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Screening Anti-influenza Agents that Target Avian Influenza Polymerase Protein PA_C from Plant Extracts Based on NMR Methods

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The highly pathogenic avian influenza A virus poses a global threat to human health. Avian influenza RNA polymerase protein PA_C was used in the screening of two herbal plant extracts for anti-influenza agents. As a result, chlorogenic acid was identified to be PA_C ligand and was discovered to inhibit polymerase activity. Hence, this work revealed a potential anti-influenza lead compound and provided an important step in the discovery of new anti-influenza drugs.

The high pathogenicity of avian influenza A virus strains of subtype H5N1 and its capability of transmission from birds to humans pose a global threat to human health.[1] The increasing geographic distribution of this epizootic virus, which causes respiratory disease to infected people with significant morbidity and mortality, has aroused serious concerns about therapeutic methods for the disease. Most current anti-influenza drugs are either neuraminidase inhibitors or M2 ion channel blockers.[2] However, the effectiveness of these drugs has been limited because of drug resistance.[3,4] Actually, a single mutation on the target protein may be sufficient to induce drug resistance. This has highlighted the urgent need for a new generation of anti-influenza agents with high efficiency as well as a low risk of drug resistance.

In the search for new anti-influenza drugs, viral RNA polymerase was reported to be a valuable target.[5] This heterotrimer, which contains PB1, PB2, and PA subunits, is responsible for viral RNA (vRNA) replication and transcription. Structural analysis of the PA subunit revealed several potential active sites.[6–10] Mutations of amino acids in these sites could reduce polymerase activity or disrupt vRNA replication. Moreover, the active sites in PA are conserved across type A, B, and C influenza viruses. This indicates that the anti-influenza agents targeting PA may be effective against most influenza strains and less susceptible to drug resistance.[8–10] In the pursuit of novel anti-influenza agents, we present a screen against the carboxy-terminal domain of PA (termed PA_C, residues 257–716).

In general, the first step of drug discovery is the screening of active compounds from chemical or natural product libraries. Many herbs have been applied to the prevention and therapy of influenza infection.[11] For example, *Flos Lonicera Japonica* (*FLJ*) is used for the treatment of viral infection such as influenza A virus,[12] and the leaves of *Eucommia Ulmoides Oliv* (*EUO*) have antimicrobial and anti-inflammatory activities.[13] Hence, *FLJ* extract and *EUO* extract were chosen in the screen of potential anti-influenza agents.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique in drug screening.[14,15] Ligand–target binding can be determined experimentally by analyzing the transverse relaxation change of the small molecule upon the addition of protein in a relaxation-edited NMR.[16] When a small molecular ligand binds to a macromolecular target, the observed transverse relaxation rate (R_2) of ligand molecule becomes much larger than that of the free-state small molecules. In order to identify the PA_C ligand from the mixtures, relaxation-edited NMR with Carr–Purcell–Meiboom–Gill (CPMG) spin-lock was applied to *FLJ* extract and *EUO* extract in the absence and presence of PA_C. As shown in Figure 1b, the application of CPMG spin-lock will reduce the ligand peaks due to its relatively fast R_2 upon binding. However, these signals were not influenced in the absence of target (Figure 1a). This difference was highlighted in the difference spectrum (Figure 1c). These attenuated peaks can be ascribed to the PA_C ligand in the extract. Thus, the ligand peaks can be "picked out" from the mixture without previous isolation. The next step is to identify which molecule in the mixture is bound to PA_C.

Based on the characteristic signals picked out by relaxation-edited NMR, structure elucidation was carried out using the

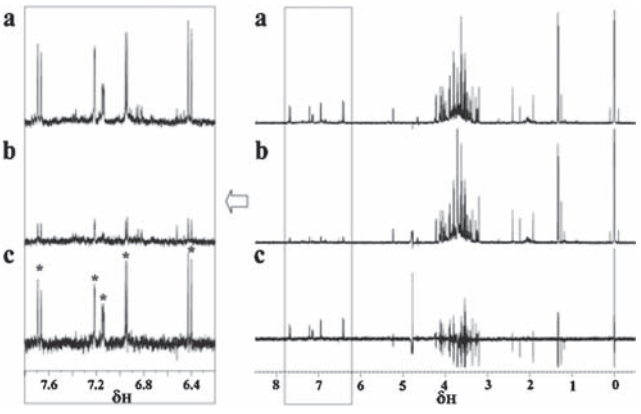
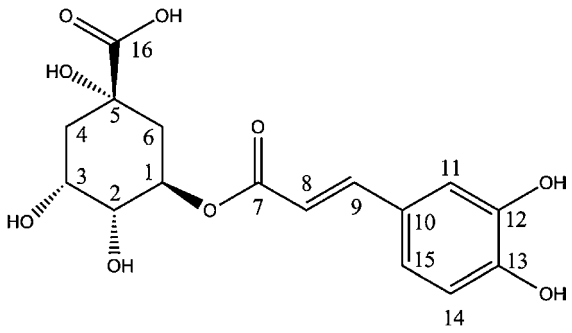


Figure 1. The relaxation-edited NMR spectra of *FLJ* in the absence (**a**) and presence (**b**) of PA_C and the difference spectrum (**c**) of **a** and **b**. The spin-lock time in **a** and **b** were both 1500 ms. Contents of *FLJ* extract were 3.3 mg mL^{-1}, and concentration of PA_C was 8.7×10^{-6} mol L^{-1}. The water peak located at δ 4.8, and 1 mM of TSP was added to the sample as a reference (δ 0). The region of δ 6.2–7.8 is broadened, and the ligand peaks attenuated upon the addition of the target were marked with "*" in plot **c**.

subsequent 2D NMR experiments, such as heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC), and total correlation spectroscopy (TOCSY). Followed by proton–carbon connections, proton–carbon long-range correlations, and proton–proton correlations provided by these 2D NMR experiments, the molecular frame of the ligand can be built. The PA_C ligand can be then identified to be chlorogenic acid (CA, Scheme 1). After these encouraging positive results, *FLJ* was replaced with *EUO* and the experiments were repeated. The PA_C ligand was also identified to be CA (SI[17]).

To confirm the interaction between CA and PA_C, the binding between the pure compound and the target was validated by relaxation-edited NMR (Figure S4, SI[17]). Additionaly, the binding affinity was evaluated by longitudinal relaxation time (T_1) simulation based on NMR (Figure 2) and by kinetics simulation in surface plasmon resonance (SPR, SI[17]). The dissociation constant (K_d) values were 5.7×10^{-4} and 9.0×10^{-4} mol L^{-1}, respectively. These results indicated that the interaction between CA and PA_C was weak binding.

A previous structure analysis of PA_C has revealed several potential active sites for anti-influenza agents.[9] To guide in the discovery of anti-influenza agents, flexible docking simulations were carried out by AutoDock (v.4.01) software to determine the binding pocket of CA in PA_C. As shown in Figure 3, the results indicated that the binding site of the ligand to PA_C most probably locates at a potential active area including G371, E372, H510, R512, D516, and K572. The H510A mutation was reported to impair endonuclease activity.[6] This result indicated that the ligand may potentially inhibit the polymerase activity.



Scheme 1. The chemical structure of CA.

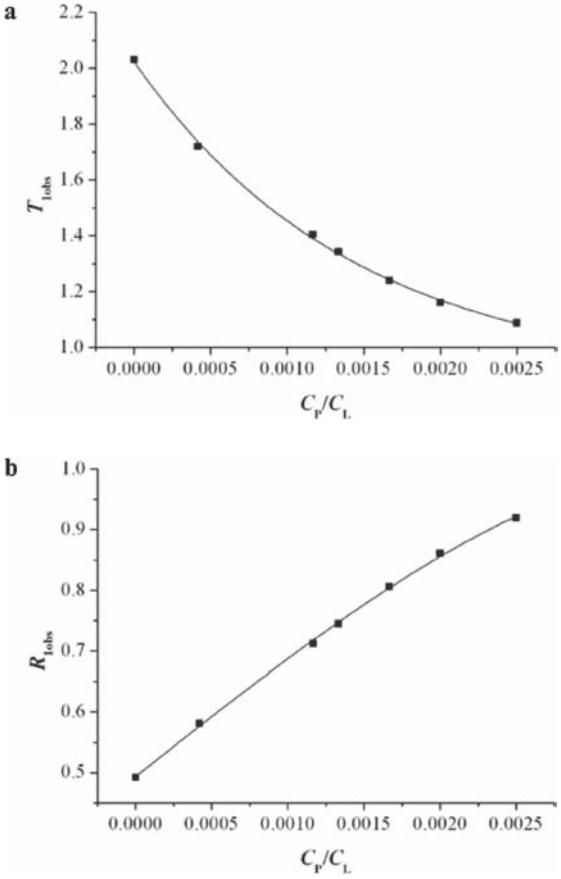


Figure 2. T_1 simulation of binding affinity between CA and PA_C based on NMR: (**a**) The plot of T_{1obs} versus C_P/C_L, (**b**) the plot of R_{1obs} versus C_P/C_L.

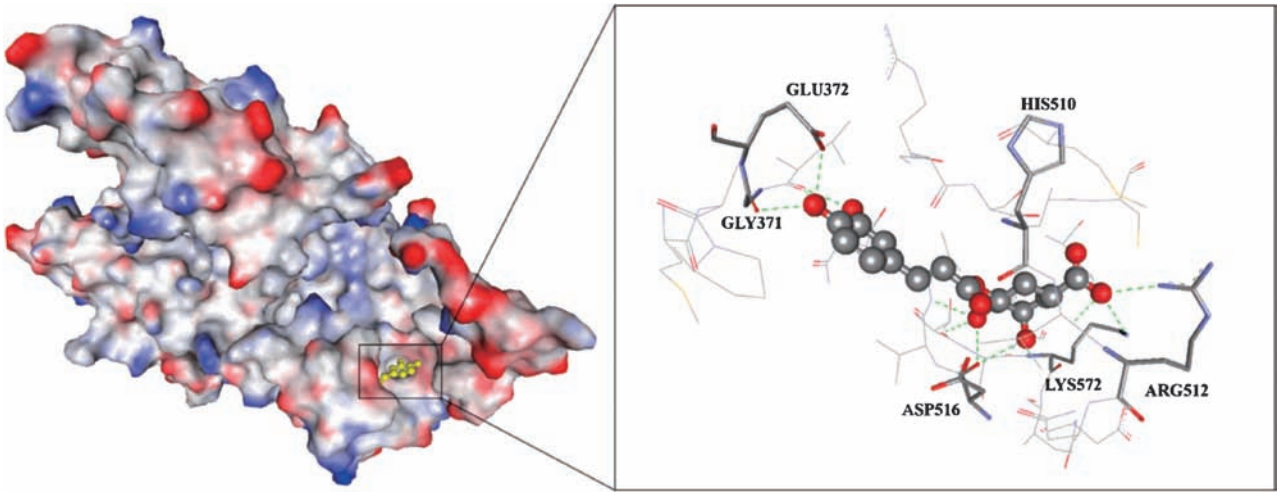


Figure 3. The binding site of CA in PA_C suggested by computer virtual docking. Hydrogen bonds between the small molecule and the target are shown by green dotted line.

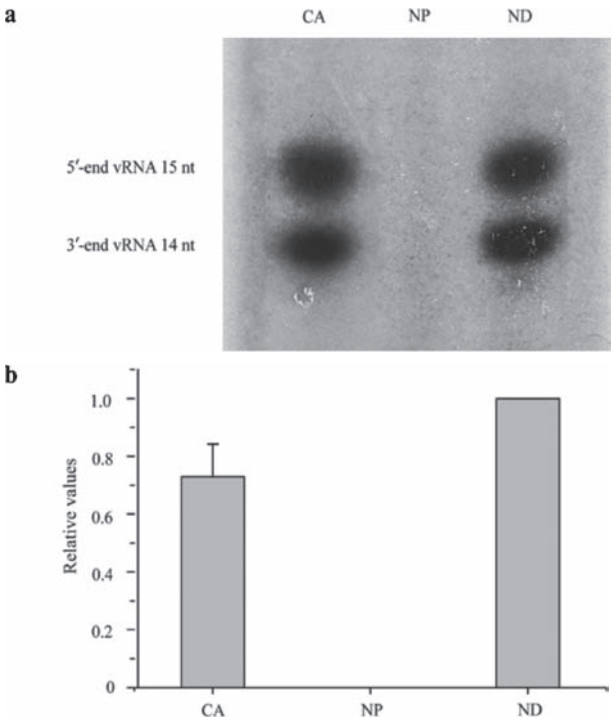


Figure 4. (**a**) The effect of CA on polymerase activity in an ApG primer extension assay. The concentration of the small molecule was 5 mM. Samples "NP" and "ND" were tested with no polymerase and no drug for negative and positive references. (**b**) The quantification of the result from plot **a** obtained by phosphorimaging analysis. The result is the average of two independent experiments and the standard derivation is shown.

The PA_C subunit is essential in RNA polymerase activity, so one may expect that PA_C ligands should inhibit polymerase activity as well. The polymerase can use ApG as a primer to synthesize complementary RNA (cRNA) from vRNA promoters; therefore, the length of cRNA can be used to judge polymerase activity. The effect of CA on polymerase activity was evaluated by an ApG primer extension assay.[18] As shown in Figure 4, CA slightly inhibited the synthesis of cRNA (inhibition rate 27%), which indicated that the binding of this compound to PA_C can reduce polymerase activity. Although this ligand was assayed at a relatively high concentration, the discovery of the effectiveness of this ligand suggests that CA could be a lead structure for a potential anti-influenza drug.

In summary, the avian influenza RNA polymerase protein PA_C, a conserved key target in the design of a new generation of anti-influenza agents, was used to screen lead compounds against anti-influenza from two herbal plant extracts. The target is valuable because the drugs discovered by this target may be effective against most influenza strains less susceptible to drug resistance due to the high conservation of the active sites in PA_C. In this work, a PA_C ligand was successfully identified in two different plant extracts by the combination of target recognition and NMR methods. The method can determine the existence of the ligand in the mixture and synchronously identify the structure of the ligand. Our work provides a very promising strategy for the fast screening of lead components from natural plant extracts. Moreover, the ligand proved to exhibit inhibition of polymerase activity, indicating that this molecule may potentially be valuable as an anti-influenza lead compound. Although CA binds to PA_C with low affinity and thus exhibits slight activity, it is promising that more PA_C ligands with higher binding affinities and also higher activities can be discovered from its analogs or by structure modifications. Further search for novel PA_C ligands from the CA derivatives is in progress. Conclusively, this work provided an important step in CA structure-based design of anti-influenza agents.

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