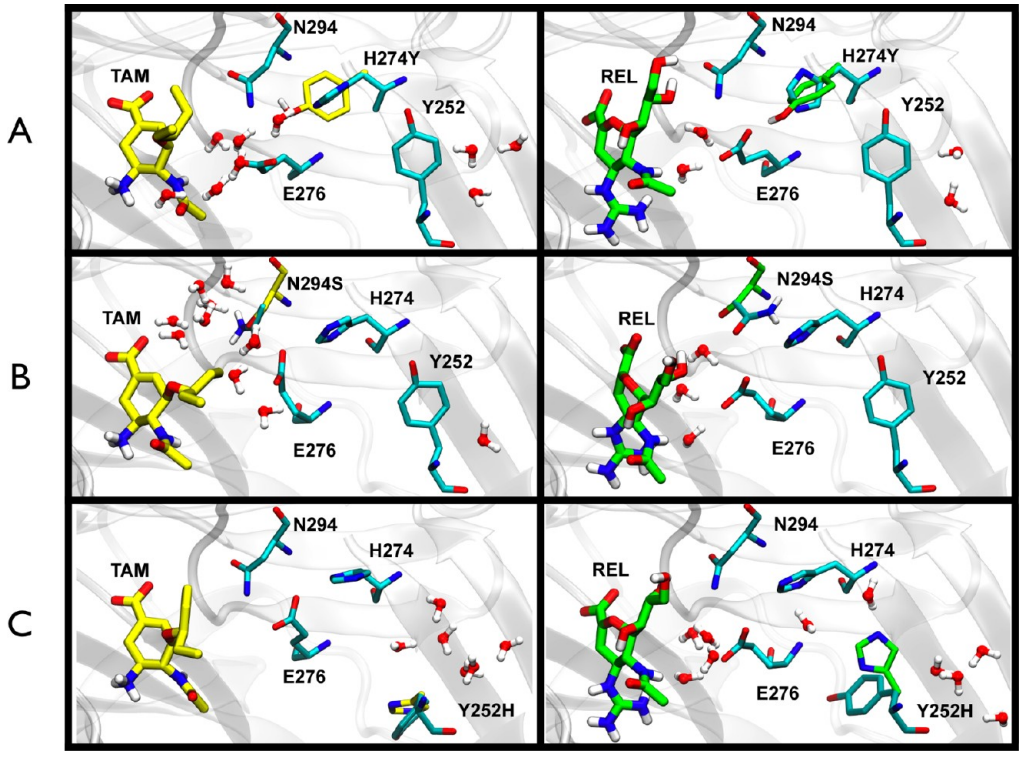

Figure 3. Infiltration of water molecules in the entrance of the active site of mutants of A/H1N1 neuraminidase. Structural characterization of the binding site of the (A) H274Y, (B) N294S, and (C) Y252H mutants associated to Tamiflu (left) and Relenza (right). The entry of water molecules into the binding site of the drugs is directly related to the conformational state adopted by the E276 residue. In case of the wild-type, no water molecules appear in this region.

the relative hydration of the drug binding pockets and the entrance to the active site (in the region of the mutants), evaluating the number of water molecules and the solvent accessible surface area (SASA) for each wild-type and mutated system. Our analyses showed that a larger number of water molecules were present in the active site of the mutants compared to that of the wild-type, suggesting that the drug affinity may depend on the hydrophobicity of the active site. In addition, the SASA calculated for all simulated systems is shown in Supporting Information Figure S2 and increases for all three mutants in comparison with the wild-type neuraminidase, thereby demonstrating that the mutants reduce the hydrophobicity of the active site; and the changes in the electrostatic potential, of the residues surrounding to the latter, are mediated by the entry of water molecules. SASA analyses per residue were also performed, revealing no significant changes in the binding pocket residues, but important modifications within the neighborhood of H274Y, N294S, and Y252H.

Analyses of the conformational changes caused by water infiltration further show that in both wild-type and mutant simulations, orientation of residues R117, E118, D150, R292, and R371 is well-conserved; however, displacement of the side chains of the other residues surrounding the active site are observed. Specifically, inspection of the H274Y mutant bound to Tamiflu reveals that the strong interaction of H274 and E276 is disrupted by the mutation H274Y, causing E276 to shift from its wild-type position, allowing water molecules to penetrate into the H274Y pocket (Figure 3A). In the case of Relenza, the mutated residue Y274 destabilizes the hydrogen bond between the amide group of the drug and the E276 residue, leading fewer water molecules to enter, compared to Tamiflu. These results lend support to the assertion that the change in electrostatic potential at the entrance of the binding site is caused by the entry of water molecules within the neighborhood of H274Y. The water molecules conspire to displace the amino acids located in this region, which implies a reorganization of the superficial charges surrounding the active site. For the N294S mutant interacting with Tamiflu and Relenza, similar changes are observed in the residues located around the drugs (Figure 3B). The N294S:Tamiflu complex, in the absence of N294, forms a hydrogen bond between E276 and S294 allowing water molecules to penetrate deeply into the N294S pocket. These water molecules destabilize the interaction of the E277 residue and the amide moiety of the drug, breaking the hydrogen bond formed between them. In N294S:Relenza complex, the hydrogen bond formed between E276 and the 2-hydroxyl group of the drug is disrupted when S294 is present and water molecules enter the cavity; however, due to the large size of Relenza, only a few water molecules manage to penetrate. Finally, for the Y252H mutant complexed with Tamiflu, we do not observe a significant influx of water molecules, while when Relenza is present, the H252 residue is oriented toward the H274 residue, hence, permitting water molecules to interact with the E276 residue (Figure 3C). Consequently, the presence of water molecules in the vestibule of the binding pocket seems to contribute significantly to the conformational change of key amino acids surrounding the active site through a modification of the electrostatic environment felt by the drugs (see Table 2). Furthermore, the E276 residue for each simulated system appears to be a vital factor for the stability of Tamiflu and Relenza, since in our mutant systems it adopts conformations that disrupt the normal accommodation of the drugs in the active site.

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CONCLUSION

In conclusion, our results reveal that the origin of the resistance to antiviral drugs for the H274Y, N294S, and Y252H mutants is caused by penetration of water molecules at the entrance of the binding site of neuraminidase. The key role played by hydration near the active site also corroborates speculation from previous studies.[26] Additionally, we find strong evidence that the hydrogen-bond network between the neuraminidase and the drugs remains relatively unperturbed among the wild-type and the three mutant structures. However, the change in the electrostatic potential arising from a spatial reorganization of the residues surrounding the binding site appears to be a major factor that governs drug selectivity toward different mutants (for more detail see Figure S9). Considering that the affinity of the drugs is determined by the electrostatic environment in the active site of the neuraminidase, Relenza has a greater number of $-NH_2$ and $-OH$ side groups than Tamiflu, lending the former a better affinity for the highly charged binding pocket. The electronegative surface observed inside of the binding cavity of the H274Y and N294S mutants is, therefore, reflected in an increase in the relative affinity of Relenza, specifically for positively charged guanidinium ($-NH_2$) group. Moreover, Relenza occupies greater physical space than Tamiflu, so that Relenza, located in the active site, prevents the ingress of water molecules, which, in the case of Tamiflu, penetrate deeply into the vestibule of the binding pocket and strongly alter the electrostatic signature of the residues pertaining to the neuraminidase active site. These results are expected to help guide the design of novel drugs with an increased antiviral efficacy.

ASSOCIATED CONTENT

Supporting Information

Details of the computational methods, system assembly, chemical structures of the drugs, RMSD analyses, thermodynamic cycle used in the free energy calculations, SASA analyses, probability distribution functions (FEP), and electrostatic potential cross sections. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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