Introduction

Influenza virus, which has high rates of morbidity and mortality, is one of the major causes of viral respiratory infections. This virus mutates frequently, spreads rapidly, and occasionally transfers from animals to humans (e.g., avian influenza A, H5N1) [1]. The increasing geographic distribution of this epizootic virus has aroused serious concerns about the therapeutic methods currently available to curb a potential pandemic of this disease. To treat influenza, two classes of anti-influenza agents, M2 ion channel blockers and neuraminidase (NA) inhibitors, have been used [2,3]. However, the results were shown in Table S2 and Figure S9, S10, S11, S12, S13, S28, and S29). The results were summarized in Table 2. From this dataset, the disubstituted caffeoylquinic acids (a, b, c, d, e) exhibited higher binding affinities to PAc relative to caffeic acid alone, quinic acid alone, and monosubstituted caffeoylquinic acids (f, g).

Results and Discussion

In previous work, we have discovered chlorogenic acid (CA) derivatives for their polymerase inhibitor activity. Among them, seven compounds were PAc ligands, and four inhibited influenza RNA polymerase activity. These results aid in the design of anti-influenza agents based on caffeoylquinic acid.
Table 1. Candidate compounds in screening.

| Candidates | Compound name               |
|------------|------------------------------|
| a          | 3,4-dicaffeoylquinic acid    |
| b          | 1,5-dicaffeoylquinic acid    |
| c          | 4,5-dicaffeoylquinic acid    |
| d          | 3,5-dicaffeoylquinic acid    |
| e          | 1,3-dicaffeoylquinic acid    |
| f          | 5-cafeoylquinic acid         |
| g          | 4-cafeoylquinic acid         |
| h          | quinic acid                  |
| i          | caffeic acid                 |

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Thus, the number and position of caffeoyls on quinic acid is an important factor in PAC binding. On the other hand, although the docking simulation predicted that candidate b would bind tightly to the target, experimentally this compound behaved oppositely in affinity evaluation by NMR and SPR. This may be due to steric hindrance of the bulky trans-disubstitution of caffeoyl at the 1- and 3-position of the quinic acid. Other disubstituted caffeoyl quinic acids bind to PAC with high affinities because the caffeoyls are either cis-disubstituted or both in equatorial positions.

In order to address specificity of the binding, the interaction between compound a and Human Serum Albumin (HSA) was evaluated by relaxation-edited NMR. As shown in Supporting Information (Figure S30), compound a did not specifically interact with HSA.

The PAC subunit is essential in influenza’s RNA polymerase activity, so one may expect that PAC ligands should inhibit RNA polymerase activity as well. The effects of these compounds on polymerase activity were evaluated by an ApG primer extension assay [24]. The polymerase can use ApG as a primer to synthesize cRNA from vRNA promoters; therefore, the length of cRNA can be used to judge polymerase activity. As shown in Figure 2, the candidate compounds a, b, c, and d inhibited the synthesis of cRNA while candidate f showed slight inhibition. The inhibition rates of compounds a, b, c, and f on polymerase activity were 75%, 54%, 81%, 67%, and 26%, respectively. Notably, candidate e enhanced the polymerase activity at a rate of 42%. Although these compounds were assayed at relatively high concentrations (5 mM), the discovery of the inhibition effectiveness on polymerase activity of these CA derivatives suggests that CA could be a lead structure for potential anti-influenza drugs. These results clearly show that substituted caffeoylquinic acids with low steric hindrance are more likely to be effective inhibitors against polymerase because they bind strongly to PAC. It was also observed that some PAC ligands did not exhibit activity in the ApG assay. This may well be due to their non-specific or weak binding to PAC.

In summary, the avian influenza viral RNA polymerase protein PAC, a conserved key target in the design of a new generation of anti-influenza agents, was used to screen lead plant-derived anti-influenza compounds. Seven compounds were identified as PAC ligands. Among them, four compounds inhibited polymerase activity. Therefore, PAC made a useful target to screen for anti-influenza agents. Based on the structure-activity relationship of CA derivatives as polymerase inhibitors, the position and number, and maybe also the synergistic effect, of the caffeoyls in quinic acid played important roles in the inhibition potential of polymerase activity. These results provided an important step in caffeoylquinic acid structure-based design of anti-influenza agents.

Materials and Methods

Materials

1,5-dicaffeoylquinic acid, 1,3-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, 5-cafeoylquinic acid, 4-cafeoylquinic acid, quinic acid, and caffeic acid were purchased from Chengdu Mansite Pharmaceutical Co., Ltd. (Sichuan, China). D2O and 3-(trimethylsilyl) propionic acid-d4 (TSP-d4) sodium salt were purchased from Sigma Co. (USA).

Protein expression and purification

Methods for the preparation of PAC protein were previously described [9]. Briefly, residues 257–716 of the PA subunit of avian H5N1 influenza A virus (A/goose/Guangdong/1/96) were cloned into a pGEX-6p vector (GE Healthcare) and transformed into E. coli strain BL21. Cells were cultured in LB medium at 37°C with 100 mg/L of Ampicillin. When the OD600 reached 0.6–0.8, the culture was induced with 0.5 mM isopropyl-thio-D-galactosidase (IPTG) at 16°C. After 20 hours of incubation, the cells expressing PAC were harvested and combined by centrifugation at 5000 rpm for 10 min. Recombinant protein was purified with a glutathione affinity column (GE Healthcare). Glutathione S-transferase (GST) was cleaved with PreScission protease (GE Healthcare), and the protein complex was further purified by Q sepharose FF ion exchange chromatography and Superdex-200 gel filtration chromatography (GE Healthcare). Methods for the preparation of the RNA polymerase were previously described [25]. Briefly, the RNA polymerase (PA, PB1, and PB2) complex was expressed in hi5 insect cell and purified by Ni-affinity column, ion-exchange column, and gel exclusion chromatography.

The affinity analysis based on virtual docking

To predict the binding affinities between candidate compounds and PAC, the simulated flexible docking of ligands was carried out using the AutoDock (v.4.01) software package. The structure of PAC was retrieved from the Protein Data Bank (PDB entries: 5CM8) and modified for visual docking. First, in the PDB file,
water molecules were removed, polar hydrogen atoms were added, and non-polar hydrogen atoms were merged using the Hydrogen module in the AutoDock Tools (ADT). Then, Kollman united atom partial charges were assigned. The grid map of the docking simulation was established in a $61 \times 61 \times 61$ cube centered on the target active sites referred to in a previous report [10]. The targets are defined as site 1: center of K328, K539, R566 and K574; site 2: center of K539, R566, K574 and N696; site 3: center of E410, K461, E524 and K536; site 4: center of F411, M595, L666, W706, F710, V636 and L640; and site 5: center of 620 and 621. There is a spacing of 0.375 Å between the grid points. When the ligand was docked to the PA$_C$ target, the Lamarckian genetic algorithm was used to optimize the conformation of the ligand in the binding pocket. The parameters were set to the following: the size of the population was 150; the number of energy evaluations was set to $1.0 \times 10^8$ as the run terminates; for clustering the conformations, the root mean square deviation tolerance was set to 2.0; fifty independent docking runs were carried out for every ligand; and other parameters were set to default. The binding affinities of the candidate compounds to the targets were summarized in Table S2, and the average p$K_a$ of the five active sites was summarized in Table 2.

Figure 1. Binder screening by relaxation-edited NMR. Spectra of 1,5-dicafeoylquinic acid (compound b) in the absence (plots a, b) and presence (plots c, d) of PA$_C$, with the CPMG spin-lock time labeled beside each spectrum. The concentration of the small molecule and PA$_C$ was $1.0 \times 10^{-7}$ mol/L and $4.4 \times 10^{-6}$ mol/L, respectively. The water peak located at $\delta$ 4.8 and 1 mM of TSP was added to the sample as a reference ($\delta$ 0). Signals of the small molecule in the absence of PA$_C$ attenuated slightly when 600 ms of CPMG spin-lock was applied (plot a). These signals were eliminated at the same spin-lock time in the presence of PA$_C$ (plot c). This difference should be ascribed to the increase in transverse relaxation rate ($R_2$) of the small molecule upon its binding to PA$_C$. The ligand peaks that attenuated when applying CPMG spin-lock in the presence of PA$_C$ were marked with ‘*’ in plots b and d.

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NMR experiments

NMR experiments were performed on a Bruker AVANCE 600 spectrometer equipped with a 5 mm BBI probe capable of delivering z-field gradients, using TOPSPIN software (Bruker, version 2.1) was used in experimental manipulating and data processing. All experiments were carried out at 298.2 K. The relaxation-edited NMR experiments utilized a \([D/\text{pre-saturation-90}_z/(\Delta-180)_y-\text{acquire}]\) pulse sequence, in which the CPMG sequence was used for the spin-lock. For all relaxation-edited experiments the following variables were used: pre-saturation water suppression was applied in pre-acquisition delay \((D = 3 \text{ s})\), \(P_{90}\) was measured and set up for each sample, \(D = 1.5 \text{ ms}\), and \(2 \times n \times \Delta = \text{total spin-lock time}\). The spectra were collected with 64 k of data points and 32 scans. Transverse relaxation times were measured by a pseudo-2D experiment using the CPMG sequence. Presaturation was applied in a pre-acquisition delay for water suppression, and 32 k and 16 data points were set for the F2 and F1 dimension, respectively. In each experiment, the following variables were used: \(D = 3 \text{ s} \) (pre-acquisition delay); \(P_{90}\) was measured and set up for each sample; \(\Delta = 1.5 \text{ ms}\; \text{and} \; 2 \times n \times \Delta = \text{total spin-lock time}\). The spectra were collected with 32 scans, and each \(T_2\) was calculated using TOPSPIN (Bruker, version 2.1) software by simulating the peak attenuation curve in different spin-lock times.

The affinity analysis based on \(T_2\) simulation

The method to evaluate \(K_d\) by \(T_2\) simulation was previously reported [26]. When a ligand \((L)\) and a protein \((P)\) form a complex \((LP)\), there is a dissociation equilibrium \(LP = L + P\) in the solution. The dissociation constant, \(K_d\), of this equilibrium is a key factor to describe the binding strength of the ligand and protein. It can be evaluated by the transverse relaxation time \((T_2)\) of the ligand in the presence of the protein. The expression describing the observed effective transverse relaxation rate \(R_{2\text{obs}} (1/T_{2\text{obs}})\) as a function of molar ratio of protein to ligand \((C_P/C_L)\) is:

\[
2(R_{2\text{obs}} - R_{2F})/(R_{2B} - R_{2F}) = 1 + (K_d/C_P)(C_P/C_L) + n(C_P/C_L) - \\
\left[\frac{1}{4} + (K_d/C_P)(C_P/C_L) + n(C_P/C_L)^2 - 4n(C_P/C_L)^2\right]^{1/2}
\]

Since the total concentration of PAC, \(C_P\), and the ligand, \(C_L\), in the experiments were known, it is possible to obtain the \(T_{2F}\) and \(T_{2B}\) from the plot of \(T_{2\text{obs}}\) versus \(C_P/C_L\). Extrapolation of the curve to \(C_P/C_L = 0\) should give \(T_{2F}\) and to infinite give \(T_{2B}\). Then, by simulating the plot of \(R_{2\text{obs}}\) versus \(C_P/C_L\) using the above equation, \(K_d\) can be simultaneously obtained.

Figure 2. Results of ApG primer extension assay. (a) The effects of candidate compounds a, b, c, d, e, f, and g on polymerase activity in an ApG primer extension assay. The concentrations of the small molecules were 5 mM. NP stands for the negative reference with “no polymerase”, and ND for the positive reference with “no drug.” (b) The quantification of the results from (a) obtained by phosphorimaging analysis. The results are the average of two independent experiments and the derivations are shown.

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To evaluate the binding affinities between the candidate compounds and PA C, a series of samples containing small molecules and PA C with different concentration ratios (shown in Figure S9, S10, S11, S12, and S13) were prepared. These T2 of the ligands were determined by the CPMG method using NMR. As we can see from Figure S9, S10, S11, S12, and S13, the observed T2 (T2obs) of the ligand decreases when the concentration ratio of protein to ligand (CP/Cp) increases and the variation trends according to exponential decay. By simulating the plot of $R_2$ vs $R_2$ using equation (6), the $k_d$ value can be obtained. The results of the simulation were summarized in Table 2.

The affinity analysis based on Surface Plasmon Resonance (SPR)

An affinity analysis of the interaction of candidate compounds a-g and PA C was carried out using an SPR spectrometer (Biacore 3000, GE Healthcare Bio-Sciences, Sweden). PA C at a concentration of 10 ng/mL in 10 mM NaAc, pH 5.5, was used to couple this protein to a CM5 sensor chip. To determine the affinity of small molecules to PA C, increasing concentrations (labeled in Figure S14, S15, S16, S17, S18, and S20) of the small molecules diluted in running buffer (130 mM PBS, pH 7.5) were injected over the sensor chip for 60 s (association phase), which was followed by dissociation for 180 s and recording of the spectra. All of the experiments were performed at 25°C, and the flow rate was 30 µL/min. The surface at the end of each experiment was regenerated using 20 mM NaOH at 30 µL/min for 10 s to remove any bound analyte. The data were analyzed using the BIAevaluation software (4.1 version) to calculate the affinity constant. The association and dissociation kinetics plots of the small molecules to PA C were displayed in Figure S14, S15, S16, S17, S18, and S19, and the results were summarized in Table 2.

Polymerase activity test

The ApG primer extension assay was previously described [24]. We performed 5 µL reactions with 2.5 µL 3P and 0.7 µm model vRNA promoter (an equimolar mixture of the 5’-end vRNA 5’-AGUAGAAACAAGGCC-3’ and 3’-end vRNA 5’-GGCCUCUUGCU-3’) in the presence of 5 mM MgCl2, 5 mM drug, 5 mM dithiothreitol, 1 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 0.1 µM [γ-32P]GTP (3,000 Ci/mmol), and 2 U/µL RNasin (Promega). Where indicated, 0.5 mM ApG (Sigma) was added to the reaction. The reaction system can be described as: 0.25 µL 0.1 M MgCl2, 0.25 µL 0.1 M DTT, 0.5 µL 10 mM ApG, 0.25 µL RNasin, 0.25 µL of TSP was added to the sample for 1 h. The loading buffer, 5 µL 2×formamide/bromophenolblue/EDTA, and the mixture were heat to 95°C for 2 min. Analysis was performed by running the samples on a 18% PAGE with 1×Trisborate-EDTA and 8 M urea followed by autoradiography.

Supporting Information

Figure S1  Binder screening by relaxation-edited NMR. Spectra of 3,4-dicaffeoylquinic acid (compound a) in the absence (plots a, b) and presence (plots c, d) of PA C. The CPMG spin-lock time of each experiment was labeled beside the spectrum. The concentration of the small molecule and PA C was 1.0×10⁻³ mol/L and 7.0×10⁻⁶ mol/L, respectively. The water peak located at δ 4.8 and 1 mM of TSP was added to the sample as a reference (δ 0). The ligand peaks that attenuated when applying CPMG spin-lock in the presence of PA C were marked with "*" in plot d. (TIF)

Figure S2  Binder screening by relaxation-edited NMR. Spectra of 4,5-dicaffeoylquinic acid (compound c) in the absence (plots a, b) and presence (plots c, d) of PA C. The CPMG spin-lock time of each experiment was labeled beside the spectrum. The concentration of the small molecule and PA C was 1.0×10⁻³ mol/L and 7.0×10⁻⁶ mol/L, respectively. The water peak located at δ 4.8 and 1 mM of TSP was added to the sample as a reference (δ 0). The ligand peaks that attenuated when applying CPMG spin-lock in the presence of PA C were marked with "*" in plot d. (TIF)

Figure S3  Binder screening by relaxation-edited NMR. Spectra of 3,5-dicaffeoylquinic acid (compound d) in the absence (plots a, b) and presence (plots c, d) of PA C. The CPMG spin-lock time of each experiment was labeled beside the spectrum. The concentration of the small molecule and PA C was 1.0×10⁻³ mol/L and 7.0×10⁻⁶ mol/L, respectively. The water peak located at δ 4.8 and 1 mM of TSP was added to the sample as a reference (δ 0). The ligand peaks that attenuated when applying CPMG spin-lock in the presence of PA C were marked with "*" in plot d. (TIF)

Figure S4  Binder screening by relaxation-edited NMR. Spectra of 1,3-dicaffeoylquinic acid (compound f) in the absence (plots a, b) and presence (plots c, d) of PA C. The CPMG spin-lock time of each experiment was labeled beside the spectrum. The concentration of the small molecule and PA C was 1.0×10⁻³ mol/L and 5.5×10⁻⁶ mol/L, respectively. The water peak located at δ 4.8 and 1 mM of TSP was added to the sample as a reference (δ 0). The ligand peaks that attenuated when applying CPMG spin-lock in the presence of PA C were marked with "*" in plots d (TIF)

Figure S5  Binder screening by relaxation-edited NMR. Spectra of 5-cafeoylquinic acid (compound g) in the absence (plots a, b) and presence (plots c, d) of PA C. The CPMG spin-lock time of each experiment was labeled beside the spectrum. The concentration of the small molecule and PA C was 1.0×10⁻³ mol/L and 8.7×10⁻⁶ mol/L, respectively. The water peak located at δ 4.8 and 1 mM of TSP was added to the sample as a reference (δ 0). The ligand peaks that attenuated when applying CPMG spin-lock in the presence of PA C were marked with "*" in plot d. (TIF)

Figure S6  Binder screening by relaxation-edited NMR. Spectra of quinic acid (compound h) in the absence (plots a, b) and presence (plots c, d) of PA C. The CPMG spin-lock time of each experiment was labeled beside the spectrum. The concentration of the small molecule and PA C was 1.0×10⁻³ mol/L and 8.7×10⁻⁶ mol/L, respectively. The water peak located at δ 4.8 and 1 mM of TSP was added to the sample as a reference (δ 0). The ligand peaks that attenuated when applying CPMG spin-lock in the presence of PA C were marked with "*" in plot d. (TIF)
8.7 × 10^{-6} \text{ mol/L}, \text{ respectively. The water peak located at } \delta \text{ 4.8 and 1 mM of TSP was added to the sample as a reference (} \delta \text{ 0). (TIF)}

**Figure S8** Binder screening by relaxation-edited NMR. Spectra of caffeic acid (compound i) in the absence (plots a, b) and presence (plots c, d) of PAC. The CPMG spin-lock time of each experiment was labeled beside the spectrum. The concentration of the small molecule and PAC was 1.0 × 10^{-3} \text{ mol/L} and 8.7 × 10^{-6} \text{ mol/L}, respectively. The water peak located at \delta 4.8 and 1 mM of TSP was added to the sample as a reference (\delta 0). (TIF)

**Figure S9** The most possible binding site of 3,4-dicaffeoylquinic acid on PAC supposed by visual docking. (TIF)

**Figure S10** The most possible binding site of 1,5-dicaffeoylquinic acid on PAC supposed by visual docking. (TIF)

**Figure S11** The most possible binding site of 4,5-dicaffeoylquinic acid on PAC supposed by visual docking. (TIF)

**Figure S12** The most possible binding site of 3,5-dicaffeoylquinic acid on PAC supposed by visual docking. (TIF)

**Figure S13** The most possible binding site of 1,3-dicaffeoylquinic acid on PAC supposed by visual docking. (TIF)

**Figure S14** The most possible binding site of 5-cafeoylquinic acid on PAC supposed by visual docking. (TIF)

**Figure S15** The most possible binding site of 4-cafeoylquinic acid on PAC supposed by visual docking. (TIF)

**Figure S16** The most possible binding site of quinic acid on PAC supposed by visual docking. (TIF)

**Figure S17** The most possible binding site of caffeic acid on PAC supposed by visual docking. (TIF)

**Figure S18** Binding affinity between 1,5-dicaffeoylquinic acid and PAC evaluated by transverse relaxation simulation. a) The plot of T_{2obs} versus C_P/C_L; b) The plot of R_{2obs} versus C_P/C_L. (TIF)

**Figure S19** Binding affinity between 1,3-dicaffeoylquinic acid and PAC evaluated by transverse relaxation simulation. a) The plot of T_{2obs} versus C_P/C_L; b) The plot of R_{2obs} versus C_P/C_L. (TIF)

**Figure S20** Binding affinity between 3,5-dicaffeoylquinic acid and PAC evaluated by transverse relaxation simulation. a) The plot of T_{2obs} versus C_P/C_L; b) The plot of R_{2obs} versus C_P/C_L. (TIF)

**Figure S21** Binding affinity between 3,4-dicaffeoylquinic acid and PAC evaluated by transverse relaxation simulation. a) The plot of T_{2obs} versus C_P/C_L; b) The plot of R_{2obs} versus C_P/C_L. (TIF)

**Figure S22** Binding affinity between 4,5-dicaffeoylquinic acid and PAC evaluated by transverse relaxation simulation. a) The plot of T_{2obs} versus C_P/C_L; b) The plot of R_{2obs} versus C_P/C_L. (TIF)

**Figure S23** Association and dissociation kinetics plot of 3,4-dicaffeoylquinic acid to PAC as determined by SPR. (TIF)

**Figure S24** Association and dissociation kinetics plot of 1,5-dicaffeoylquinic acid to PAC as determined by SPR. (TIF)

**Figure S25** Association and dissociation kinetics plot of 4,5-dicaffeoylquinic acid to PAC as determined by SPR. (TIF)

**Figure S26** Association and dissociation kinetics plot of 3,5-dicaffeoylquinic acid to PAC as determined by SPR. (TIF)

**Figure S27** Association and dissociation kinetics plot of 1,3-dicaffeoylquinic acid to PAC as determined by SPR. (TIF)

**Figure S28** Association and dissociation kinetics plot of 5-cafeoylquinic acid to PAC as determined by SPR. (TIF)

**Figure S29** Association and dissociation kinetics plot of 4-cafeoylquinic acid to PAC as determined by SPR. (TIF)

**Figure S30** Binder screening by relaxation-edited NMR. Spectra of 3,4-dicaffeoylquinic acid (compound a) in the absence (plots a, b) and presence (plots c, d) of HSA. The CPMG spin-lock time of each experiment was labeled beside the spectra. The concentrations of small molecule and HSA were 1.0 × 10^{-3} \text{ mol/L} and 7.0 × 10^{-6} \text{ mol/L}, respectively. The water peak located at \delta 4.8. The peaks of small molecule were not eliminated at a long spin-lock time, indicated that compound a did not specifically interact with HSA. (TIF)

**Table S1** Structure of candidate compounds. (DOC)

**Table S2** Binding affinities of chlorogenic acid to different active sites of PAC evaluated by virtual docking. (DOC)

**Author Contributions**
Conceived and designed the experiments: LL SHC JFX QL HHL YFL. Performed the experiments: LL SHC QL. Analyzed the data: LL SHC JFX QL. Contributed reagents/materials/analysis tools: LL SHC. Wrote the paper: LL SHC.
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