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NMR identification of anti-influenza lead compound targeting at PA_C subunit of H5N1 polymerase

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Abstract

PA_C subunit from avian influenza (H5N1) viral RNA polymerase was used in this work as a target in the screening for anti-influenza agents from licorice-derived compounds. As a result, 18β-glycyrrhetinic acid was suggested to be PA_C ligand by flexible docking, and was then confirmed by relaxation-edited NMR. The result of ApG primer extension assay indicated that this PA_C ligand can inhibit the polymerase activity, and thus may potentially be valuable as anti-influenza lead compound. This work validated the possibility of screening polymerase inhibitors by using PA_C as a target, and provided a starting point for the further discovery of new anti-influenza drugs.

Keywords: PAC; RNA polymerase inhibitor; Anti-influenza agent; Licorice-derived compounds

Influenza virus is one of the major causes of respiratory viral infection, and carries high rates of morbidity and mortality. Since the first report of chicken-to-human transmission of avian influenza (H5N1) in 1997 [1], the increasing geographic distribution of this epizootic virus this epizootic virus has posed a global threat to human health. Thus, there are serious concerns about the effective therapeutic methods of the disease. To treat influenza, two kinds of medicines, the M2 ion channel blockers and the neuraminidase inhibitors, have been used [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 induce drug resistance. Obviously, alternative anti-influenza agents that are effective with low risk of drug-resistance are urgently needed.

In the search for a new generation of anti-influenza drugs, the RNA polymerase is reported to be a valuable target [5,6]. This heterotrimer, which contains PB1, PB2, and PA subunits, is a specific enzyme catalyzing influenza viral RNA replication and transcription [2,7]. The polymerase inhibitors can directly block this step and thus disrupt the viral replication. Structural analysis of the PA subunit revealed several potential active sites. Mutations of certain

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Table 1
Binding affinities of GHA to different active sites of PA_C evaluated by flexible docking.

	Site 1[a]	Site 2	Site 3	Site 4	Site 5
pK_i	6.33	6.43	6.21	6.26	6.28

[a] 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; site 5: center of 620 and 621.

residues in these sites could significantly reduce the polymerase activity and thus disrupt vRNA replication [6,8,9]. Moreover, the highly conserved active sites in PA indicated that the anti-influenza agents targeting at PA may be effective against most influenza strains and less susceptible to drug resistance problems [8]. Herein, we present a screen for anti-influenza agents against the carboxyl-terminal domain of PA (termed PA_C, residues 257–716).

In general, the first step of drug discovery is to identify active compounds by screening chemical libraries or natural products. Traditional Chinese medicines (TCM) have long been used to treat influenza and are increasingly drawing attention as potential sources for development of new anti-influenza drugs [10,11]. For example, some components from *Glycyrrhiza* species were reported to inhibit influenza virus [10,12], indicating that licorice-derived compounds may potentially be anti-influenza lead compounds.

1. Results and discussion

In the visual screening for PA_C ligand carried out by AutoDock software package (version 4.01), 18β-glycyrrhetinic acid (GHA), a licorice-derived compound, was discovered to be a possible ligand. The active pockets in flexible docking were set to potential active sites according to the structure analysis of PA_C [8]. The binding affinities of GHA to different active pockets were summarized in Table 1. Wherein pK_i is the negative logarithm of the dissociation constant of binding complex, higher pK_i values indicate stronger binding affinities. The result indicated that GHA is a strong binder of PA_C and the binding site most probably locate at site 2 including GLU656, LYS574, TRP577, ARG583, *etc*. (Fig. 1). Nevertheless, the interaction between 18β-glycyrrhetinic acid and PA_C needs further confirmation.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique in the screening based on bio-target recognition, because of its unique ability to provide information on the structural, thermodynamic, and kinetic aspects of the binding reactions between ligands and targets [13,14]. Transverse relaxation rate (R_2) of small molecule is an attractive probe of ligand biding. When small molecular ligand binds to macromolecular target, the R_2 of target and bound ligand is much larger than that of free ligand ($R_{2,\ \text{bound}} \gg R_{2,\ \text{free-ligand}}$). As a result, the observed R_2 ($R_{2,\ \text{obs}}$) of bound molecule, which is the weighted average of $R_{2,\ \text{bound}}$ and $R_{2,\ \text{free-ligand}}$, will become much larger (faster) relative

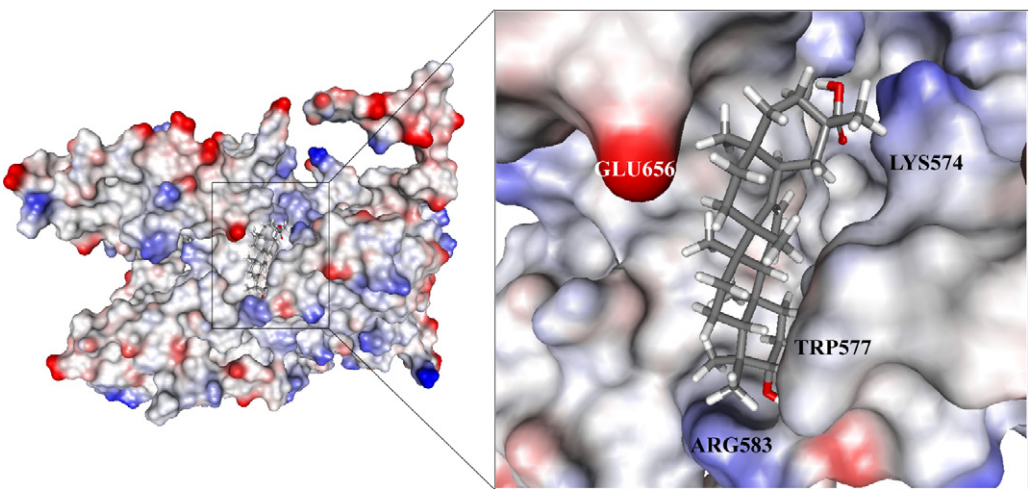

Fig. 1. The most possible binding pocket of 18β-glycyrrhetinic acid on PA_C suggested by flexible docking.

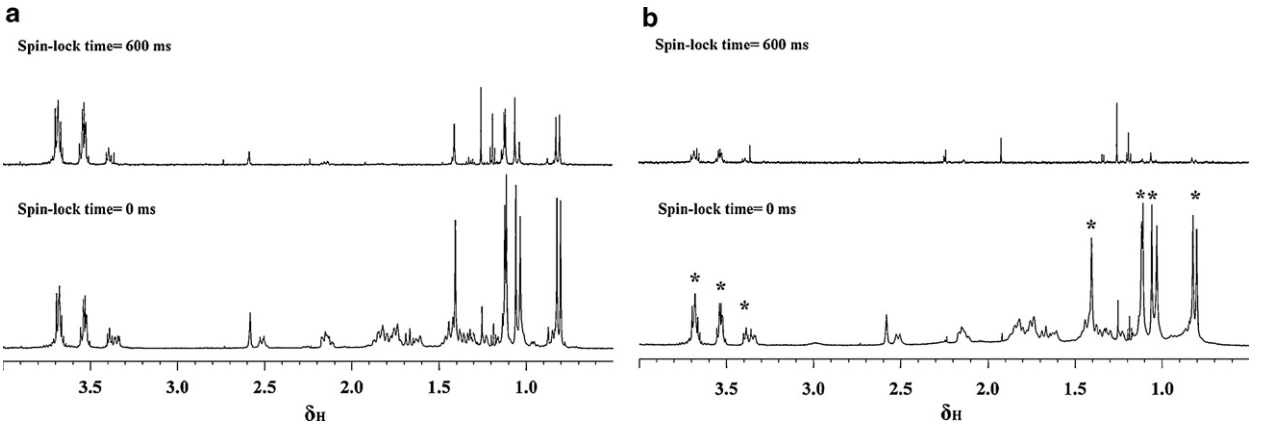

Fig. 2. The relaxation-edited NMR spectra of GHA in the absence (a) and presence (b) of PA_C. The spin-lock time of each experiment was labeled beside the spectra. The concentrations of GHA and PA_C were 1.5×10^{-3} mol/L and 1.5×10^{-5} mol/L, respectively. The ligand peaks attenuated when applying CPMG spin-lock in the presence of PA_C were marked with "*" in (b).

to free small molecule. Herein, ligand peaks in relaxation-edited NMR will attenuate upon the addition of the target, because the applying of Carr–Purcell–Meiboom–Gill (CPMG) spin-lock will reduce or even eliminate fast relaxing resonances. As shown in Fig. 2, the adding of PA_C to the small molecule significantly increased the attenuation of ligand peaks (marked with "*" in Fig. 2b). The transverse relaxation time ($T_2 = 1/R_2$) of the ligand peaks also confirmed this change, as we can see from Table 2, T_2 of most ligand peaks decreased over 50%. These results indicated that GHA is a ligand of polymerase protein PA_C.

The PA_C subunit is essential in RNA polymerase activity, so one may expect that PA_C ligands should inhibit polymerase activity as well. To examine the inhibition of GHA against polymerase activity, ApG primer extension assay was then carried out [15]. In the presence of 5′-end vRNA (at least 15 nt) promoter and 3′-end vRNA (at least 13 nt) promoter, influenza polymerase could use ApG as primers and use UTP, ATP, CTP, and [α-^{32}P]GTP as substrates to synthesize cRNA from the vRNA template. Therefore, the length of cRNA can be used to judge polymerase activity. As shown in Fig. 3, GHA significantly inhibited the synthesis of cRNA, indicated that this compound can inhibit the polymerase activity (inhibition rate 80%). This result validated the proposal that the PA_C ligand may inhibit the polymerase activity. Moreover, the discovery of this polymerase inhibitor revealed a lead compound against influenza virus.

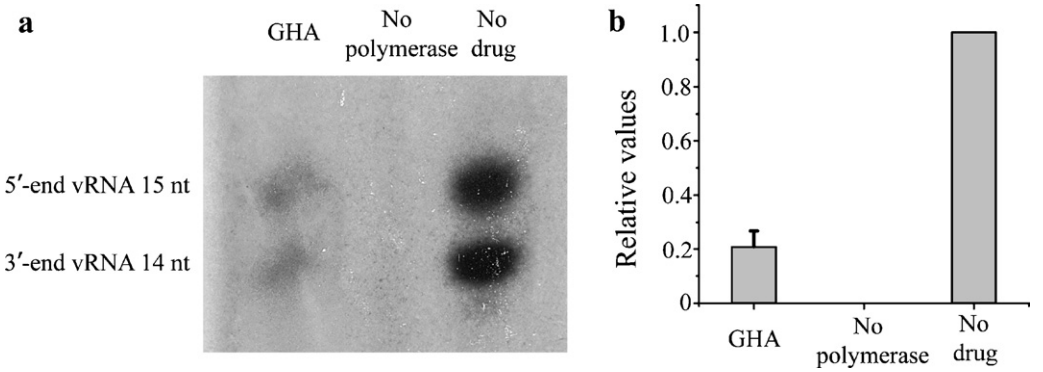

Fig. 3. (a) Effects of GHA on polymerase activity in ApG primer extension assay. Wherein the concentration of the small molecule was 5 mmol/L, samples with "No polymerase" (only small molecule was added) and "No drug" (only polymerase was added) were tested for negative and positive references, respectively. (b) Quantification of the result from a obtained by phosphorimaging analysis. The result is the average of two independent experiments, and the standard derivation is shown.

Table 2
T_2 of GHA peaks in the absence (sample a) and presence (sample b) of PA_C.

Protons	δ 3.7	δ 3.5	δ 3.4	δ 1.4	δ 1.1	δ 1.0	δ 0.8	
T_2 (ms)	Sample a	423	435	174	201	234	225	231
	Sample b	210	222	108	75	87	99	96

2. Conclusion

In this work, avian influenza RNA polymerase protein PA_C, which was proposed to be a key conserved target for the design of new generation of anti-influenza agents, was used as a new target in the screening of lead compounds against influenza virus from licorice-derived compounds. As a result, 18β-glycyrrhetinic acid was suggested to be PA_C ligand by flexible docking, and was then confirmed by relaxation-edited NMR. The result of ApG primer extension assay indicated that this PA_C ligand can inhibit the polymerase activity. Thus, this ligand may be potentially valuable as anti-influenza lead compound. Furthermore, it is promising to find more polymerase inhibitors from its analogues or by structure modifications. Conclusively, this work validated the possibility of screening polymerase inhibitors by using polymerase protein PA_C as a target, and thus provided a starting point for the further discovery of new anti-influenza drugs.

Acknowledgments

Experimental section is available in Supporting Information. This work was supported by the National Natural Science Foundation of China (No. 81072576) to Y.L. Tang, the National Natural Science Foundation of China (No. 30925011) and the Ministry of Science and Technology 863 Project (No. 2006AA02A314) to Y.F. Liu.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2011.09.006.

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