Establishment of a protein thermal shift chip (PTSC) for COVID-19 and exploration of the future of protein chips in pharmacology

Graphical abstract

Highlights

• The concept of a protein thermal shift chip (PTSC) is proposed.

• The first PTSC chip to screen COVID-19 drug targets was prepared.

• The applications of the COVID-19 PTSC were tested.

Authors

Peng Chen, Zhao Cui, Caifeng Li, Shiwen Deng, Hongjun Yang

Correspondence

sdzpchenpeng@qq.com (P. Chen); hongjun0420@vip.sina.com (H. Yang)

In brief

A protein thermal shift chip enables high-throughput screening of protein targets and drugs in a label-free manner.
Establishment of a protein thermal shift chip (PTSC) for COVID-19 and exploration of the future of protein chips in pharmacology

Peng Chen\textsuperscript{a,b,*1}, Zhao Cui\textsuperscript{c,1}, Caifeng Li\textsuperscript{a,b}, Shiwen Deng\textsuperscript{a,b}, Hongjun Yang\textsuperscript{a,b,*}

\textsuperscript{a}Beijing Key Laboratory of Traditional Chinese Medicine Basic Research on Prevention and Treatment, Experimental Research Center, China Academy of Chinese Medical Sciences, Beijing, China
\textsuperscript{b}Robot Intelligence Laboratory of Traditional Chinese Medicine, Experimental Research Center, China Academy of Chinese Medical Sciences, Beijing, China
\textsuperscript{c}Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China

\textsuperscript{1}These authors contributed equally.

*Correspondence: sdzpchenpeng@qq.com (P. Chen); hongjun0420@vip.sina.com (H. Yang)

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Traditional protein chips are based on solid chips and cannot enable drug and target screening in a label-free manner. Herein, a protein thermal shift chip (PTSC) based on fluorescence signals is proposed, which enables low-cost, high-throughput, label-free screening. We developed a PTSC for COVID-19, containing 12 SARS-CoV-2 and host target proteins. A series of quality-control tests were performed for small-molecule drugs, macromolecular antibodies, and herbal-medicine extracts. This chip enabled high-throughput screening of COVID-19 drugs and thus may serve as a tool for screening drug targets clinically effective drugs.

protein thermal shift chip, COVID-19, drug target, pharmacology

COVID-19 has posed major challenges to public health worldwide for more than 2 years. Cases have been confirmed in every country and region worldwide. The current consensus opinion is that humans must live with the long-term existence of COVID-19. At present, the main methods for mitigating the pandemic rely primarily on social-distancing restrictions and immunization through vaccines [1]. Hundreds of vaccines have been investigated to date, several of which have been authorized by the World Health Organization, including inactivated vaccines, adenovirus vectored vaccines, recombinant protein vaccines, and mRNA vaccines [2].

Although the above strategies are effective, these measures to prevent the epidemic remain imperfect. Because of differences in the economies and the social and cultural contexts among regions, social-distancing limitations and travel restrictions vary worldwide. In addition, many types of mutant viruses exist, and new mutations appear to be acquired once per week on average. Because of differences in economic and social development, the speed of vaccination also varies among regions, thus leading to a risk of insufficient vaccination protection rates [3]. Therefore, the methods for ultimately end the COVID-19 pandemic may depend on specific drugs [4, 5].

Drug screening and pharmacology research on COVID-19 continue to be performed [6]. Early in the COVID-19 pandemic, researchers developed \emph{in vitro} viral infection models based on multiple cell lines [7]. Later, additional models were developed and established in animals, such as rats, mice, or monkeys [8, 9]. Although some drugs derived from natural products have been demonstrated to inhibit viruses in \emph{in vitro} models, but their drug targets remain unclear. A variety of compounds listed in the guidelines for the treatment of COVID-19 in China [10] have been found to significantly inhibit SARS-CoV-2 replication [11]. Targets of small-molecule drugs are usually screened through labeling with chemical probes. Protein targets are enriched and traced by probes, and identified by methods including mass spectrometry and biochips [12]. Traditional protein chips are based on glass substrates and rely on probe labeling to obtain detection signals, thus greatly limiting the speed of their application in drug and target screening. New forms of protein chips are therefore needed to support pharmacology research.

Thermal stability is an important aspect of protein stability. Protein structure is destroyed during heating. The temperature at which half the protein is in an unfolded state is defined as the protein melting temperature.
(Tm), which is usually associated with conformational stability. The method initially used to determine the Tm value of proteins was differential scanning calorimetry. Later, scientists used the polarity sensitive dye 1-anilinonaphthalene-8-sulfonic acid (ANS) to analyze the folding state of proteins, on the basis of the gradual exposure of proteins’ internal hydrophobic amino acids during the unfolding process and their subsequent interaction with hydrophobic dyes, thus producing fluorescence [13]. The discovery of hydrophobic residues bound to dyes has enabled automatic detection of the thermal stability of proteins through PCR [14]. This method, called differential scanning fluorescence, has wide applications in protein science, including the optimization of stable buffer conditions, crystallization parameters, or ligands to determine the binding constants. On the basis of this technical principle, we propose a new form of protein chip, PTSC, which, on a microscale, uses a protein cluster with specific functions in liquid solvent to characterize the binding between proteins and drugs through thermal stability. PTSC is a low-cost, high-throughput, label-free, and rapid method. Compared with traditional protein chips, PTSC do not require labeling of drugs, and can directly detect interactions between drugs and proteins. Simultaneously, the conformation of proteins in the liquid phase is well maintained.

COVID-19 was chosen to provide a proof of PTSC. We placed drug targets on a PCR plate and constructed a PTSC to demonstrate the chip’s application potential. We optimized the reaction parameters and implementation conditions, and automatically detected the thermal stability of COVID-19 drug target proteins, thus indicating whether the drug molecule bound the protein.

This chip was divided into two upper and lower regions: the drug test area and the blank control area. Each region contained 12 COVID-19 target proteins, as described in the methods section. Each target protein was analyzed at least four times independently.

The prototype PTSC chip has 12 target proteins when 96-well plates are used with four wells per protein. However, this platform strategy could be extended to incorporate as many as more than 1000 protein targets at a time. To date, PCR chips have been used with more than 5,000 samples per plate (Smart Chip, Wafergen Biosystems)—a number is far exceeding the known number of human drug targets, thus reaching the proteomic level. Therefore, the PTSC has broad application potential. Specific target information and methods are provided in the supplementary materials.

First, we tested reaction buffers including water, PBS buffer (pH=7.4, 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl), PBS buffer with disaccharide (1% trehalose, a disaccharide added to increase protein stability), PBS buffer with 1% trehalose and 0.1% bacteriostatic agent (5-chloro-2-methyl-4-isothiazolin-3-one2-methyl-4-isothiazolin-3-one, 3:1), and PBS buffer with 0.02% NaN₃. After testing, the PBS buffer had the same Tm value as that of water, but the fluorescence signal obtained with PBS was stronger. After addition of the bacteriostatic agent, the Tm value of the protein greatly differed from that with the PBS buffer. The presence of bacteriostatic agents can affect the stability of some enzyme proteins and decrease the Tm value. Careful consideration should be exercised when using bacteriostatic agents in the reaction buffer. PBS, the primary buffer used in this experiment, resulted in less interference. PBS is recommended for short-term storage (Figure 1).

According to the conventional targets of SARS-CoV-2, we designed and developed the chip, whose targets and functions are shown in Figure 2a. We screened the protein concentrations used in the chip, as shown in Figure 2b, by using Nfkb1-p50 as a representative target. Finally, we determined that 0.045 μg/μL was the ideal protein concentration for the chip. We conducted quality-control testing and measured the standard curves of all proteins on the chip (Figure 2c-d). Different proteins exhibited markedly different dissolution-curve patterns. Differences in Tm values are associated with the properties of proteins, such as structural conformation, the number of subunits, and the number of disulfide bonds. The same protein showed a single peak pattern, thus indicating the purity of the protein and the experimental replicability. Therefore, the chip can be used for applications in molecule-drug screening.

The applications of this chip mainly involve three aspects. The first aspect is the screening of small-molecule drugs. We tested small molecules with known targets. Previously, we found that the small-molecule drug chlorogenic acid (CLA) interacts with its active protein targets, such as annexin A2 protein, thus changing the protein’s conformation and consequently its thermal
stability [15]. In Figure 3a, the red curve represents the thermal stability curve of annexin a2 protein without CLA, and the green curve represents the thermal stability curve of annexin a2 bound to CLA. The thermal stability of the annexin a2 protein was altered after CLA binding. The change in the Tm value after CLA binding was significant, on the basis of analysis of three independent repeated experiments with a t test.

Second, the chip could be used to detect macromolecular drugs and to assess whether binding occurs. The interaction between an antigen and antibody also changes the thermal stability of each component. In this case, we successfully detected the binding between the nucleocapsid protein and its antibody (Figure 3b). The thermal stability curve of the nucleocapsid protein is shown (red curve), and the binding of the specific antibody increased the thermal stability. The blue curve represents the thermal stability of nucleocapsid protein during antibody binding; because the antibody is also a protein, another thermal stability curve for the antibody (green curve) is shown.

Herein, we propose a new concept of a PTSC based on a PCR chip. We chose 12 potential targets for COVID-19 treatment, screened the chip buffer, and optimized the protein concentration. This protein chip is the first device enabling high-throughput detection of the protein stability of COVID-19. Its potential applications are reflected in the following aspects. First, the protein thermal stability principle was used to achieve low-cost, high-throughput screening of COVID-19 drugs (small molecules, macromolecules, or complex herbal medicines), which may be valuable in the global COVID-19 pandemic. This chip can also be used for pharmacological research, and it provides a tool for screening drug targets of complex herbal drugs.

Third, the chip could be used in pharmacological research to identify unknown targets of small molecules. One of the most remarkable features of this chip is its ability to efficiently identify protein targets of complex chemical composition systems. In the literature, many herbs have been demonstrated to be clinically effective against COVID-19. The China Food and Drug Administration has approved three compound drugs for the clinical treatment of COVID-19. Pinellia ternata is a component in traditional Chinese medicine. Pinellia ternata is widely used in the clinical treatment of COVID-19 in China, but its molecular mechanism is unclear [16]. We tested the potential application of our chip with aqueous extract of Pinellia ternata, which we
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found to contain chemical components that strengthen the thermal stability of the SARS-CoV-2 receptor ACE-2. In a control experiment, the aqueous extract of *Pinellia ternata* did not change the thermal stability of another viral receptor (AXL) protein (Figure 4).

ACE-2 is the main receptor of SARS-CoV-2 in the respiratory system, and ACE2 expression is extremely low in many human tissues, particularly those in the respiratory tract [17]. AXL specifically interacts with the N-terminal domain of the SARS-CoV-2 spike protein, thereby suggesting that the mechanism of *Pinellia ternata* may involve blocking viral invasion in the respiratory system but not in other tissues that are dependent on the AXL receptor [18]. Thus, *Pinellia ternata* may include small molecules that specifically bind ACE-2 receptors. This finding may provide directions for further drug discovery and pharmacological research. The components of *Pinellia ternata* could be tested to determine which small molecules are responsible for its effects. This method, if combined with high-throughput automatic equipment, such as robots, could profoundly influence the discovery of natural drugs. Our future research efforts will be focused on combining these techniques.

This chip is a method for preliminary screening of drugs and targets that provides the advantage of high-throughput, rapid drug screening, which cannot be achieved by isothermal titration calorimetry, surface plasmon resonance, and other technologies. However, this method also has several disadvantages, and the drugs identified through preliminary screening require verification through various methods. We suggest that

Figure 3 | Testing of small-molecule and macromolecule applications.
(a) Chlorogenic acid is a small molecule that has been reported to bind annexin a2. We tested thermal stability before and after binding. Binding of chlorogenic acid to annexin changed its thermal stability ($P<0.05$). (b) Similarly, we tested the thermal stability of the N protein of SARS-CoV-2 and anti-N protein antibody before and after binding. The stability of N protein clearly increased after antibody binding ($P<0.05$). Independently repeated experiments were performed to determine the curves for each protein.

Figure 4 | Testing of the chip’s application to a complex chemical system.
(a) The extract of *Pinellia ternata* contains chemical components that can increase the thermal stability of the SARS-CoV-2 receptor ACE-2. (b) In a control experiment, the extract of *Pinellia ternata* did not change the thermal stability of another viral receptor (AXL) protein.
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this chip be combined with isothermal titration calorimetry and other technologies to achieve rapid and accurate screening of drugs and targets.

DECLARATION OF COMPETING INTERESTS

The authors declare no conflicts of interest.

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