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Another look at the molecular mechanism of the resistance of H5N1 influenza A virus neuraminidase (NA) to oseltamivir (OTV)

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ABSTRACT

In the context of a recent pandemic threat by the worldwide spread of H5N1 avian influenza, the high resistance of H5N1 virus to the most widely used commercial drug, oseltamivir (Tamiflu), is currently an important research topic. Herein, molecular bases of the mechanism of H5N1 NA resistance to oseltamivir were elucidated using a computational approach in a systematic fashion. Using the crystal structure of the complex of H5N1 NA with OTV (PDB ID: 2hu0) as the starting point, the question, how mutations at His274 by both smaller side chain (Gly, Ser, Asn, Gln) and larger side chain (Phe, Tyr) residues influence the sensitivity of N1 to oseltamivir, was addressed and correlated with the experimental data. The smaller side chain residue mutations of His274 resulted in slightly enhanced or unchanged NA sensitivity to OTV, while His274Phe and His274Tyr reduced the susceptibility of OTV to N1. In contrast to the binding free energies, the net charges of Glu276 and Arg224, making charge–charge interactions with Glu276, were established to be more sensitive to detecting subtle conformational differences induced at the key residue Glu276 by the His274X mutations. This study provides deeper insights into the possibility of developing viable drug-resistant mutants.

1. Introduction

Based on a recent pandemic threat by the worldwide spread of the highly pathogenic H5N1 avian influenza, the World Health Organization has shown its profound concern regarding the possibility of having the virus spread among humans in the near future. The virus resistance to two approved anti-influenza drugs, oseltamivir (Tamiflu) and zanamivir (Relenza), targeting NA enzyme of the virus, as well as the lack of adequate vaccine have raised the urgent question of developing new anti-viral drugs [1]. The structure of H5N1 NA is particularly attractive because it offers a wide spectrum of new opportunities for drug design [2]. However, a fundamental secret associated with the high resistance of H5N1 to OTV is still unrevealed.

A key reason underlying the drug-resistance has been ascribed to different hydrophobic interactions of lipophilic side chains with influenza A and B NAs having a complete homology in active site [3]. In the context of the resistance of H5N1 to some existing NA inhibitors, the same reason has been considered using homology modeling [4]. The oseltamivir-resistance of H5N1 NA has been suggested to be caused by the mutations of residues at the positions 119, 152, 274, and 292 [5]. The Tyr252His mutation in H5N1 NA with the His274Tyr substitution has been hypothesized to be responsible for an increased affinity of NA for oseltamivir [6]. The only molecular mechanism helping to understand the N1 NA resistance to OTV has been proposed by Moscona [7], and has indicated that the N1 active site must reorient itself in order to accommodate the bulky side chain of OTV, whereas such a change is not needed for zanamivir. This reorientation has been reflected through the rotation of Glu276 and its bonding to Arg224. The proposed rearrangement of N1 active site has been based on the X-ray structure of OTV with N9 NA [7]. To better understand the OTV-resistance of H5N1 virus, a systematic structure-activity relationship study is currently indispensable [8]. By starting from the OTV/H5N1 NA crystal structure, in this paper the molecular bases of H5N1 NA resistance to oseltamivir are explored using contemporary principles of conformational analysis.

2. Computational methods

The crystal structure of the complex of H5N1 NA with oseltamivir (PDB ID: 2hu0) was taken from the Protein Data Bank [2]. The substitutions of His274 were carried out by the Mutate subroutine that was implemented in the HyperChem 5.02 program [9]. Flexible docking calculations for this letter were performed using the AScore/ShapeDock protocol from the ArgusLab 4.0.1 suite of programs [10]. Charge distribution was evaluated using the semi-empirical AM1 method. Figures shown in this letter were generated by PyMol [11].

2.1. The ArgusLab4/AScore/ShapeDock flexible docking

Docking problem is a complicated optimization or an exhaustive search problem involving many degrees of freedom. The goal is to find

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Table 1
Binding free energies (ΔG), fifty percent inhibitory concentrations (IC_{50}), and inhibition constants (K_i) for various N1 NA mutants in the complex with oseltamivir

PDB ID: NA subtype inhibitor	Residue 274	$\Delta G_{binding}$ [kcal mol^{-1}]	IC_{50}^{a} [nM]	K_i^{b} [nM]	Net charge [e]		
					Arg224	Glu276	Glu276 side chain
2hu0: N1 oseltamivir	His274 (2hu0)	−9.57	90.0	0.3 (wt)	+9	−14	−18
	His274Gly	−9.66	82.2	0.2	+4	−14	−18
	His274Ser	−9.60	87.4	0.1	+1	−17	−18
	His274Asn	−9.60	87.4	0.1	+1	−17	−18
	His274Gln	−9.60	87.4	0.3	+1	−17	−18
	His274Phe	−8.69	165	86	−27	−27	−20
	His274Tyr	−8.84	152	105	−26	−31	−20
	His274Tyr Tyr252His [c]	−8.84	152	–	−22	−31	−20

[a]Estimated values on the basis of the correspondence between the experimentally determined ranges of binding free energies and 50% inhibitory concentrations [14].
[b]Experimental values for wild type (wt) and mutant influenza A/WS/33 (H1N1) NA [5].
[c]There is a hypothesis that the Tyr252His mutation in H5N1 NA with the His274Tyr substitution could be responsible for an increased NA affinity for oseltamivir [6].

the optimal ligand/protein configurations, and accurately as well as consistently predict their binding free energies without relying on formal statistical mechanics approaches. To computationally accomplish the objective within a reasonable time framework, an empirical scoring function (AScore) and a docking engine (ShapeDock) were developed through the ArgusLab program [10].

A Score is based on the decomposition of the total protein–ligand binding free energy:

$$\Delta G_{binding} = \Delta G_{vdw} + \Delta G_{hydrophobic} + \Delta G_{H\text{–}bond} + \Delta G_{H\text{–}bond\,(chg)} + \Delta G_{deformation} + \Delta G_0. \quad (1)$$

The distinct contributions account for the van der Waals interaction between the ligand and the protein, the hydrophobic effect, the hydrogen bonding between the ligand and the protein, the hydrogen bonding involving charged donor and/or acceptor groups, the deformation effect, and the effects of the translational and rotational entropy loss in the binding process, respectively. The intra-ligand van der Waals energy is included in the overall VDW term. The ShapeDock docking engine approximates a complicated search problem. Flexible ligand docking is available by describing the ligand as a torsion tree. Groups of bonded atoms that do not have rotatable bonds are nodes, while torsions are connections between the nodes. Topology of a torsion tree is a determinative factor influencing efficient docking. The AScore/ShapeDock protocol is fast, reproducible, and formally explores all energy minima.

3. Results and discussion

Since two approved anti-influenza drugs, oseltamivir and zanamivir, have come to market, clinically relevant resistance development and possible unknown side effects have become the topics of vital importance. Clinical experience of more than 8 years has not provided evidence that the use of zanamivir would result in viable mutants [1]. The indication may be a consequence of the limited use of this drug. A much wider use of OTV has provided evidence that a viable resistant mutant has emerged [12]. There is a serious indication that mutations at position 274 in H5N1 NA may give rise to the OTV-resistance of H5N1 [5]. To shed more light on the mechanism by which mutations at His274 alter the sensitivity of N1 NA to OTV, His274 was mutated by both smaller side chain (Gly, Ser, Asn, Gln) and larger side chain (Phe, Tyr) amino acid residues.

The list of all the active site residues is available as part A of the Supplementary material. Traditional residues within the active site throughout the family of NA protein structures are highly conserved in all the H5N1 NA mutants, with the only exception that His274Phe and His274Tyr cause to have the bulky Phe and Tyr present in two NA active sites. All the binding free energies are within 1 kcal mol^{-1} (Table 1). For the smaller side chain residue mutations, the energies are about −9.6 kcal mol^{-1} and correspond to an estimated IC_{50} of about 85 nM. Thus, the substitution of His274 by Gly, Ser, Asn, and Gln resulted in slightly enhanced or essentially unchanged the NA sensitivity to OTV. For the larger side chain residue mutations, the energies are roughly −8.8 kcal mol^{-1} and correspond to an estimated IC_{50} of approximately 158 nM. Therefore, the replacement of His274 with Phe or Tyr reduced the NA sensitivity to OTV. An IC_{50} of 152 nM for the His274Tyr NA mutant (Table 1) is in satisfactory agreement with an experimental value of 200 nM [5]. The trend of both the binding free energies and the corresponding IC_{50} values is in accordance with that of the experimentally determined inhibition constants K_i (Table 1).

The interactions of oseltamivir with the active site residues of each N1 mutants are available as part B of the Supplementary Material. Since detected interactions of OTV with the His274X (X=Gly, Ser, Asn, Gln) N1 mutants are similar, a representative case is shown in Fig. 1b. Due to its capability to rotate around the single bond between oxygen and alkyl chain R, the –O–R group of OTV is able to take a comfortable position relative to its environment. By focusing on the position of the –O–R group, the substitution of His274 by a smaller side chain residue, such as Ser, causes a flip of OTV by 180°(Fig. 1). In the original complex there are 8 electrostatic interactions between OTV and the active site residues (Fig. 1a). In the complex of N1 with His274Ser, OTV makes 3 electrostatic interactions with Asp151, Arg152, and Arg371 respectively, besides 2 strong interactions (1.20 and 1.45 Å) with Arg292 and Tyr347 respectively (Fig. 1b). The two strong interactions of OTV are characteristic for all the complexes of OTV with the N1 mutants containing His274X (X=Gly, Ser, Asn, Gln) (Supplementary Material, B). The strong interactions can be viewed as a compensation for fewer electrostatic interactions of OTV with the His274X (X=Gly, Ser, Asn, Gln) NA mutants than with the His274 NA, thus helping to rationalize essentially unchanged the NA sensitivity to OTV (Table 1).

The OTV's particular rotation around the –O–R single bond was established to affect the orientation of the nearest amino acid residues, Trp178, Arg224, Glu227, Glu276, and Arg292 [13]. Glu276 plays a key role by adopting an alternative conformation, which is stabilized by an electrostatic interaction with Arg224 [1,12]. To further investigate the sensitivity of N1 NA to OTV, it is useful to analyze how various side chain volumes of amino acid residues at position 274 affect distinct conformations adopted by Glu276. Note that there are always two electrostatic contacts between Glu276 and Arg292 regardless of particular mutations at position 274 (Supplementary material, B, C). Fig. 2 displays the spatial orientation of Glu276 relative to both oseltamivir and the nearest residues (Arg224, Glu277, Arg292) in the NA active site with His274 and with His274Ser, respectively. There are two electrostatic contacts between Glu276 and Arg224 in the original N1 NA (Fig. 2a), while only one is present in the mutated N1 NA (Fig. 2b). The sole Arg224-Glu276 contact is in common for all the smaller side chain residue substitutions at position 274 (Supplementary material, Figs. S10–S13). The difference relative to the original complex is visible in the trend of the net charges of Glu276

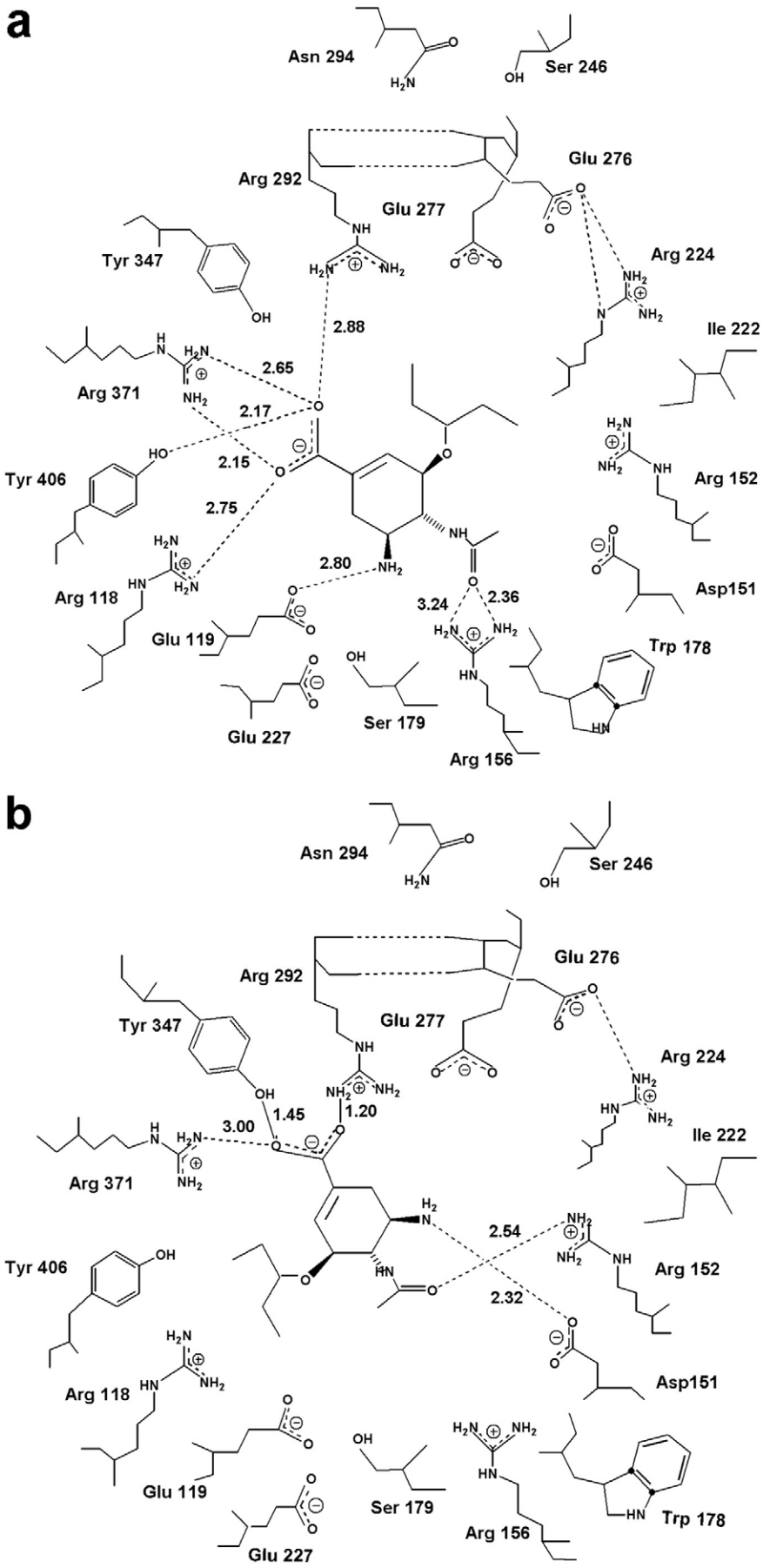

Fig. 1. The interactions (in Å) of oseltamivir with the residues in the active site of N1 NA with His274 (a) and His274Ser (b). Ser is a smaller side chain amino acid residue.

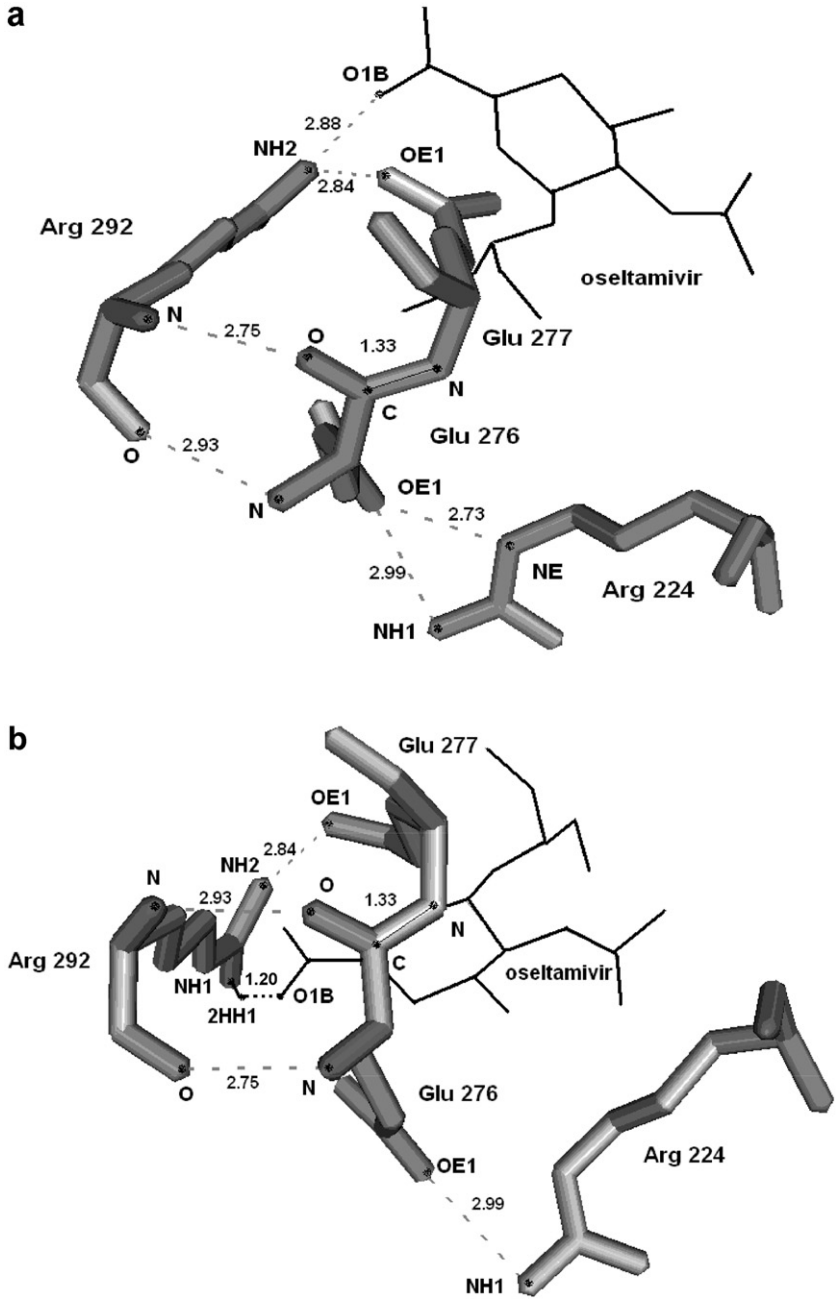

Fig. 2. The spatial orientation of Glu276 relative to both oseltamivir and the nearest amino acid residues (Arg224, Glu277, Arg292) in the active site of N1 NA with His274 (a) and His274Ser (b). The particular orientation (b) of Glu276 is in common for all N1 NAs with the smaller side chain residue (Gly, Ser, Asn, Gln) mutations at position 274 (Supplementary material, Figs. S10-S13).

and Arg224 (Table 1). More negative Glu276 and less positive Arg224, as well as unchanged charge on the side chain of Glu276 are the consequences of the His274X (X = Gly, Ser, Asn, Gln) mutations. Note in Fig. 2 that OTV is clearly behind Glu276 in the mutated complex and is shifted more towards Arg292 by making a strong interaction of 1.20 Å in length. The spatial orientation of Glu276 is unique for all the mutants having the smaller side chain amino acid residues at position 274 (Supplementary material, Figs. S10–S13). This conformation adopted by Glu276 is in agreement, to some extent, with the previously proposed mechanism of OTV-resistance of N1 [7].

His274Phe causes to have Phe involved in the NA active site (Fig. 3a). OTV is rotated a bit more towards left with respect to OTV in the original complex (Fig. 1a), and its –O–R group makes a strong interaction (1.58 Å) with Arg224, besides other 5 electrostatic contacts with Arg118, Glu119, and Trp178. Although Phe274 does not make interactions with Glu276, it most likely influences Glu276 by being in the immediate vicinity (Fig. 3a). Glu276 makes a charge-charge interaction with Arg224 (Fig. 3b). The conformational change of Glu276 is reflected through a substantial negative charge (−27 e) of Glu276 and of Arg224 (Table 1). His274Tyr causes to have Tyr involved in the NA active site (Fig. 4a). By focusing on the –O–R group, OTV is rotated to right by about 90°(Fig. 4a) and is flipped in a horizontal position (Fig. 4b) with respect to OTV in the original complex (Figs. 1a and 2a). There is a strong Glu276-Tyr274 bond (1.75 Å), besides two Glu276…Arg224 and Tyr274…Arg224 electrostatic interactions (Fig. 4a). The conformational change of Glu276, due to the His274Tyr mutation, is visible through substantial negative charges (−31 and −26 e) of Glu276 and Arg224 (Table 1). Note that net charge of the

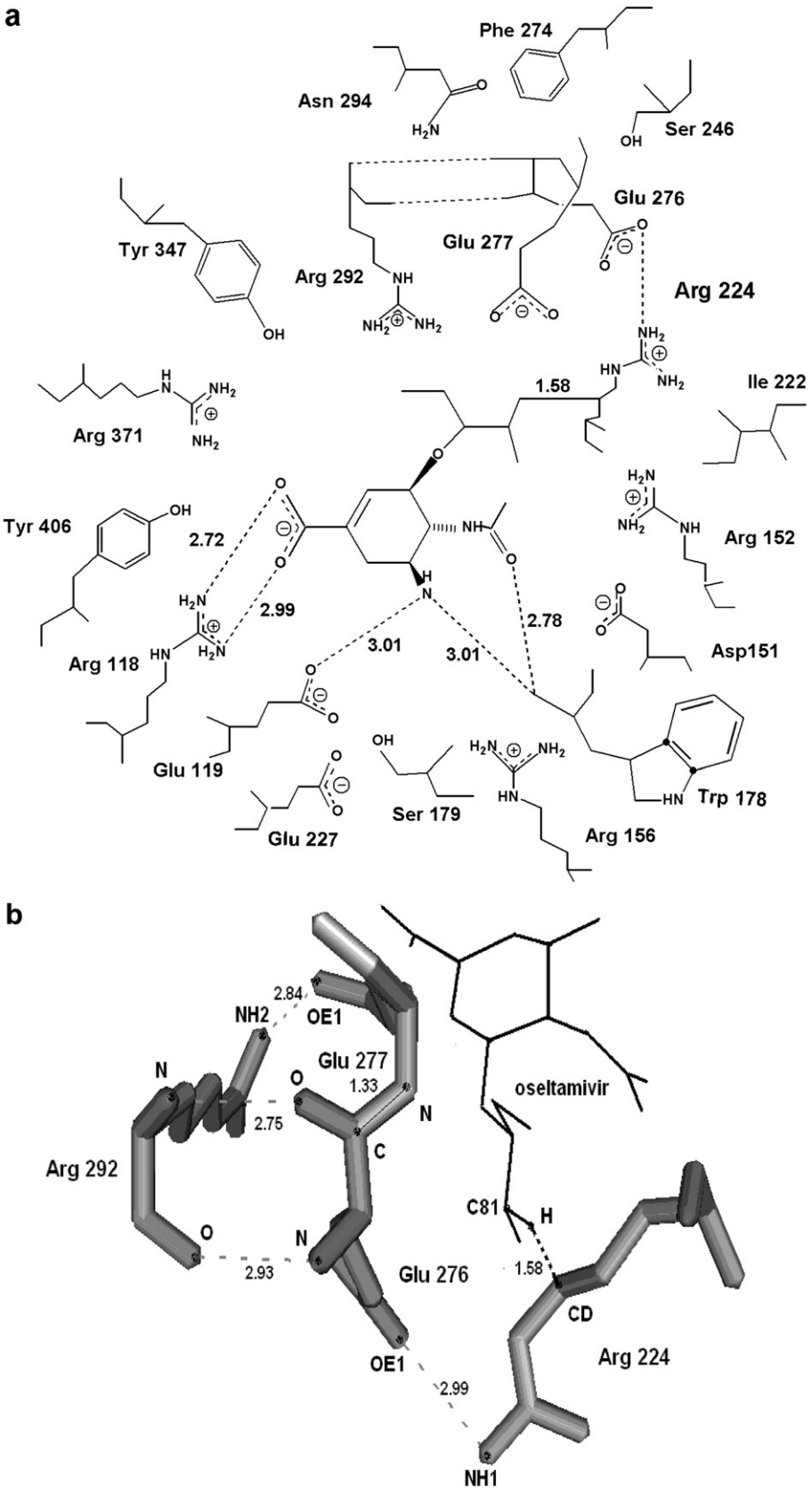

Fig. 3. (a) The interactions (in Å) of oseltamivir with the residues in the active site of N1 NA with His274Phe. Phe is a larger side chain amino acid residue. (b) The spatial orientation of Glu276 relative to both oseltamivir and the nearest amino acid residues (Arg224, Glu277, Arg292).

Glu276 side chain does not reflect to great extent the conformational change of Glu276 due to the His274Phe and His274Tyr mutations (Table 1).

More recently, the crystal structures of oseltamivir-resistant influenza virus neuraminidase mutants were reported by Collins et al. [15]. The structure of the H5N1 mutant His274Tyr-oseltamivir

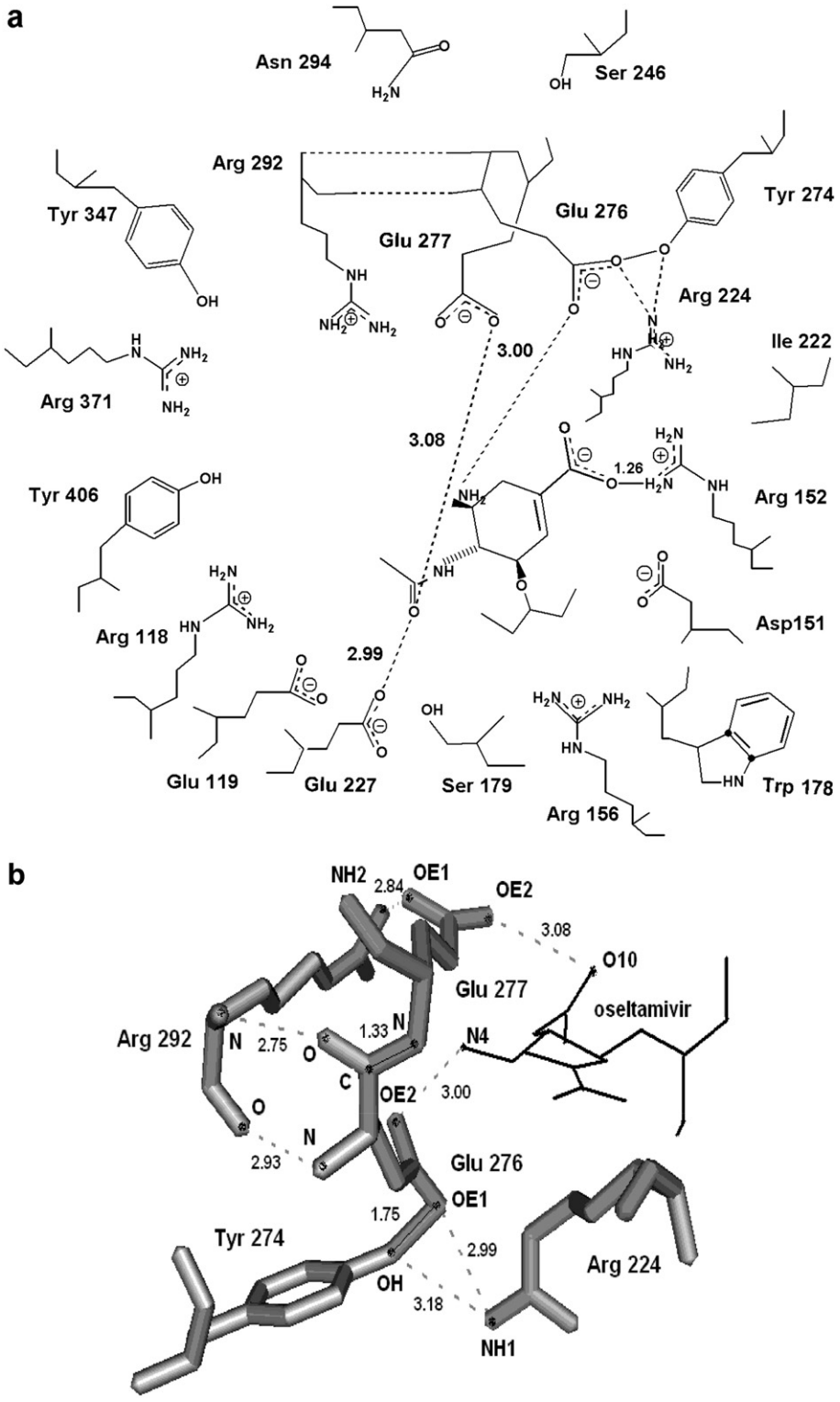

Fig. 4. (a) The interactions (in Å) of oseltamivir with the residues in the active site of N1 NA with His274Tyr. Tyr is a larger side chain amino acid residue. (b) The spatial orientation of Glu276 relative to both oseltamivir and the nearest amino acid residues (Arg224, Glu277, Arg292).

complex (PDB ID: 3cl0) showed that substitution of His by the bulkier Tyr pushes the carboxyl group of Glu276 2 Å farther into the binding site. In this position the charged group disrupts the otherwise hydrophobic pocket that normally accommodates the 3-pentyloxy substituent of oseltamivir and causes a change in the conformation of the inhibitor such that its C9 and C91 carbons move about 2.5 Å from the wild-type NA-bound position. The experimentally determined binding mode of oseltamivir relative to the nearest residues of the mutant His274Tyr is shown in Fig. 5. Interestingly, the particular binding mode is substantially different from the computationally predicted one (Fig. 4b). To rationalize this standpoint, note that the crystal structure of the complex of H5N1 NA with OTV (PDB ID: 2hu0) was the starting point for identifying the active site amino acid residues (Supplementary material, Table S1). His274Tyr caused to have the bulkier and hydrophobic Tyr involved in the N1 active site, thus making Tyr274 able to directly influence the Glu276

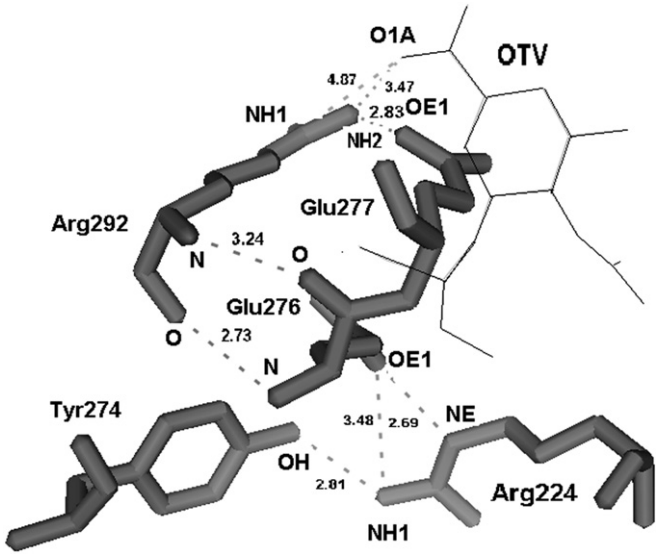

Fig. 5. The experimentally determined binding mode of oseltamivir relative to the nearest residues of the mutant His274Tyr (PDB ID: 3cl0), which was recently reported [15].

conformation (Fig. 4b). In contrast to this, the present computational approach, if applied onto the crystal structure of the H5N1 mutant His274Tyr-oseltamivir complex (PDB ID: 3cl0) reported by Collins et al. [15], properly identifies all the active site amino acid residues, except Tyr274. The experimental binding mode of OTV given in Fig. 5 is thus more similar with that shown in Fig. 2a. Also it is important to note that the experimental binding mode of OTV to the original crystal structure of the H5N1 His274-oseltamivir complex (PDB ID: 3cl2F), reported by Collins et al. [15], is quite similar to that shown in Fig. 5.

The Tyr252His mutation in H5N1 NA with the His274Tyr substitution was hypothesized to be responsible for an increased NA affinity for oseltamivir [6]. However, additional mutation Tyr252His does not make any difference in both the binding free energy of OTV (Table 1) and the interaction of OTV with N1 NA (Figs. S8 and S16).

4. Summary

The resolved question, how volumes occupied by the side chains of various amino acid residues at position 274 influence NA sensitivity to oseltamivir, contributed to several novel insights into the OTV-resistance of H5N1 NA. The smaller side chain residue (Gly, Ser, Asn, Gln) mutations of His274 resulted in slightly enhanced or unchanged NA sensitivity to OTV, while His274Phe and His274Tyr reduced the susceptibility of OTV to N1 NA. The key difference is due to the fact that His274Phe and His274Tyr caused to have the bulkier and hydrophobic residues (Phe, Tyr) involved in the N1 NA active sites, thus directly affecting conformations adopted by Glu276. Hence, OTV-resistance may be ascribed to different hydrophobic interactions of lipophilic side chains with influenza NA. A previous hypothesis that the Tyr252His mutation in H5N1 NA with His274Tyr could be responsible for an increased NA affinity for oseltamivir was not found to hold. Since the binding of OTV appears to be more dependent on interactions with the active site amino acids than on the active site amino acid reorientation, the possibility of escaping H5N1 mutants might be increased by maintaining a clear resemblance of novel inhibitors to sialic acid Neu5Ac, a natural substrate from which zanamivir is directly derived with minimal functional modifications.

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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.bpc.2008.06.003.

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