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Original Article

Docking and molecular dynamics: simulation of the inhibition of H5N1 influenza virus (Anhui 2005) neuraminidase (NA) by chlorogenic acid (CHA)

Jiayi Ren[1,2*], Junqing Huang[3*], Bing Yang[4], Shujian Lin[4], Junqi Li[4], Huaxin Liao[4,5], Xiaohui Yuan[4,6], Xiaomin Wu[7], Shiyi Ou[8]

[1]School of Traditional Chinese Medicine, Jinan University, Guangzhou 510632, Guangdong, China; [2]Zhuhai College of Jilin University, Zhuhai 519041, Guangdong, China; [3]Formula-Pattern Research Center, School of Traditional Chinese Medicine, Jinan University, Guangzhou 510632, Guangdong, China; [4]Institute of Biomedicine, Jinan University, Guangzhou 510632, Guangdong, China; [5]Guangdong Provincial Key Laboratory of Bioengineering Medicine, Guangzhou 510632, Guangdong, China; [6]Zhuhai Trinomab Biotechnology Co., Ltd., Zhuhai 519040, Guangdong, China; [7]College of Life Science, Huaibei Normal University, Huaibei 235000, Anhui, China; [8]Department of Food Science and Engineering, Jinan University, Guangzhou 510632, Guangdong, China. [*]Equal contributors and co-first authors.

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Abstract: Background: Chlorogenic acid (CHA) is an effective component of many traditional Chinese medicines for clearing away heat and detoxification. Studies have shown that CHA can inhibit the influenza virus neuraminidase (NA) and thus exert direct anti-influenza virus action, but the mechanism of inhibition of CHA in NA is not clear. The molecular docking method plays an important role in the study of the mechanism of traditional Chinese medicine active ingredients or natural products against influenza, and provides an effective tool for the discovery and optimization of compounds. Objective: To explore docking and molecular dynamics of the Inhibition of H5N1 Influenza Virus (Anhui 2005) Neuraminidase (NA) by Chlorogenic Acid (CHA). Methods: This study mainly used the molecular docking calculation method to obtain the interaction model between CHA and NA molecules, and then molecular dynamics (MD) simulations were performed to optimize a global 3-D model. The molecular dynamics trajectory data can also be used to calculate the binding free energy between CHA and NA using the MM-PBSA calculation. The key amino acids of the CHA-NA interaction have been analyzed. The mechanism of inhibition of CHA against influenza virus was described from the perspective of structure, energy and amino acid virtual mutation. Results: It was found that as the same as most NA inhibitors, CHA interacted with hydrogen by Arg292, Arg371, Arg118, Glu119 and Glu276 in the surface-active cavity of NA. In addition, CHA also produced a weak interaction with Arg152 of the NA-150-cavity in the form of hydrogen bonds, but did not link with other amino acids of the NA-150-cavity. Conclusion: CHA and NA have non-binding interactions with the traditional surface-active cavity and NA-150-cavity, thereby preventing the flu virus from further invading the host cell. CHA can be an ideal compound, providing broad research prospects for further drug molecular design against the influenza virus NA, especially for the NA-150-cavity.

Keywords: Molecular dynamics (MD) simulation, MM-PBSA, binding free energy, mean square deviation (RMSD), virtual mutation

Introduction

Active ingredients of traditional Chinese medicines or other natural products and their modified derivatives are biologically active, and the basic reasoning is their binding to the receptor, thereby changing the behavior and characteristics of the receptor [1, 2]. The key factors determining the efficacy are the combinations of the active ingredients of traditional Chinese medicine or a natural product inhibitor with a receptor, and its binding degree [3]. The interaction and mechanism of plant-derived inhibitors and receptors are studied from their three-dimensional structural properties and energy, which is more conducive to the optimization of the compounds [4]. Chlorogenic acid (CHA) is also called caffeoyl guinic acid, with chemical name 3-O-caffeoylquinic acid (**Figure 1**). CHA is a carboxylic acid composed of caffeic acid and quinic acid. Its molecular weight is 354.30 kDa, and is an effective component of many traditional

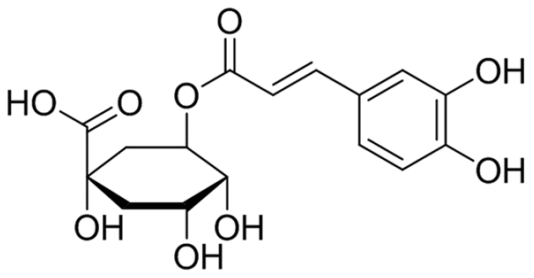

Figure 1. Chemical structure of chlorogenic acid used in this study (CAS_ID: 327-97-9).

Chinese medicines such as eucommia ulmoides, and honeysuckle, etc. [5]. Studies have shown that CHA has an anti-influenza virus effect [6], especially in inhibiting the influenza virus neuraminidase (NA), CHA can directly inhibit influenza virus [3]. However, the mechanism of inhibition of influenza virus neuraminidase by CHA is not clear.

High pathogenic avian influenza (HPAI) can acquire the ability to spread horizontally between mammals or between humans through mutation or recombination, resulting in a pandemic and serious social harm [7]. H5N1 (Anhui 2005) is a Chinese epidemic strain (11C) that was isolated from a pregnant woman infected with a highly pathogenic H5N1 avian influenza virus in Anhui Province in 2005 [8]. The structure-based drug molecular design of the NA protein of H5N1 (Anhui 2005) is of great significance for the prevention and control of H5N1 in China.

Molecular docking calculation is a molecular simulation method for studying the interaction between receptor and ligand through spatial matching and energy matching. It predicts the binding-conformation of small molecule ligands to the appropriate target binding site.

This study first used the molecular docking method [9] to dock CHA with H5N1 (Anhui 2005) influenza virus NA molecules through simulating the actual docking process in which the ligand-protein pairwise interaction energies are calculated. Then, molecular dynamics simulation was carried out, and the mechanism of action of CHA against the influenza virus was proposed from the aspects of binding free energy calculation, key amino acid residue decomposition and virtual mutation. This work is of important significance for the design of novel anti-influenza drugs using CHA as a lead compound.

Materials and methods

Molecular structure preparation and optimization

The CHA structure was obtained from the Chemical Book database (CAS number: 327-97-9, molecular formula C16H1809). Dmol3 quantum chemistry in the Discovery Studio software [10] was used for calculation. The quantum chemical optimization was optimized by the density functional theory B3LYP method. The maximum SCF cycle was set to 300, and the non-bond interaction value was set to 14 angstroms.

For the H5N1 (Anhui 2005) neuraminidase sequence (Genebank_ID: HM172189) [11], the Modeler module in the DS software [12] was used for modeling with 3CKZ_A (homology: 96%, resolution: 1.9), 3B7E_A (homology: 91%, resolution: 1.45) and 3TI4_B (homology: 90%, resolution: 1.602). The Ca^{2+} ions in the active site and the surrounding water molecules were retained. The NA active cavity was defined according to literature reports. The 3D-sequence compatibility and stereochemical rationality of the model were evaluated using the Profile-3D tool [13] and the Ramachandran Plot tool [14].

Molecular docking

The CDocker module under the Discovery Studio 4.5 (DS) platform was used to molecularly dock the CHA and NA active chambers. For the CDocker module in DS, the ligand was docked to the receptor binding site based on Charmm's molecular dynamics (MD) protocol. High-temperature MD simulation was then used to generate a random ligand conformation. By random selection with the receptor binding site and rigid body rotation, simulated annealing was performed to minimize the energy of the entire complex to obtain the final accurate receptor-ligand complex. First, the active cavity of the NA was defined, and then the CHA and NA all-atom Charmm27 force field was given [15]. The steepest descent method (SD) was used to optimize the 500 steps, and then conjugate gradient method (CG) was used to optimize until the whole system convergence criterion was reached 0.4184 kJ/(mol·nm). CHA was used as a ligand, and NA was used as a receptor to run CDocker. The Momany-Rone method was used to allocate atomic partial

charge; The Grid Extension was set to 8.0 angstroms; the random MD target temperature was set to 1000K. After simulated annealing, the highest scored docking model was used as the final composite conformation. For the virtual mutant model, the Mutation tool under the Discovery Studio 4.5 platform was adopted. Based on the docking model, a virtual mutant complex model was constructed.

Molecular dynamics simulation

Molecular dynamics simulations of docking models and mutant models were performed using the Gromacs 5.1.2 software package [16]. The small molecule ligands in the complex are first processed, and the topology file was generated by the antechamber program [17]. Then, in the Amber99sb force field [18], the periodic cube box was defined, and the corresponding SPC/E water solvent model was added. Neutral ions were added to the system using the Genion program in the Gromacs software package to balance the entire system to be electrically neutral. The protein molecules in the system were optimized by the steepest descent method (SD) of 500 steps to eliminate unreasonable van der Waals positional conflicts. Then, the isothermal isovolumetric ensemble (NVT) and the isothermal isostatic ensemble (NPT) were used to perform a 1 ns position-restricted MD simulation of the water solvent and sodium ions in the system (2 ns in total). Finally, a 100 ns unrestricted MD simulation was performed at 310 K with an atmospheric pressure NPT ensemble [19] and periodic boundary conditions, and repeated three times. The PME algorithm was used to calculate the electrostatic interaction. All the bond lengths were limited by the LINK algorithm [20], the time integration step was 2 fs, and the constellation was acquired every 20 ps. A total of 1000 constellations are acquired for each MD track.

MM-PBSA binding free energy calculation

For the kinetic trajectory generated by Gromacs, the equilibrium phase conformation was selected, and the g_mmpbsa tool [21] was executed, and the MM-PBSA method was used to calculate the binding free energy of NA-CHA. In MM-PBSA, the entangalment of the system was calculated by molecular mechanics (MM); the contribution of the polar part to the free energy in the solvent effect was calculated by the method of solving the PB equation; the contribution of the non-polar part to the free energy in the solution effect was calculated by the molecular surface area (SA). The basic principle was as shown in the following formula.

$$\Delta G_{bind}=\Delta E_{MM}+\Delta\Delta G_{sol}-T\Delta S$$

Where ΔG_{bind} is the binding free energy, ΔE_{MM} is the intramolecular energy difference under a vacuum, $\Delta\Delta G_{sol}$ is the solvation free energy difference, T is the absolute temperature, and ΔS is the entropy change. ΔE_{MM} is calculated by molecular mechanics methods, and $\Delta\Delta G_{sol}$ consists of a polar solvation free energy difference and a non-polar solvation free energy difference. The polar part was obtained by solving the finite difference PB equation, and the non-polar part was obtained by estimating the solvent accessible surface area (SA). The $T\Delta S$ was calculated using the regular model method.

Results

H5N1 (Anhui 2005) NA structure and active cavity

The monomer of NA is a beta sheet propeller structure whose active position is located in the annular region at the top of the propeller. It typically consists of catalytic residues (Arg118, Asp151, Arg152, Glu276, Arg292 and Arg371) that interact directly with the inhibitor. In addition, the framework region residues (Glu119, Asp198, Ile222, His274, Glu277, Asn294 and Glu425) inhibitor-NA complexes have a stabilizing effect. According to the results of homology modeling, Arg118, Asp151, Arg152, Glu276, Arg292 and Arg371 were defined as active compartments of the NA protein (**Figure 2**).

The Profile_3D program is generally used to quantitatively evaluate the compatibility between an amino acid sequence and its corresponding three-dimensional structure, especially for spatial structure applications obtained by theoretical calculations. The evaluation results are shown in **Figure 3A**. In the figure, the amino acid with a Compatibility Score above the 0-line was considered to be reasonable and acceptable. The Ramachandran Plot tool was used to compare the structural parameters such as the bond length, the bond angle and the dihedral angle of the model with the corresponding parameters of the template structure

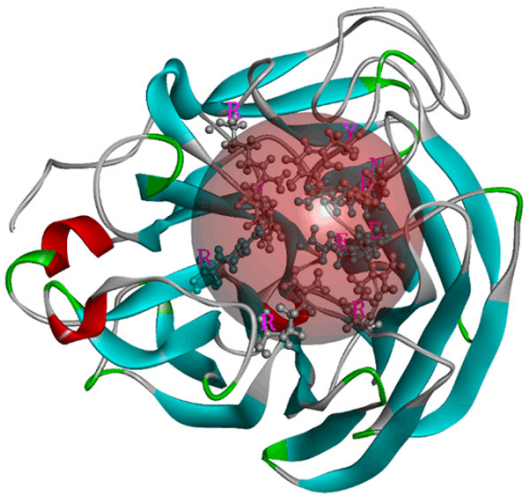

Figure 2. Three-dimensional structure of NA molecules of H5N1 (Anhui 2005). NA is displayed in the form of a secondary ribbon, the active cavity is represented by a red sphere and the main active sites are labeled with a purple amino acids.

to check whether there was an unreasonable conformation. As a result, more than 95% of the atoms fall in the first quadrant, the second quadrant and the third quadrant, indicating that the bond length, bond angle and dihedral angle of the entire molecule are reasonable (**Figure 3B**).

Analysis of the combination mode of chlorogenic acid and NA

The CDocker program was used to molecularly dock CHA and NA to obtain the highest docking pose (**Figure 4**). CHA had the strong interaction with catalytic residues on the active cavity of NA, such as formation of hydrogen bonds with sites Arg292, Glu276 and Glu119. It attracted the charge formed by Arg371 and Arg118, and played a major role in the combination of CHA and NA. The whole molecule was embedded in the active cavity of the NA molecule. It was also found that CHA formed a hydrogen bond interaction with Arg152 in the 150-cavity of NA, but did not interact with other amino acids in the 150-cavity.

By monitoring the hydrogen bond formation of the NA-CHA complex, 12 intermolecular hydrogen bonds were formed between the NA-CHAs in the docking model (**Table 1**). These hydrogen bonds had an average length of about 2.8 angstroms, indicating that they played a key role in the binding of the NA-CHA during complex formation. From the analysis of the NA-CHA complex configuration, during the interaction between CHA and NA, it had a good combination with the catalytic residues of the NA active cavity and 150-cavity, in which hydrogen bonding played an important role.

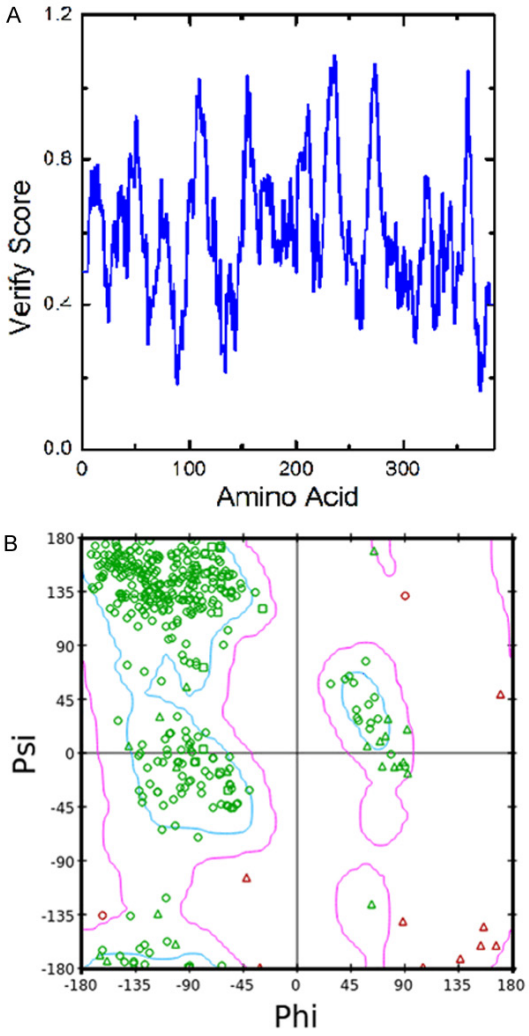

Figure 3. Structure evaluation of H5N1 (Anhui 2005) NA. A. Profile of the 3D verification result of the NA structure with residues exhibiting reasonable folding. B. The Ramachandran plot shows phi-psi torsion angles of all residues in the structure.

Stability of the docking compound

As a global optimization tool, molecular dynamics simulation can be used to solve the energy barrier problem that EM calculation can't cross, that is, to search for the optimal structure of the initial structure under the current conditions in a certain MD simulation time. Secondly, after the MD simulation has obtained the opti-

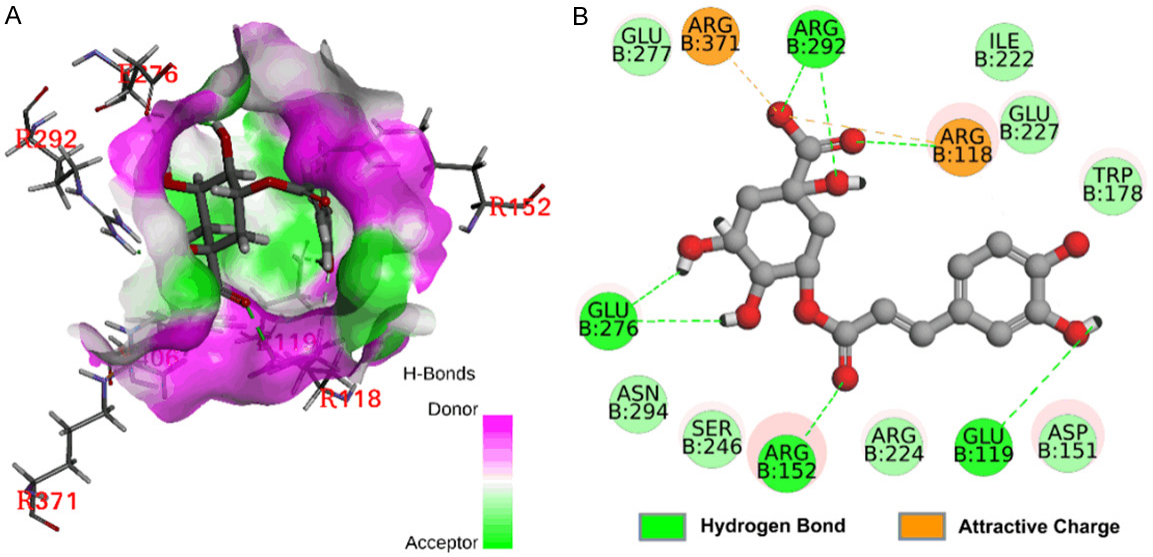

Figure 4. Interaction of chlorogenic acid (CHA) with H5N1 (Anhui 2005) NA. A. Display of the receptor surfaces with H-bond contacts. B. 2-D Diagram of the CHA and H5N1 (Anhui 2005) NA active sites.

Table 1. The hydrogen bonds in the docking complex

Donor (hydrogen donor)	Acceptor (hydrogen acceptor)
NA:ARG118:HH11	CHA:O21
NA:ARG118:HH12	CHA:O22
NA:ARG152:HH22	CHA:O10
NA:ARG292:HH12	CHA:O23
NA:ARG292:HH22	CHA:O22
NA:ARG371:HH11	CHA:O22
NA:ARG371:HH21	CHA:O22
NA:ARG118:HD2	CHA:O24
CHA:H38	NA:GLU276:OE1
CHA:H39	NA:GLU276:OE1
CHA:H41	NA:GLU119:OE1
CHA:H36	NA:GLU276:OE1

mal conformation, it will maintain a balanced conformation, which can be used for the conformational selection of the MM-PBSA calculation. For the NA-CHA complex system, a 100 ns molecular dynamics simulation was performed under the Amber99sb-spce model and repeated three times (named MD-1, MD-2, MD-3, respectively), and then from the backbone of the whole protein. The root-mean-square deviation (RMSD) of the molecular and NA active cavities was used to analyze the stability of the docking complex.

For the molecular kinetic trajectory of the 100 ns NA-CHA complex system, three replicate MD calculations exhibited similar RMSD curves. The skeleton RMSD showed that the entire system reached a balance of around 40 ns (**Figure 5A**). The goal of this study was to investigate the interaction between CHA and NA. Therefore, the NA active cavity catalytic residues (Arg118, Glu119, Arg152, Glu276, Arg292 and Arg371) and the outer framework residues (Asp198, Ile222, His274, Glu277, Asn294 and Glu425) were defined using the indexing tool in Gromacs, which together formed the interaction group with CHA (**Figure 5B**). The RMSD curve of this group of atoms had been stable and kept fluctuating within 0.1 nm (1 angstrom), indicating that the CHA and NA docking complex was very stable under the kinetic model of Amber99sb-spce.

In the molecular dynamics simulation of the initial model of NA-CHA molecular docking, in addition to the CHA and NA wild-type complex NA-CHA (WT), six NA-CHA complex mutants (alanine replacement) were designed. They were NA-CHA (292), NA-CHA (371), NA-CHA (118), NA-CHA (119), NA-CHA (152), and NA-CHA (276). For the mutant complex, we performed a 20 ns MD simulation. For the wild-type and mutant complexes of NA-CHA, 20 ns was sufficient to bring the entire molecule to equilibrium (**Figure 6**). Moreover, when most of the MD simulations were carried out to 5-7 ns, the system had basically reached equilibrium. The trajectory of the equilibrium state was used for sampling for the MM-PBSA calculation.

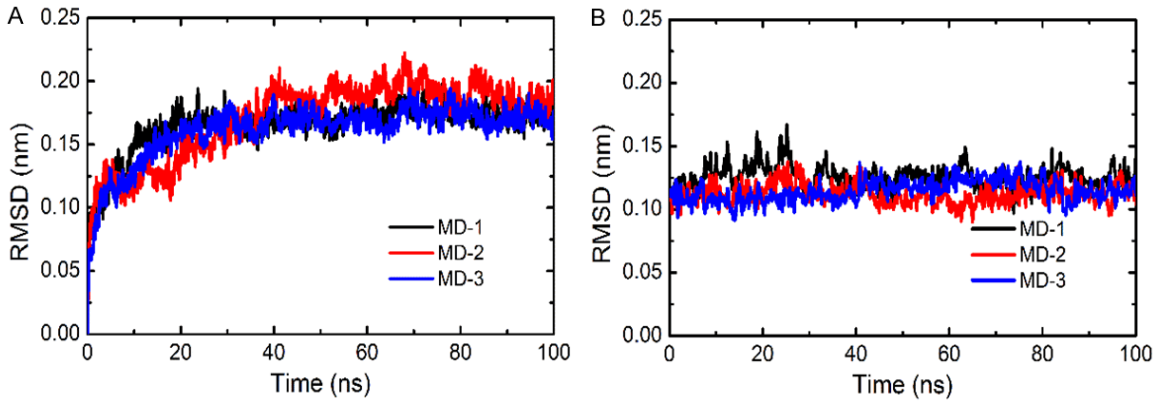

Figure 5. The root-mean-square deviation (RMSD) as a function of the NA-CHAMD simulation time. A. Backbone-atom RMSD of whole the H5N1 (Anhui 2005) NA structure. B. Catalytic residues at the active site and the residues in the framework regions RMSD.

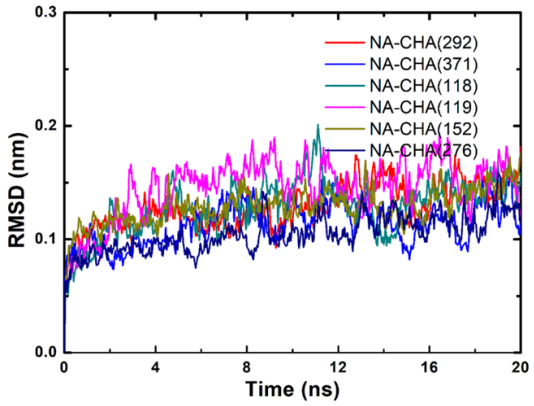

Figure 6. The root-mean-square deviation (RMSD) as a function of the NA-CHA mutants MD simulation time. The catalytic residues of the six active sites (Arg118, Glu119, Arg152, Glu276, Arg292 and Arg371) shown in different color curves.

Binding free energy calculation and energy decomposition

The MM-PBSA calculation of CHA and influenza virus NA protein was performed using the g_mmpbsa tool and the conformation was sampled by MD simulation trajectory, combined with the analysis tool in Gromacs. The residue obtained from the wild-type molecular dynamics trajectory was decomposed, and the energy values obtained from the three MDs were averaged. The energy contributions of the amino acid sites on the five polypeptide segments were examined respectively, namely VAL116-ILE122 containing 150-cavity and catalytic residues VAL149-PRO154, HIS274-CYS278, VAL290-ASV294 and ASN369-GLY373 containing active chambers. The major key amino acid positions were negative (**Figure 7**). It was indicated that these amino acids were mainly involved in the energy contribution of binding to CHA.

Since g_mmpbsa did not include entropic calculations in the current version, it calculated relative binding free energy. However, for the same NA system, it was comparable to calculate the binding free energy of small molecule inhibitors with the same NA system and its mutants. As shown in **Table 2**, for the six mutants of NA-CHA, the calculated binding free energy -31.429±22.483 kJ/mol for NA-CHA (292), and -28.295±23.027 kJ/Mol for NA-CHA (371), -51.443±21.124 kJ/mol for NA-CHA (118), -39.639±23.023 kJ/mol for NA-CHA (119), -50.450±20.501 kJ/mol for NA-CHA (152), and -50.279±28.549 kJ/mol for NA-CHA (276).

CHA interacted closely with the catalytic residues Arg292, Arg371 and Glu119 on the active cavity of NA molecules. The mutation of these three amino acid sites can cause the binding of mutants to CHA, so these three sites were the key amino acid sites where NA binds to CHA. The two amino acids Arg118 and Glu276 contributed to the binding of NA to CHA, but the energy of the mutant was not much decreased. In addition, CHA had a weak interaction with Arg152 on the 150-cavity of the NA molecule.

Discussion

Chlorogenic acid (CHA) is an active component of many Chinese medicines such as eucommia ulmoides and honeysuckle [22]. Many studies

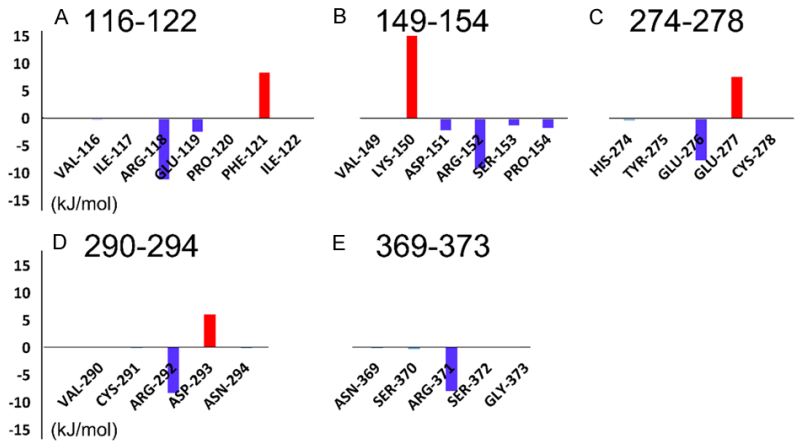

Figure 7. Amino acid energy decomposition with the five peptides in NA containing six catalytic residues in the active sites. A. Peptide VAL116-ILE122. B. Peptide VAL149-PR0154. C. Peptide HIS274-CYS278. D. Peptide VAL290-ASV294. E. Peptide ASN369-GLY373.

Table 2. Binding Free Energy of NA-Chlorogenic acid Complexes

NA-CHA	Binding free energy (kJ/mol)
NA-CHA (WT)	-52.015±19.987
NA-CHA (292)	-31.429±22.483
NA-CHA (371)	-28.295±23.027
NA-CHA (118)	-51.443±21.124
NA-CHA (119)	-39.639±23.023
NA-CHA (152)	-50.450±20.501
NA-CHA (276)	-50.279±28.549

have shown that CHA can directly inhibit influenza virus, especially the influenza virus neuraminidase, and thus prevent it from infecting new host cells [23, 24]. In computer aided drug design, influenza virus NA is a very important target. The anti-influenza drug oseltamivir is one such successful case [25]. In this study, we used molecular docking to explore the association between CHA and NA, and then used molecular dynamics and MM-PBSA-based methods to calculate the energy decomposition of the bound free energy and key interacting amino acids. Furthermore, a theoretical discussion was made on the mechanism of action of CHA against the influenza virus.

The model used in this study is the highly pathogenic avian influenza virus, H5N1 (Anhui), which is of great significance for the prevention and control of H5N1 in China. Since a suitable template can be found in the PDB database, this study uses a homology modeling approach to model the NA spatial structure.

Studies have shown that the NA active cavity is highly conserved among different types of influenza viruses [26]. This study used the molecular docking CDocker to obtain the interaction model between CHA and NA and found that there are non-bonded interactions that act between the traditional active cavity of CHA and NA. At the same time, CHA also forms a weak hydrogen bond interaction with Arg152 in the 150-cavity of NA. This provides a new direction for the mechanism of active constituents of traditional Chinese medicine against the influenza virus and the design of influenza virus NA inhibitor molecules based on CHA as a leading compound.

Molecular dynamics simulation can not only overcome the energy barrier problem that molecular mechanics can't overcome, but it also can be used as a global optimization tool to globally optimize the NA-CHA complex model obtained by molecular docking, in search for conformation and finding a globally optimum solution [27]. At the same time, the trajectory of the equilibrium molecular dynamics simulation can provide sampling for the MM-PBSA method, and further clarify the energy contribution of the above key amino acids by energy decomposition. Using the Amber99sb-spce model, this study performed 100 ns molecular dynamics simulations and established an independent analytical index with active catalytic residues and CHA. The CHA-NA structure remained stable during the MD process. The results of the free energy calculation are consistent with that of the molecular docking calculation. The results indicated that the mechanism of CHA against the influenza virus was mainly through the hydrogen bonding interactions with Arg292, Arg371, Arg118, Glu119, Arg152 and Glu276 on the NA protein. The binding free radical decomposition of NA key amino acid virtual mutants indicated that CHA can interact with hydrogen via the catalytic residues Arg292, Arg371, Arg118, Glu119 and Glu276 of the NA

active cavity. It also had a weak interaction with the NA-150-cavity of Arg152 in the form of hydrogen bonds, but did not associate with other amino acids of NA-150-cavity. This suggested that for the NA-150-cavity, CHA can be an ideal leading compound, providing a broad space for further drug design against the influenza virus NA.

In this study, molecular docking, molecular dynamics simulations, free energy calculations and virtual mutations were used to explore the interaction patterns of CHA and NA and the key amino acids involved, and to explore the mechanisms of action of CHA against the influenza virus. This is instructive for the design of novel anti-influenza drugs using CA and CHA as leading compounds.

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Disclosure of conflict of interest

None.

Address correspondence to: Xiaomin Wu, College of Life Science, Huaibei Normal University, No. 100, Dongshan Road, Huaibei 235000, Anhui, China. Tel: +86-0561-3083237; E-mail: wuximi0@163.com; Shiyi Ou, Department of Food Science and Engineering, Jinan University, No. 601, Huangpu Road West, Guangzhou 510632, Guangdong, China. Tel: +86-020-85220130; E-mail: tosy@jnu.edu.cn

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