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Journal of Chromatography B 1162 (2021) 122469

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Short communication

Cell membrane chromatography for the analysis of the interaction between chloroquine and hydroxychloroquine with ACE2 receptors

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ARTICLE INFO

Keywords:
Cell membrane chromatography
COVID-19
ACE2 receptor
Chloroquine
Hydroxychloroquine

ABSTRACT

The recent emergence of the novel pathogenic coronavirus disease 2019 (COVID-19) is responsible for a worldwide pandemic. In sight of this, there has been growing interest in the use of chloroquine (CQ) and hydroxychloroquine (HCQ) as potential treatments. In this study, we use angiotensin converting enzyme 2 (ACE2) over-expressed cell membrane chromatography (CMC) to study the interaction of CQ and HCQ with ACE2 receptor. Both CQ and HCQ were retained on the ACE2/CMC column. Then we analyzed the binding character of CQ and HCQ to ACE2 by CMC frontal analysis, ionic force investigation and competitive binding experiment. Results showed that CQ and HCQ KD values obtained from the CMC frontal analysis method were $8.22(\pm0.61) \times 10^{-7}$ M and $11.70(\pm2.44) \times 10^{-7}$ M. Compare to CQ, HCQ has the weaker affinity with ACE2. The action force of CQ, HCQ and ACE2 is mainly ionic force. CQ and HCQ have different degrees of competitive binding relationship with ACE2. Our study revealed the interaction of CQ and HCQ with ACE2 receptor, which provides new insights for the use of CQ and HCQ in the treatment of COVID-19. Moreover, this biomimetic drug screening method is expected to open the door for rapid targeting and separating bioactive ingredients active towards ACE2 receptor.

1. Introduction

Since the first case was reported in Wuhan, China in late December 2019, humans have been threatened by COVID-19 caused by novel coronavirus SARS-CoV-2 [1,2]. At present, it is generally believed that coronavirus spike glycoprotein (Spike) and receptor ACE2 are the key binding sites for severe acute respiratory syndrome (SARS-CoV). The new coronavirus uses its own Spike protein to interact with the ACE2 protein to invade the human body [3–5]. Therefore, blocking or antagonizing the ACE2 signal pathway of susceptible cells should be helpful to prevent COVID-19 virus infection [6]. ACE2 is a membrane-bound aminopeptidase that has a vital role in the cardiovascular and immune systems, and the size of ACE2 is 92.4 kDa [7]. In addition, ACE2 has been identified as a functional receptor for coronaviruses, including SARS-CoV and SARS-CoV-2 [8,9]. Develop inhibitors for ACE2, which can effectively cut off the binding of two proteins, thereby blocking the virus from invading human cells, and preventing infection.

So far, there is no effective treatment for COVID-19 drugs. New treatments or vaccines take time to develop. Recently, CQ and its derivative HCQ are considered to be promising repurposed drugs against COVID-19, based on pathophysiological considerations and *in vitro* results [10,11]. CQ and HCQ have been used to treat malaria and chronic inflammatory diseases, such as systemic lupus erythematosus and rheumatoid arthritis [12,13]. It has been reported that CQ interferes with ACE2, inhibits viral spike glycoprotein/ACE2 interaction, and inactivates ACE2 receptor glycosylation, thereby restricting virus entry into target cells [14,15]. It has been reported that HCQ interferes with the terminal glycosylation of ACE2 [16], which acts as both SARS-CoV [8] and SARS-CoV-2 [9] membrane receptor, HCQ can play a role in several steps of the coronavirus replication cycle [16]. Therefore, a method is urgently needed to study the interaction between CQ and HCQ with ACE2 receptors.

CMC is a new affinity chromatography technology for studying the interaction between receptor and drug, which was created by Professor He Langchong in 1996 [17]. As a biomimetic affinity chromatography, CMC is characterized by the ability of cell membrane stationary phase to

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https://doi.org/10.1016/j.jchromb.2020.122469
Received 12 October 2020; Received in revised form 18 November 2020; Accepted 19 November 2020
Available online 29 November 2020

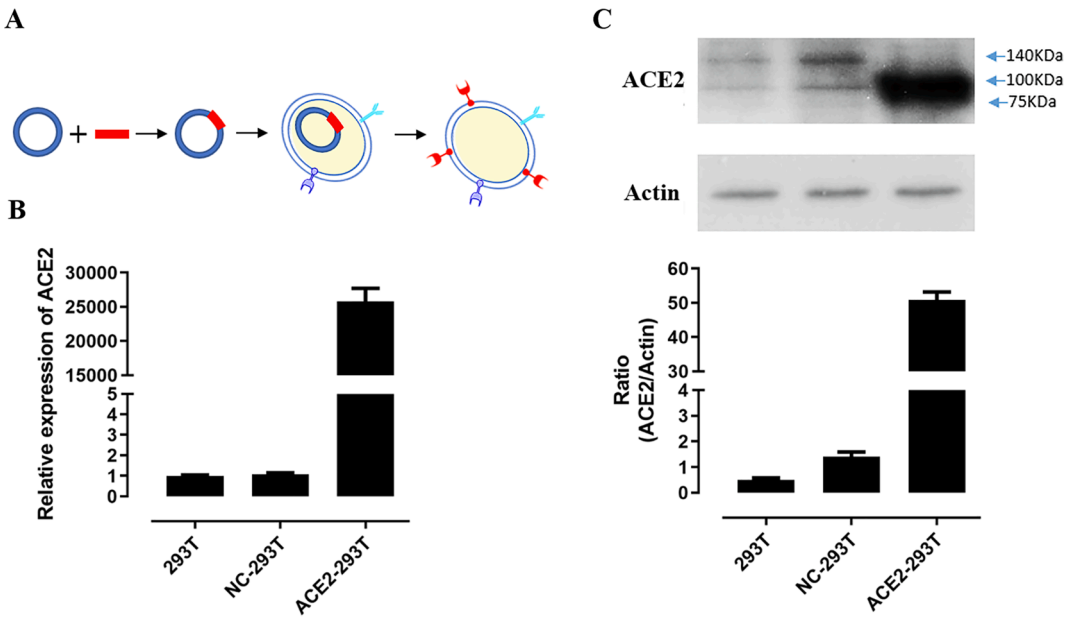

Fig. 1. Construction of over-express ACE2-293T cell line. A: Schematic diagram of the construction of over-express ACE2-293T cell line; B: qPCR detection of ACE2 gene expression; C: Western blot analysis of the expression levels of ACE2 protein in 293T cells.

retain the three-dimensional configuration and biological activity of membrane receptors to a large extent, and accurately reveal the interaction process between drugs and membrane receptors [18,19]. CMC has been widely used in the interaction characteristics of compounds and membrane receptors [20,21].

In this study, the ACE2 over-expression cell membrane chromatography system was established, and the action characteristics of CQ, HCQ and ACE2 were compared. These results will provide valuable information and useful models for a better understanding of drug receptor interactions.

2. Methods

2.1. System suitability of ACE2/CMC model

For each ACE2/CMC column, the reproducibility was studied by repeatedly injecting CQ and the retention time was used as an indicator. For different ACE2/CMC columns, the reproducibility was tested by injecting CQ into different ACE2/CMC columns. Different drugs acting on different receptors were used to examine the specific selectivity of the ACE2/CMC column.

2.2. Frontal analysis

Series concentrations of CQ and HCQ were prepared by saline buffer (50 mM, pH 7.4) were separately flushed through the ACE2/CMC column at a flow rate of 0.2 mL min^{-1} to generate breakthrough curves. In the CMC model, the K_D value is investigated by using frontal analysis with the following equation [22]:

$$\frac{1}{m_{Lapp}} = \frac{K_D}{m_L}\cdot\frac{1}{[A]} + \frac{1}{m_L} \quad (1)$$

m_{Lapp} represents the number of moles of analyte needed to reach the midpoint of the curve under a given molar concentration of analyte; [A] represents molar concentration of analyte; m_L represents total moles of bound receptors on the cell chromatography column; K_D represents equilibrium dissociation constant. According to the obtained breakthrough curve, the value of the formula m_{Lapp} can be calculated, and then according to formula (1), a plot of $1/m_{Lapp}$ versus 1/[A] should produce a linear relationship for a system, and the ratio of slope to intercept is the K_D value.

2.3. Stoichiometric displacement model in CMC

A stoichiometric displacement model for retention (SDM-R) [23] of small solutes based on CMC was presented. Using the SDM-R as the model to study the interaction characteristics of CQ, HCQ with ACE2. According to the structural properties of sodium chloride, various concentrations of saline buffer (pH 7.4) in the range of 5 to 30 mM was separately pumped through the ACE2/CMC column. Then CQ (1×10^{-3} M) and HCQ (1×10^{-3} M) were injected separately for analysis. According to the theory of SDM-R and formula (2), linear regression of lg [D] with lgk′ can obtain the information of affinity parameters [24].

$$\lg k' = \lg I - Z\lg[D] \quad (2)$$

where k′ is the capacity factor; lgI is a constant related to the affinity of the solute and the stationary phase, [D] is the concentration of the displacing agent in the mobile phase, when [D] changes in a small range. The stoichiometric displacement parameters, Z and lgI could be obtained from the slope and the intercept of the linear plots of lgk' vs. lg [D].

2.4. Competitive binding experiment

Competitive binding experiments were performed using CQ as a site-specific probe, the mobile phase that contained CQ and HCQ were prepared by saline buffer (8 mM, pH 7.4) at various concentrations in the range of 1×10^{-7}–8×10^{-7} M were separately pumped through the ACE2/CMC column. The breakthrough curves of ligands at several different concentrations were recorded. Afterwards, 5 μL injections of 1×10^{-3} M CQ was propelled towards the column. According to the theory of SDM-R and formula (2), linear regression of lg[D] with lgk′ can obtain the information of affinity parameters.

2.5. Molecular docking

For better understanding the binding mode of CQ and HCQ with the active sites of ACE2 domain (PDB ID: 6M0J), the molecular docking test

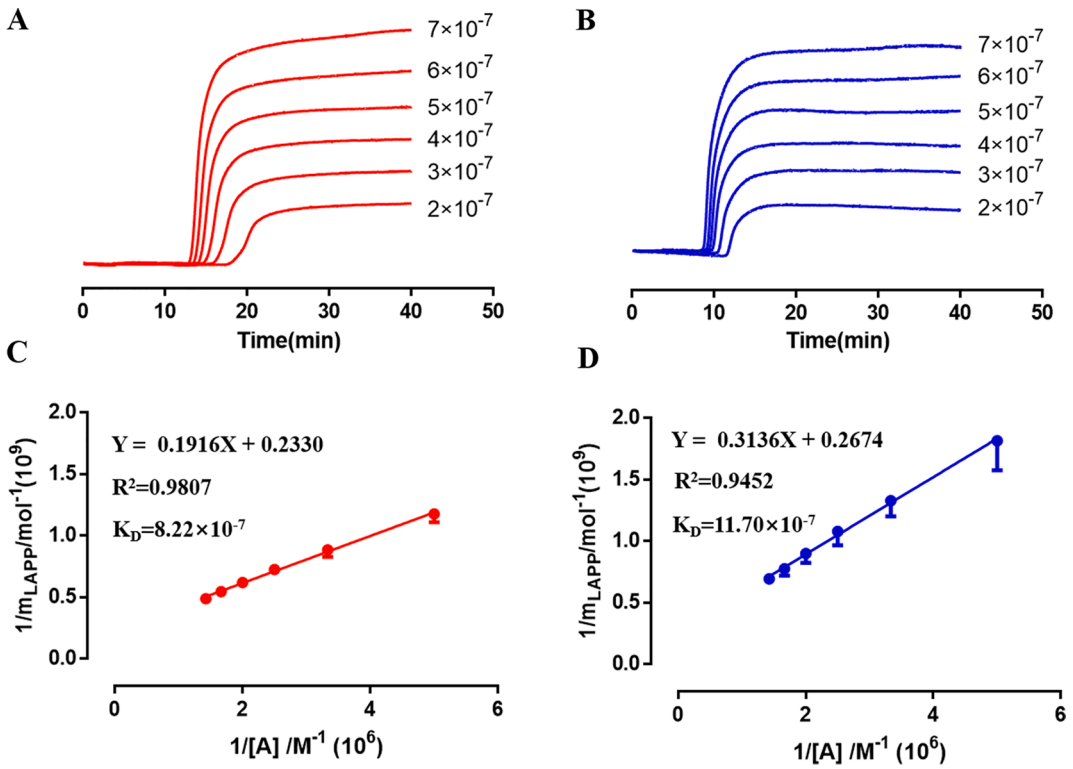

Fig. 2. The K_D values obtained by CMC frontal analysis method. ACE2/CMC breakthrough curves of CQ (A) and HCQ (B). The regression curves achieved by plotting m_{Lapp} versus 1/[A] of CQ (C) and HCQ (D). The concentrations of drugs were 2×10^{-7}, 3×10^{-7}, 4×10^{-7}, 5×10^{-7}, 6×10^{-7} and 7×10^{-7} M, respectively.

was performed using the Surflex-dock program in Sybyl X-2.0 version. First, CQ and HCQ were sketched in SYBYL-X, and use Tripos force field and Gasteiger-Huckel charge for energy minimization. The convergence criterion is 0.005 kcal/(Åmol) and the maximum iteration time is 1000 times. Secondly, download the structure file of ACE2 protein from PDB. org (PDB ID = 6M0J). The water molecules was removed, hydrogen was added, and minimization of protein molecules was performed using AMBER7 FF99 Force field and Gasteiger-Huckel charges. The 5.0 Å residue around the original ligand is defined as the active capsule. Finally, using other parameters as default values, the constructed molecule is docked to the active site of the protein.

3. Results and discussions

3.1. Expression of ACE2

In 293T cells, we over-express ACE2 receptors and Fig. 1A shows the schematic diagram of the construction of ACE2 over-expression 293T cell line. Expression of ACE2 has been investigated with real time PCR and western blotting. The gene expression of ACE2 in 293T transfected with PGMLV-PA6-ACE2 plasmid was about 2500 times higher than that of transfected with empty vector PGMLV-PA6 plasmid 293T (Fig. 1B). The cell lysates were directly analysed by Western blot. The over expression of ACE2 in 293T cell line was about 2500 times higher than that of NC-293T group (Fig. 1C). The above results indicate the successful construction of ACE2 over-expressing 293T cells.

3.2. System suitability of ACE2/CMC model

The retention time of CQ was used to evaluate the reproducibility of ACE2/CMC column. The relative standard deviation (RSD) was 2.6% (n = 5) on a single ACE2/CMC column and 7.3% (n = 3) on different columns. To verify the selectivity of the ACE2/CMC column, benzhexol hydrochloride acting on m-cholinergic receptor, prazosin hydrochloride acting on α_1 receptor, and CQ and HCQ were tested on the ACE2/CMC column and the results are reported in Fig. S1. Only CQ and HCQ were retained on ACE2/CMC column which suggests the better selectivity of ACE2/CMC column.

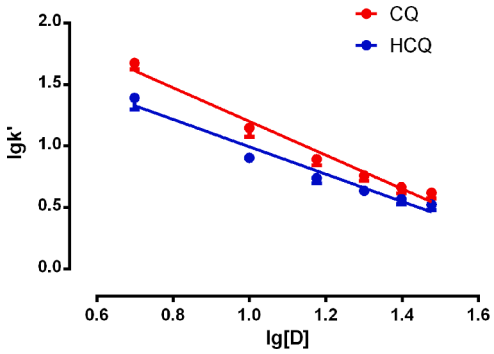

Fig. 3. ACE2/CMC SDM-R method to determine the effect of ionic force on the combined action of CQ and HCQ.

3.3. Reference K_D values obtained from the CMC frontal analysis method

As illustrated in Fig. 3, typical breakthrough curves were acquisitioned by frontal analysis. CQ (Fig. 2A) and HCQ (Fig. 2B) presented a negative corresponding relationship between the breakthrough time and the drug concentrations in mobile phases. The KD values of CQ and HCQ determined by frontier analysis were 8.22(±0.61) × 10^{-7} M and 11.70 (±2.44) × 10^{-7} M. Compared with HCQ, CQ has stronger affinity with ACE2 receptor. CMC is a powerful method for elucidating protein-drug interaction.

Table 1
CQ, HCQ and ACE2 interaction affinity parameters.

Ionic affinity parameters				Competitive replacement affinity parameters			
Name	lgI	Z	R	Name	lgI	Z	R
CQ	2.57	2.11	0.98	CQ	0.18	−0.25	0.93
HCQ	1.37	1.11	0.97	HCQ	0.38	−1.28	0.95

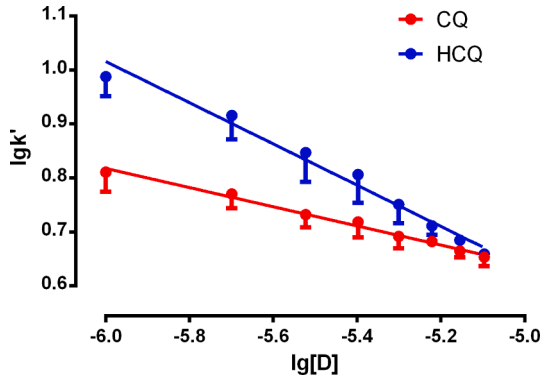

Fig. 4. The displacement of the CQ and HCQ binding to the specific site of ACE2 receptor.

3.4. SDM-R to study the interaction characteristics of CQ, HCQ with ACE2

The binding ability of small molecule drugs to replace sodium chloride and ACE2 was investigated by the principle of stoichiometric displacement. According to the theory of solvent metering displacement and formula (2), linear regression of lg[D] with lgk′ to obtain Fig. 3. The information of affinity parameters was listed in Table 1. The z values of CQ and HCQ are 2.11 and 1.11 in the presence of different concentrations of sodium chloride, respectively. The action force of CQ, HCQ and ACE2 is mainly ionic force. Compared with HCQ, CQ and ACE2 have a stronger ion force.

3.5. Site-specific competition with ACE2

Then competitive binding experiments were performed. According to the theory of SDM-R and formula (2), linear regression of lg[D] with lgk′ (Fig. 4) can obtain the information of affinity parameters (Table 1). The magnitude of the z value in the quantitative replacement model can illustrate the possibility of the competitor and ligand acting on the same site of the membrane receptor from another perspective. As shown in Table 1, the z values of CQ are 0.25 and 1.28 in the presence of different concentrations of displacer CQ and HCQ, respectively, indicating that CQ and HCQ have different degrees of competitive binding relationship with ACE2.

3.6. Potential binding target of CQ and HCQ

The binding mode of CQ and HCQ with ACE2 was studied by molecular docking. As shown in Fig. 5, CQ (Fig. 5A, C) had two hydrogen bonds with THR371 and GLU375 and the bond distances were 2.32 Å and 1.80 Å, respectively. HCQ (Fig. 5B, D) formed two hydrogen bonds with GLU375 and ALA348 and the bond distances were 2.06 Å and 1.94 Å, respectively. The docking results indicated that both CQ and HCQ bind well to ACE2. Both CQ and HCQ form hydrogen bonds with GLU375 and act on the same amino acid residue, which is consistent with the results of competitive experiments.

4. Conclusion

In this study, we established an ACE2/CMC model to analyze the effect of CQ and HCQ binding to ACE2 receptor. Both CQ and HCQ could act on ACE2 receptor. Compare to HCQ, CQ has the stronger affinity with ACE2. The interaction between CQ, HCQ and ACE2 is mainly ionic

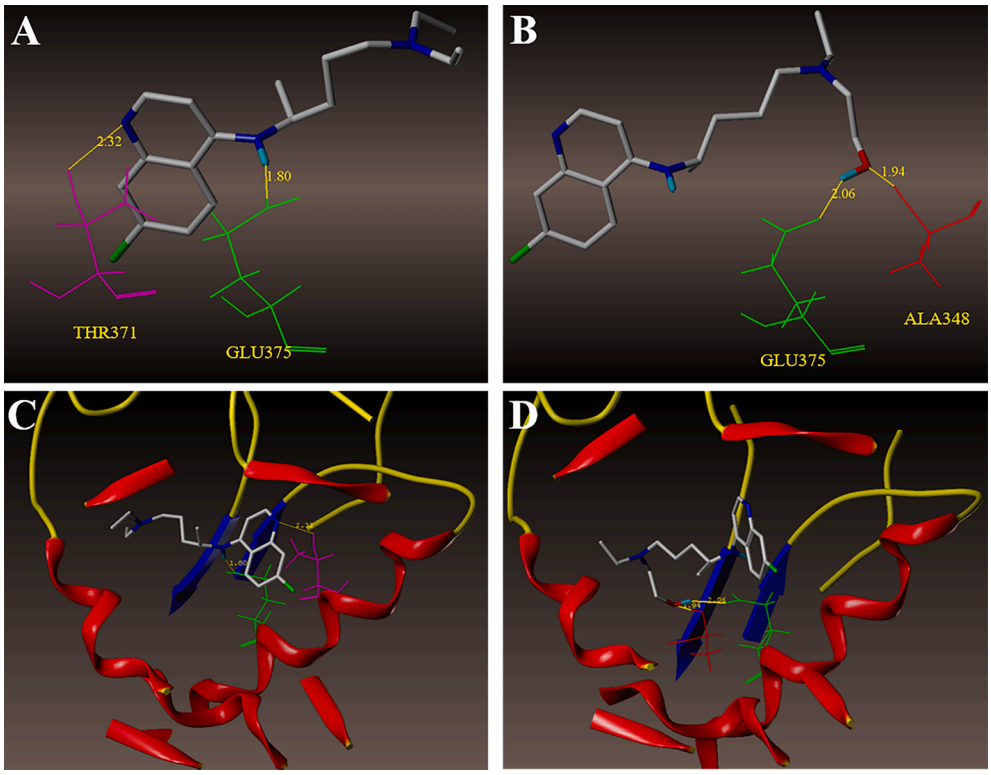

Fig. 5. The molecular docking of CQ (A and C) and HCQ (B and D) with ACE2.

force. CQ and HCQ have different degrees of competitive binding relationship with ACE2. Our study revealed the interaction of CQ and HCQ with ACE2 receptor, which provides a new insight for the use of CQ and HCQ in the treatment of COVID. Moreover, this biomimetic drug screening method is expected to open the door for rapid targeting and separating bioactive ingredients active towards ACE2 receptor.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by National Natural Science Foundation of China (Grant Numbers: 81973278 and 81930096), National China Postdoctoral Science Foundation Funded Project (Grant Numbers: 2019T120923 and 2018M641003), Natural Science Foundation of Shaanxi Province (Grant Number: 2020SF-309).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2020.122469.

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