A Quantitative Assay of Recombinant Malarial Lactate Dehydrogenase as a Platform for Screening Inhibitors from Crude Herbal Extracts

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Abstract: The potential of malaria parasite (Plasmodium falciparum) generating resistance to currently used antimalarial drugs is a major scourge for malaria patients. The multiple targets on which antimalarial drugs act hinder the occurrence of such resistance. As an essential metabolic enzyme involved in energy production in the parasite, the lactate dehydrogenase of P. falciparum (PfLDH) provides an alternative target for parasite killing. In the present study, the PfLDH gene was amplified from the FCC1/HN isolate of PfLDH and functionally expressed in Escherichia coli, which had been subsequently identified by Western blotting with the recombinant PfLDH protein-raised rabbit polyclonal antiserum, and further confirmed by the hybridization with lytic suspension of cultured parasites. The recombinant PfLDH protein was purified for uniform measurement of enzymatic activity and immobilized on 96-well plates for detection of PfLDH-targeted inhibitors. Hematin (HT) and chloroquine (CQ), two well-known inhibitors of PfLDH, were chosen for evaluating the feasibility of the in vitro colorimetric enzyme assay. The results showed that the enzymatic activity of PfLDH sharply dropped in a concentration-dependent pattern, as HT was involved, but only slightly dropped in the presence of CQ. Furthermore, this enzyme assay was applied to screening inhibitory component(s) of PfLDH from the crude extracts of medicinal plants, Pueraria lobata, Amomum villosum, Dichroa febrifuga, and Polygonum cuspidatum, in which the suppression of malaria growth was previously observed for P. lobata and D. febrifuga. Consequently, the correlation of enzyme inhibition with growth suppression was observed for the crude extracts of P. lobata although further investigations should be pursued for elucidation of the exact compound exhibiting inhibitory effect to PfLDH. The present recombinant PfLDH-based enzymatic activity determination provides a simplified, reproductive and economic platform for screening inhibitors of PfLDH, which contributes to the discovery of inhibitory compounds from either pure chemicals or crude extracts of medicinal plants, animals, and fungi towards development of novel antimalarial drugs.

Key Words: malaria; lactate dehydrogenase; recombinant protein; enzyme inhibitor; screening platform

Malaria caused by P. falciparum is a fatal epidemic disease in the tropical regions of the World, especially in Africa and Southeast Asia[1]. Although an effective antimalarial drug, artemisinin (qinghaosu), has been used for years without resistance induction in the parasite, a potential trend towards artemisinin tolerance associated with the mutation of SERCAs has been demonstrated[2]. It is clear that the multiple attacks on malaria by various antimalarial drugs are crucial to overcome or abolish multi-drug resistance (MDR). Therefore, the combination of artemisinin with other antimalarial drug(s) is a reasonable option for interrupting the infection and transmission by MDR malaria.

As an essential metabolic enzyme responsible for energy production in the parasite, PfLDH catalyzes dehydrogenation of lactate and generates pyruvate by using NAD⁺ as a cofactor. The inhibition of PfLDH in the parasite leads to parasite death, suggesting a potential antimalaria target[3]. A polyphenolic binaphthyl disesquiterpene, gossypol, found in cottonseed was identified as a potent antimalarial compound[4], which was later elucidated to be a potent inhibitor of PfLDH[5].
Unfortunately, its side effects of hypokalemia and possibly permanent infertility make it unacceptable as an antimalarial drug although attempts for improvement are continuously pursued[6,7].

As a most commonly used malarial therapeutic agent, CQ did not necessarily link its PfLDH inhibitory role to the malaria-killing effect. In spite of uncertain antimalaria mechanism, CQ was previously identified as a competitive inhibitor of PfLDH[9]. The resistance to CQ, however, is noted worldwide in the cases of P. falciparum and P. vivax, revealing an incredible trend that it is no longer to use alone for malarial transmission control[9]. Recently, a series of heterocyclic azole-based inhibitors that bind selectively to the active sites of PfLDH and exhibit effective antimalarial activity in vitro and in vivo has been identified[10,11], confirming that PfLDH is still a usable target for screening and designing novel antimalarial drugs.

In order to establish a platform for high throughput screening inhibitors of PfLDH, we amplified PfLDH gene from the FCC1/HN isolate of P. falciparum and cloned into pET-29a(+) vector for expression in E. coli BL21(DE3) strain, by which we developed a solid-phase colorimetric enzymatic activity assay for quantitative measurement of PfLDH. To test the effectiveness and reproducibility of the present system, we chose HT and CQ as positive inhibitors for testing. Furthermore, we also used this system for screening inhibitors of PfLDH in extracts of four rationally chosen medicinal plants, P. lobata, D. febrifuga, A. villosum, and P. cuspidatum.

When applied to in vitro evaluation of antimalarial activity, only the extract from P. lobata displays a capacity to block parasite growth. This study would pave a path to the high throughput screening of PfLDH-targeted inhibitors in the development of novel antimalarial prodrugs.

1 Methods

1.1 Amplification, cloning and sequencing of PfLDH gene

Plasma from a malaria patient naturally infected by the FCC1/HN isolate of P. falciparum was washed twice with STE buffer (100 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA). The parasite pellet was suspended in 100 μL of DNA extract buffer (2% SDS, 2 mol/L NaOH, 0.5 mol/L EDTA) and boiled before centrifugation. The supernatant was applied to amplification of PfLDH gene in 50 μL of reaction mixture, consisting of 1×LA PCR buffer II (Mg²⁺ plus, TaKaRa), 2.5 μL LA Taq (TaKaRa), 800 μmol/L primer each as a final concentration. The forward primer, pLDH-a, is designed, and synthesized, and referred as DQ198261: 5'-GGCGGATCCATGGGACCCAAAAGCAAAAATC-3' and 5'-GTCGCGGTTAGCTAATGCCTTCATTCTC-3'. The PCR profile is as follows: 94°C for 5 min, followed by 30 s at 94°C, 1 min at 56°C, 3 min at 72°C, and 10 min at 72°C for 30 cycles. The amplified fragment was run on agarose gel, recovered by gel extraction, and ligated to pGEM-T plasmid (Promega) for sequencing.

For construction of an expression vector, cloned PfLDH gene in pGEM-T was re-amplified by using an alternative pair of primers, i.e. pLDH-a with an Nco I site and pLDH-b with an Xho I site. Their sequences are as follows: pLDH-a, 5'-GGC CCATGGGACCCAAAAGCAAAAATCG-3', and pLDH-b, 5'-CTACTGTAGAGCT ATGCGCTTCAATCTC-3'. The PCR was performed at 94°C for 5 min, followed by 30 s at 94°C, 30 s at 52°C, 90 s at 72°C for 30 cycles. The amplified fragment was recovered by extract of gel, which was digested with Nco I and Xho I and cloned into pET-29a(+) (Novagen) to give rise to a recombinant fusion expression construct, pET-PfLDH, which was then transformed into E.coli BL21(DE3) strain for re-sequencing and inducible expression.

1.2 Expression and purification of recombinant PfLDH

BL21(DE3) cells transformed by pET-PfLDH were grown at 37°C in 2×YTA medium supplemented with kanamycin (30 μg/mL). One absorbance at 600nm of wavelength (A600) approximately equivalent to 0.8–1.0, isopropyl-D-thiogalactopyranoside (IPTG) (Promega) was added to the medium at a final concentration of 1mmol/L and incubated for an additional 6h. After harvesting by centrifugation, cell pellet was resuspended in binding buffer (300 mmol/L NaCl, 50 mmol/L sodium phosphate buffer, 10 mmol/L imidazole, pH 8.0) at a proportion of 2 mL/g wet weight. For cell lysis, lysozyme (0.1 mg/mL), RNase A (10 μg/mL), and DNase I (5 μg/mL) were added simultaneously and incubated in room temperature prior to repeated freezing/thawing treatent and centrifugation. The supernatant containing a 6×His tagged fusion protein was then purified with Ni-NTA His Bind Resin (Novagen) according to the manufacturer’s instruction. Finally, the eluted fusion protein was quantified by a classic procedure (Bradford 1976) and separated by 12% SDS-PAGE.

1.3 Preparation of polyclonal antiserum and Immunoblotting of PfLDH

For primary immunization, 0.8 mg of purified protein was diluted in 1 mL of PBS and mixed with 1 mL of Freud’s Complete Adjuvant (Sigma). The mixture was injected subcutaneously (6–8 sites/rabbit, 0.2 mL/site) and in muscle (0.25 mL/leg). The boost immunizations were performed on the 2nd, 4th, 6th, and 8th weeks with the same antigen mixed with equal volume of Freud’s Incomplete Adjuvant (Sigma). On the 7th day after the 3rd immunization, 1 mL of blood was collected from the ear vein of a rabbit for titer determination by the enzyme-linked immunosorbent assay (ELISA). Two weeks after the 4th immunization, whole blood was collected by neck artery bleeding.

The recombinant protein sample to be tested was separated by SDS-PAGE and electronically transferred to a nitrocellulose membrane, which was first soaked with blocking buffer (10% W/V defatted milk powder and 0.3%
TWEEN-20 in PBS) overnight at 4°C, and subsequently incubated in a 1:1 000 blocking buffer-diluted antiserum at 37°C for 30 min with gentle mixing. After being washed for three times with washing buffer (1% TWEEN-20 in PBS) and incubated in a 1:250 diluted secondary antibody (goat anti-rabbit IgG horseradish peroxidase conjugate) with blocking buffer at 37°C for 30 min with gentle mixing, the membrane was developed with DAB staining.

For preparing natural PfLDH from cultured parasite, 100 µL of parasite-infected erythrocyte was washed with 1mL of RPMI1640 and 1 mL of PBS before centrifugation. The cell pellet was then mixed with 1mL of 0.05% saponin (Sigma) in PBS and PMSF (Sigma, FW: 174.20) at 1 mmol/L of a final concentration. Followed by 3 500 r/min spinning at 4°C for 15 min, the pellet was washed with pre-cold PBS for another three times to achieve pure and intact parasites. To release the extracts of medicinal plants were prepared according to the previously described method[13]. Briefly, for P.lobata, A.villosum, and D.febrifuga, 100 g of ground powder was extracted with 70% ethanol by heating and circulating for 2 times and 1.5 h for each time. All ethanol extracts for each plant were mixed, filtered and condensed to a total volume of 50 mL for P.lobata and A.villosum or 150 mL for D.febrifuga by a spin evaporator. For P.cuspidatum, 100 g of crude powder was decocted in boiling water for 3 times and 1 h for each time. The water decocts were mixed, filtered and collected to a total volume of 150 mL, in which 350 mL of 95% ethanol was added with continuous stirring. After standing at room temperature for 4 h, the mixture was filtered and condensed to 50 mL in a spin evaporator.

1.4 Preparation of crude extracts from medicinal plants

The extracts of medicinal plants were prepared according to the previously described method[13]. Briefly, for P.lobata, A.villosum, and D.febrifuga, 100 g of ground powder was extracted with 70% ethanol by heating and circulating for 2 times and 1.5 h for each time. All ethanol extracts for each plant were mixed, filtered and condensed to a total volume of 50 mL for P.lobata and A.villosum or 150 mL for D.febrifuga by a spin evaporator. For P.cuspidatum, 100 g of crude powder was decocted in boiling water for 3 times and 1 h for each time. The water decocts were mixed, filtered and collected to a total volume of 150 mL, in which 350 mL of 95% ethanol was added with continuous stirring. After standing at room temperature for 4 h, the mixture was filtered and condensed to 50 mL in a spin evaporator.

1.5 In vitro evaluation of antimalarial activity in crude herbal extracts

The in vitro culture of malaria parasite was performed according to a conventional protocol[14]. The parasites maintained in erythrocyte culture were synchronized by treatment of D-sorbitol[15]. The drug sensitivity test was carried out by using a modified version of the standard in vitro microassay recommended by World Health Organization (WHO)[16]. Briefly, 10 µL of each medicinal extract was added into one well, and the sample-filled plate was covered and sealed for storage at 4°C. For testing, 90 µL of a mixture of culture medium with parasite-containing blood suspension was added into each well and covered after gentle shaking for 10 s. After incubation at 37°C for 24 h, thick blood smear slides were fixed in methanol and Giemsa stained for evaluation of parasite development by microscopy. No schizont counted in 100 parasites was judged as (−), 1% schizont judged as (+), and more than 1% schizont judged as (++).

1.6 Pre-incubation of PfLDH and coating with reaction mixture

A stock solution of HT (20 mmol/L) was prepared by dissolving 12.7 mg of HT (Sigma, FW: 633.51) in 1 mL PBS (pH 8.0) and stored at 4°C. 132mg of CQ (Sigma, FW: 515.9) was dissolved in 2 mL of distilled water to prepare a stock solution of 128 mmol/L and stored at −20°C. A series concentration of HT (2 µL), CQ (2 µL), or a medicinal extract (4 µL) was pre-incubated with 8 µL of purified PfLDH (0.112 mg/mL for HT and CQ, 2.5 mg/mL for medicinal extracts) in a 96-well plate for 20 min at room temperature. The reaction mixture was then coated overnight at 4°C with 100 µL 0.05 mmol/mL Tris-HCI (pH 7.5). After repeated washing with 150 µL of the same buffer, pre-treated PfLDH samples were applied to determine enzymatic activity in situ. All samples were tested in duplicate or triuplicate.

1.7 Enzymatic activity assay for inhibitory component screening

The reagent kit used for detection was purchased from Jiancheng Biotech Co., which mainly includes NAD+, pyruvate and substrate buffer. In testing, NAD+ in detection well is replaced by distilled water in detection blank well. 8µL of 2 µmol/L pyruvate was filled in standard wells, while pyruvate was substituted by water in standard blank wells. For all wells, 25 µL of substrate buffer was supplemented. When mixed thoroughly and incubated at 37°C for 15 min, 25 µL of DNPH was filled in wells and incubated at 37°C for 15 min, and then 250 µL of 0.4 mol/L NaOH was added to the mixture in each well and incubated at least 3 min. The A450 was measured in a Bio-Tek spectrophotometer.

One unit of the enzyme activity is defined as the yield of 1 µmol pyruvate after incubation with substrate buffer containing lactate at 37°C for 15 min in a total volume of 1 000 mL reaction mixture. The calculating formula is as follows: [(A450 of detection well−A450 of detection blank well)/(A450 of standard well−A450 of standard blank well)]×2 µmol/mL (concentration of standard well)×1 000 mL×dilution folds of sample prior to detection. The 50% inhibitory concentration (IC50) for inhibitors was calculated by the following formula: Antilog [B+(50−<50% inhibition rate/ >50% inhibition rate−<50% inhibition rate)]×C in which A=log>50% inhibitor concentration, B=log<50% inhibitor concentration, and C=A−B. The inhibition rate was caculated from A450 by the following formula: 1−(A450 of sample/A450 of control)×100%.

2 Results and analysis

2.1 Cloning and recombination of PfLDH gene

The intronless character of PfLDH gene is beneficial to its PCR amplification directed from the plasmodial genome without RNA extract and reverse transcription steps. By using
a pair of primers flanked by 16 bp of recognition sequence of restriction enzymes, \textit{Nco I} and \textit{Xho I}, an approximately 1 kb fragment equivalent to the actual size of PfLDH gene (951 bp) was amplified from a malaria positive sample carrying FCC1/HN isolate of \textit{P. falciparum}. The PfLDH fragment flanked by \textit{Nco I} and \textit{Xho I} sites was then digested by double digestion and cloned into pET-29a(+) with digestion of same restriction enzymes for bacterial expression. The sequence result obtained from this study was submitted to GenBank of National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov). Comparison of our sequence data (DQ825436) with that accessed in GenBank (DQ198261) indicates two base pairs of difference, which lead to a consequence of two point mutations by base conversion: one is from T to C at 407 bp (Leu→Ser), and another is also T to C at 563 bp (Leu→Pro). Whether these modification features would affect the normal catalytic activity of PfLDH is awaiting further investigation, such as comparative studies of catalytic kinetics and dynamics between natural and recombinant enzymes.

2.2 Expression and purification of PfLDH

We originally designed the fusion product comprising a large size of peptide (33 kD), a short S-tag of 26 amino acid residues, a thrombin site at the N-terminal, and a 6-histidine tag at the C-terminal, which are beneficial to affinity purification and enzymatic cleavage of fusion protein. After induction by IPTG, a condensed protein band with a size slightly larger than 36 kD was expected to be observed on the gel for lysed BL21(DE3) cells harboring pET-PfLDH. Although the 36 kD protein was mainly retained in precipitate, a large quantity of soluble protein appeared in suspension. For affinity purification, cleared lysate from bacterial cells was mixed with Ni-NTA His Bind Resin and followed by washing and elution, from which a main band with other minor bands was seen in the gel through SDS-PAGE (Fig. 1). After several rotated elution, an individual clear band was obtained, which has the same size and location with the condensed band after electrophoretic separation of cleared lysate.

2.3 Immunological verification of recombinant PfLDH

The potency titer of polyclonal antiserum raised against purified PfLDH was evaluated by ELISA. The colorimetric result of gradient dilution was examined as a titer of 1:12 800. When confirmed by Western blotting, a hybridized band larger than the estimated size of 36 kD was detected in BL21(DE3) cells transformed with pET-PfLDH (Fig. 2A, lanes 1 and 2), and another band of approximately 33 kD showing natural PfLDH from suspension of cultured parasite came into view (Fig. 2B, lanes 1 and 2).

Furthermore, blood samples collected from malaria patients also showed a positive binding reaction with polyclonal antiserum raised against the recombinant protein (unpublished data), confirming immunological binding capacity of either artificial or natural PfLDH with specific polyclonal antiserum.

2.4 Enzymatic activity determination of recombinant PfLDH

The chromatographically purified PfLDH protein was sampled in a liquid-phase for determination of enzymatic activity (u). After total amount of protein (mg/mL) was measured by a Coomassie blue method, the specific enzymatic activity (u/mg protein) was calculated. The average values with their standard deviation (SD) are listed in Table 1.
cells also displays relatively higher enzymatic activities comparatively equal to the purified protein, which may be used to determine colorless inhibitor without following coating.

**Table 1** The mean enzymatic activity and specific activity of recombinant protein preparations

<table>
<thead>
<tr>
<th>Samples</th>
<th>$A_{450}$</th>
<th>Activity /u</th>
<th>Total protein amounts / (mg/mL)</th>
<th>Specific activity / (u/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.631</td>
<td>3042.8</td>
<td>1.5</td>
<td>2028.5</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.865</td>
<td>5191.8</td>
<td>2.5</td>
<td>2076.7</td>
</tr>
<tr>
<td>$\bar{x} / \bar{x} \pm SD$</td>
<td>0.748</td>
<td>4117.3 ± 1519.6</td>
<td>2.0</td>
<td>2058.7</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.386</td>
<td>83.9</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.245</td>
<td>169.0</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>$\bar{x} / \bar{x} \pm SD$</td>
<td>0.315</td>
<td>126.5 ± 60.2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ND: not detected.

### 2.5 Inhibition of PfLDH activity by model inhibitors

To evaluate the inhibitory effects of HT, the purified PfLDH was pre-incubated with HT and coated on the plate. As illustrated in Fig. 3, the inhibition of PfLDH by HT was easily distinguished from the gradual colorimetric change. Because of repeated washings, colorful components in tested samples did not interfere with the detection.

Indeed, more HT (dark brown) does not mean deeper color, that is, a higher dosage of HT gives shallower brown color (well 8), while a lower dosage of HT gives deeper brown color (well 3). For quantitative evaluation of HT and CQ inhibition to PfLDH, $A_{450}$ was determined after incubation of PfLDH with serial dilution of HT or CQ. Because the value of $A_{450}$ is actually correlated with the activity unit of the enzyme, we use $A_{450}$ as the unit of apparent enzyme activity. The results were demonstrated in Fig. 3.

The statistical analysis of the above result showed that the inhibition of PfLDH by HT displays a significant difference from control at a 0.05 level, but that of PfLDH by CQ is not significantly different from control. Therefore, we conclude that the activity of PfLDH is potently inhibited by HT, but only slightly inhibited by CQ. In addition, from results of the inhibition percentage estimated from $A_{450}$ in proportion with a serial dilution of HT, 50% inhibitory concentration (IC$_{50}$) was calculated as 6.131 mmol/L.

In general, the solid-phase enzyme determination system should be advantageous for large-scale screening of inhibitors from both colored and colorless natural or artificial products. In addition, the suspension of lyzed bacterial cells expressing PfLDH can be directly applied to evaluate the effects of colorless inhibitors on PfLDH activity without need of any coating steps.

### 2.6 In vitro microassay for antimalaria effect

To elucidate the relationship between the inhibitory effect and the antimalarial activity, these medicinal extracts were evaluated in vitro for their roles on suppressing malarial growth. The results are shown in Fig. 4.

![Fig. 3](image-url)  
**Fig. 3** The inhibition of enzymatic activity of PfLDH by HT (A) and CQ (B)  
The quantity of purified PfLDH in each well is 0.112 mg/mL. Treatments in serial concentrations of 0.25, 0.5, and 2 mmol/L (except for 1 mmol/L) of HT have significant difference from control ($P<0.05$, $n=3$), Treatments in all concentrations in CQ group have no significant difference from control ($P>0.05$, $n=3$).
Fig. 4 Antimalarial activity of medicinal extracts represented by a proportion of 1% schizont counted under a 100 × oil objective

A is a image of parasites grown without any medicinal extract and evaluated as (++); B is a image of parasites grown with extract from *A. villosum* and evaluated as (+); both C and D are images of parasites grown with extracts from *P. lobata* and *D. febrifuga* and evaluated as (-). Note: counting result is not available for *P. cuspidatum* due to its unclear image in thick blood smears.

As compared with control, the schizonts in treatment by extracts of *P. lobata* and *D. febrifuga* were counted as (−), and that in treatment by extracts of *A. villosum* was counted as (+). As to *P. cuspidatum*, schizont-counting result is not available due to unclear image in thick blood smears. In summary, only extract from *P. lobata* was evaluated as an inhibitor of recombinant enzyme and a suppressor of parasite growth, while the extract from *D. febrifuga* was evaluated as a suppressor of growth but not an inhibitor of enzyme. In contrast, the extract from *A. villosum* is an inhibitor but not a suppressor.

2.7 Screening inhibitory components of PfLDH from medicinal herb extracts

For evaluation of availability of solid-phase PfLDH activity detection system described above, the purified PfLDH (2.5 mg/mL) was pretreated with medicinal extracts for detection of PfLDH activity. The values of $A_{450}$ representing inhibition of PfLDH were determined for extracts from *P. lobata*, *D. febrifuga*, *A. villosum*, and *P. cuspidatum*, demonstrating significant inhibitory effects with the exception of extract from *D. febrifuga*. The result is shown in Fig. 5.

Although three medicinal extracts exhibit inhibition to PfLDH, they show vary degree of potency with *A. villosum* and *P. cuspidatum* being the most potent kinds, while *P. lobata* being the least potent type.

3 Conclusions

The PfLDH gene, containing no intron and located on chromosome 13 in a single copy, is transcribed as a full-length 1.6 kb mRNA and translated into a 316 amino acid protein in all asexual blood stages of malarial parasite[17]. We amplified an almost completely homologous PfLDH gene (DQ825436) to the only accessible sequence in GenBank (DQ198261), except for two substitutions of amino acid residues. Whether this mutation actually affects the function of PfLDH needs a comparative study of their catalytic features. As a pioneering work, PfLDH gene was first cloned[18], and subsequently expressed in *E. coli*[19]. After several years, over-expression of fully active PfLDH was achieved, which helps in deciphering the high resolution X-ray structure of the enzyme[20]. The recombinant PfLDH protein was utilized to raise rabbit antiserum for immunological confirmation of the identity for bacteria synthesized 33 kD protein[21]. By choosing a pET vector system, we have successfully expressed a large quantity of active 33 kD PfLDH in a soluble state, which was reflected by the presence of a highly solubilized PfLDH in suspension of lysed bacterial cells expressing PfLDH gene. Due to its higher specific enzymatic activity, the enriched protein is most likely to be a sort of soluble form of expression product without dominant formation of inclusion bodies.
All tests of inhibitors of PfLDH usually adopt the soluble enzyme as reaction phase, which are interfered by the color of inhibitory materials to be tested. Here, we report a solid-phase enzyme detection system suitable for large-scale screening of inhibitory components in any crude extracts of either color or colorless samples. In an initial trial, we found that the effectiveness of this inhibitor detection platform is largely dependent on the coating process of purified PfLDH. In general, if the enzyme was pre-coated on the well, no color change occurred, presuming a blockage of active site of the enzyme by the pre-coating step (unpublished result). So we optimized the immobilization procedure by coating a mixture of the enzyme with inhibitors directly on the surface of wells. The simultaneous coating may allow the inhibitor ready access to enzyme surface and also accommodate the inhibitor in the active pocket of enzyme.

A simple method for specifically measuring the activity of PfLDH in the presence of human LDH (hLDH) has been devised[22], which is based on APAD\(^+\), an analog of NAD\(^+\), as a cofactor of PfLDH. The PfLDH converts APAD\(^+\) to APADH in the presence of lactate, and APADH in turn reduces colorless NBT to blue formazan that regenerates APAD for another cycle of reaction with PfLDH. This produces a colorimetric detection that remains linear over long periods of time due to the ample supply of substrates. However, measurement of PfLDH activity in the whole blood of patients was complicated by the presence of hemoglobin, which interferes with the colorimetric reaction[23]. To remove the interference of hemoglobin, a monoclonal antibody-bound immunocapture assay of PfLDH has been introduced for diagnosis of malarial infection, which exploits a panel of immunocapture antibody of PfLDH and allows for the quantitation and speciation of malaria infections without interference by the red color of blood[24]. This test, however, is inconvenient for determination of the enzymatic activity of PfLDH in a large-scale and at room temperature. In particular, bacterial produced recombinant PfLDH was used in the present study as noted, so no interference of human erythrocytes as well as human LDH should be considered. On the other hand, the cost of enzyme test for this assay is estimated to be as low as 0.03 US dollar for each well, which would facilitate large-scale screening of inhibitors, including small molecules or even biological macromolecules such as peptides and nucleotides.

HT is a toxic byproduct of hemoglobin digested by malaria parasite, but the parasite is able to detoxify HT by polymerizing it as hemozoin. HT strongly binds and hence inhibits malarial PfLDH, its binding to PfLDH is about 100-fold tighter than bovine LDH[27]. We have detected pronounced inhibition of recombinant PfLDH by HT at the range from 0.125 mmol/L to 2 mmol/L, which has significant difference at a statistical level when compared with the control. On the other hand, some evidence for CQ binding to PfLDH was informative, but inhibition of PfLDH by CQ was not observed[28]. Inhibition studies confirm that CQ acts as a weak inhibitor of PfLDH. The low level of inhibition by CQ correlates with the limited interactions validated between CQ and the enzyme[22]. While being consistent with these reports, we have also assessed minor inhibition of PfLDH by CQ in the concentration from 0.025 mmol/L to 4 mmol/L range. These results emphasize that our inhibitor-coupled enzyme evaluation procedure is sufficiently accurate for both noncompetitive inhibitors like HT and competitive inhibitors like CQ.

Discovery of antimalarial produgs from natural products is eagerly explored[28,29], but none of these antimalarial components is targeted toward PfLDH. Therefore, we focus our screening of potential antimalarial compounds on the inhibitors of PfLDH. Interestingly, a kind of alkaloid (\(\gamma\)-dichroine) from D. febrifuga (Chang-Shan, in Chinese) was identified as an antimalarial component about 50 years ago[30], so we chosen it for evaluating the correlation of enzyme inhibition to malaria growth repression. As expected, its ethanol extracts completely block the parasite growth, but do not inhibit the activity of PfLDH. P. lobata (Ge-Gen), was chosen because it belongs to legume plants, and we expect that its unique leghemoglobin constituent may inhibit PfLDH like HT. As to A. villosum (Sha-Ren), there have been reports indicating their arresting parasite development due to the presence of limonene[31]. The resveratrol rich in P. cuspidatum (Hu-Zhang) is able to decrease activity of hLDH by an uncertain mechanism[32], so we chose it as one of the tested samples.

It is clear from in vitro tests that only the extract from P. lobata can be evaluated as both an enzyme inhibitor and a suppressor of malaria growth. Whether this substantial inhibition is due to the action of leghematin or other compounds needs extensive investigation by employing other analytical tools like GC-MS, which may prove to be the starting point for antimalarial drug development. The extract from D. febrifuga only acts as a growth suppressor but not an enzyme inhibitor, suggesting an expected role of dichroine in attacking the malarial parasite[30]. Although demonstrating a significant inhibition to PfLDH, the extract from A. villosum exhibits only mild suppression to malaria growth, implying a weak block role by limonene in the selected concentration. However, it is difficult to quantitatively ascertain the exact role played by limonene because we did not use the pure limonene standard in the test.

In conclusion, the present study has provided a reliable, economic and universal procedure for high throughput screening highly specific inhibitors of PfLDH, which enables a rational design and modification of antimalarial drugs.
collected from natural sources and synthesized in the combinatory chemical library, for example, the crude extract of *P. lobata* discussed in this study. The preliminary results presented here have addressed the importance of the introduced rapid enzyme inhibition measurement in combination with *in vitro* microassay of parasite growth suppression would greatly facilitate the discovery and development of urgently needed novel antimalarial prodrugs.

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