Identifying the Naphthalene-Based Compound 3,5-Dihydroxy 2-Napthoic Acid as a Novel Lead Compound for Designing Lactate Dehydrogenase-Specific Antibabesial Drug

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Human babesiosis is caused by apicomplexan Babesia parasites, including Babesia microti, Babesia crassa, Babesia sp. MOI, Babesia divergens, Babesia duncani, and Babesia venatorum. Among them, B. microti is the most common cause of human and rodent babesiosis. Currently, no vaccine is available, and drugs for the treatment have high failure rates and side effects. Due to lack of a traditional tricarboxylic acid cycle (TCA cycle) and its dominant dependence on anaerobic metabolism to produce ATP, B. microti lactate dehydrogenase (BmLDH) was assumed to play a critical role in B. microti ATP supply. Our previous study demonstrated that BmLDH is a potential drug target and Arg99 is a crucial site. Herein, a molecular docking was performed based on the crystal structure of BmLDH from a series of gossypol derivatives or structural analogs to find the potent inhibitors interacting with the residue Arg99, and three naphthalene-based compounds 2,6-naphthalenedicarboxylic acid (NDCA), 1,6-dibromo-2-hydroxynapthalene 3-carboxylic acid (DBHCA), and 3,5-dihydroxy 2-napthoic acid (DHNA) were selected for further tests. Enzyme activity inhibitory experiments show that DBHCA and DHNA inhibit recombinant BmLDH (rBmLDH) catalysis with ~109-fold and ~5,000-fold selectivity over human LDH, respectively. Surface plasmon resonance (SPR) assays demonstrate that DHNA has a lower K_D value to BmLDH (3.766 × 10^{-5} M), in contrast to a higher value for DBHCA (3.988 × 10^{-8} M). A comparison of the kinetic parameters [association constant (k_a) and dissociation constant (k_d) values] reveals that DBHCA can bind the target faster than DHNA, while the complex of DHNA with the target dissociates slower than that of DBHCA. Both DBHCA and DHNA can inhibit the growth of B. microti in vitro with half-maximal inhibitory concentration (IC_{50}) values of 84.83 and 85.65 μM, respectively. Cytotoxicity tests in vitro further indicate that DBHCA and DHNA have selectivity indexes (SI) of 2.6 and 22.1 between B. microti and Vero cells, respectively. Although the two naphthalene-based compounds only display modest...
inhibitory activity against both rBmLDH and the growth of *B. microti*, the compound DHNA features high selectivity and could serve as a novel lead compound for designing LDH-specific antibabesial drug.

**Keywords:** *Babesia microti*, lactate dehydrogenase, naphthalene-based compound, human babesiosis, growth inhibition

**INTRODUCTION**

* Babesia species are tick-borne intraerythrocytic parasites and could cause babesiosis in humans and many animals globally (Bock et al., 2004; Westblade et al., 2017). *Babesia microti*, the smallest apicomplexan, is transmitted by ticks in both rodents and humans; however, human infection with the parasite could also be induced by blood transfusion, placenta, and solid organ transplantation (Cornillot et al., 2012; Villatoro and Karp, 2018). At present, *B. microti* is considered as an emerging zoonotic disease and widely distributed in United States of America, northeastern Eurasia, Japan, and so on (Tsui et al., 2001; Zamoto et al., 2004; Hersh et al., 2012). No vaccines or miracle drugs are available to control and cure the parasitosis, and in general, the recommended therapies for *B. microti* are the combinations of antibiotic and antimalarial drugs, such as the combination of atovaquone with azithromycin for the treatment of mild infection or clindamycin and quinine for the treatment of severe infection (Centers for Disease Control, 1983; Krause et al., 2000). Due to the low efficiency of these drugs, the resulting drug-resistant parasite often causes relapsing and deterioration of *B. microti*, suggesting the urgency to discover new drug targets and produce new drugs against the disease (Wormser et al., 2010).

Most apicomplexan parasites derive energy from the Embden-Meyerhof pathway (EMP), and in the anaerobic metabolism, lactate dehydrogenase (LDH), an essential enzyme of anaerobic metabolism, plays a crucial role in catalyzing the reversible reaction between pyruvic acid and lactic acid, with reduced nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide (NAD⁺) serving as cofactors, respectively (Kavanagh et al., 2004). Available studies demonstrated that the LDH enzymes of protozoans are an attackable target for drug development (Razakantoanina et al., 2000; Dando et al., 2001; Conners et al., 2005), and inhibiting the activity of these LDH enzymes can result in the death of the parasites, including *Plasmodium* spp., *Toxoplasma gondii*, *Babesia* spp., and *Cryptosporidium*. (Al-Anouti et al., 2004; Bork et al., 2004; Vivas et al., 2005; Zhang et al., 2015). Interestingly, the enzyme of *B. microti* (BmLDH) acquired by a lateral gene transfer event has been identified as a mammalian-like LDH enzyme, and is significantly different from all the other protozoa in the unusual event (Cornillot et al., 2012; Silva et al., 2016). Our previous study with the crystal structures of BmLDH indicated that the enzyme activity of BmLDH could be dramatically inhibited by gossypol and oxamate, and the residue Arg99 was crucial in the catalysis of BmLDH, but not in Human LDH-A (Yu et al., 2019). All the available reports indicate that the BmLDH could serve as a novel drug target for the development of new strategies to treat the human parasitosis.

Gossypol, a natural phenolic aldehyde extracted from the cotton plant, displays various biological activities, including antiviral, anti-parasitic, and male contraceptive effects (Radloff et al., 1986; Royer et al., 1986; White et al., 1988). In mammalian LDHs, gossypol was observed to be a non-selective LDH inhibitor competitive with NADH binding, and the inhibition constant (Ki) values were determined as 1.9 µM for LDH-A4, 1.4 µM for LDH-B4, and 4.2 µM for LDH-C4. In *Plasmodium falciparum*, the enzyme activity of *P. falciparum* lactate dehydrogenase (PfLDH) was inhibited competitively by gossypol with a Ki value of 0.7 µM and the growth of the parasite in vitro was also inhibited by gossypol with an half-maximal inhibitory concentration (IC50) value of 15.3 µM (Vivas et al., 2005). In *Babesia bovis*, gossypol inhibited BbLDH enzymatic activity to cause the death of *B. bovis* and its Ki and IC50 in vitro values were determined as 0.085 and 50 µM (Bork et al., 2004). In *B. microti*, the catalytic activity of BmLDH was inhibited by gossypol at 0.67 µM (Ki), and the IC50 value of gossypol against *B. microti* in vitro was 7.07 µM (Yu et al., 2019). Previous studies showed that these derivatives of gossypol exhibited obviously lower cytotoxicity toward mammalian cells than the parent compound (Royer et al., 1991; Royer et al., 1995). Therefore, the naphthalene-based compounds, the core of the gossypol structure, could have the potential use for screening selective inhibitors of BmLDH.

Previous research on the derivatives or structural analogs of the phenolic aldehyde gossypol revealed several chemical compounds as selective human LDH inhibitors, such as the substituted 2,3-dihydroxy-1-naphthoic acid family with 200-fold selectivity over dihydroxynaphthoic acids with substituents at the 4- and 7-positions (Yu et al., 2001). In malaria parasites, although the gossypol was observed to non-selectively inhibit the catalytic activity of human LDHs and PfLDH with inhibitory constants in the low micromolar range, its derivative, 8-deoxyhemigossylic acid, has been developed to selectively inhibit PfLDH compared with human LDHs (Gomez et al., 1997).

Owing to the dependence of parasites on glycolysis system for energy generation, these enzymes play crucial roles as potential drug targets for development of anti-parasitic agents (Dunn et al., 1996). Thus far, many compounds have been reported to produce a significant effect on the enzymatic activity of the protozoan LDHs, including gossypol, derivatives or structural analogs of gossypol, oxamic acid, and azole-based compounds (Cameron et al., 2004; Choi et al., 2007; Rai et al., 2017). Therefore, screening and identifying new BmLDH inhibitors
would facilitate the discovery of novel treatment strategies. The objective of the present study was to find the potent inhibitors interacting with the residue Arg99 via a molecular docking based on the crystal structure of BmLDH from a series of gossypol derivatives or structural analogs. Then, the screened inhibitors (naphthalene-based compounds) were further investigated by enzyme inhibition and growth inhibition experiments and cytotoxicity tests in vitro.

MATERIALS AND METHODS

Compliance With Ethical Standards
This study was approved by the Scientific Ethic Committee of Huazhong Agricultural University (permit number: HZAUMO-2018-007). All mice were handled in accordance with the Animal Ethics Procedures and Guidelines of the People’s Republic of China.

Molecular Docking
For the structure-based study, the complex crystal structure of BmLDH was obtained from Protein Data Bank (PDB accession no. 6K13) for CDOCKER docking. The structure model was further optimized by using the software (Discovery Studio 2018 Client), including removing water molecules, dopant atoms, and original ligands, cleaning geometry, adding hydrogen atoms, and defining the active site. In the CDOCKER protocol, the values of top hits, random conformations, and the orientations to refine were set as 10, respectively. In addition, the parameter of simulated annealing was switched to true and all the other parameters were maintained as defaults. In the docking study, a series of commercially available gossypol derivatives or structural analogs (16 species) were used for ligands, including 2,6-naphthalenedicarboxylic acid (PubChem CID: 14357), naphthalene-1,5-disulfonamide (PubChem CID: 96237), 1,6-dibromo-2-hydroxynaphthalene 3-carboxylic acid (PubChem CID: 74502), 2,6-naphthalene disulphonic acid (PubChem CID: 11390), 3,7-dihydroxy-N-[(4-nitrophenyl)methyl] naphthalene-2-carboxamide (PubChem CID: 481499), 3,7-dihydroxy-6-(hydroxymethyl) naphthalene-2-carboxylic acid (PubChem CID: 123699198), 6,6-dithiodinicotinic acid (PubChem CID: 85040), 3,5-dihydroxy 2-naphthoic acid (PubChem CID: 66837), 1,6-dihydroxynaphthalene (PubChem CID: 68463), methyl 1,6-dihydroxynaphthalene-2-carboxylate (PubChemCID: 131307105), 1-bromo-2-(bromomethyl) naphthalene (PubChem CID: 37828), dimethyl 2,6-naphthalenedicarboxylate (PubChem CID: 61225), naphthalene-1,4-dithiocarboxamide (PubChem CID: 73995732), 4,8-dimethoxy-1-naphthaldehyde (PubChem CID: 612187), 2-[5-(benzylxoxy)-1H-indole-3-yl]-1,4-naphthoquinone (PubChem CID: 101865177), 2-amino-8-methoxy-1,2,3,4-tetrahydro-naphthalene-2-carboxylic acid (PubChem CID: 12562606). All the structure data format (SDF) files were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov). The software permission of discovery studio files were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov). The software permission of discovery studio 2018 client (v18.1.100.18065) was provided by State Key Laboratory of Agricultural Microbiology of Huazhong Agricultural University (Wuhan, Hubei, China).

Parasites
B. microti ATCC PRA-99™ strain was provided by the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Shanghai, China) and preserved in liquid nitrogen with the additive of dimethyl sulfoxide (DMSO) in the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, China.

Preparation of Genomic Deoxyribonucleic Acid
Blood sample was collected from the tail of BALB/c mouse into 1.5 ml centrifuge tubes containing EDTA-K2 (Sigma, Shanghai, China) at day 7 post-infection. Genomic DNA (gDNA) of B. microti was extracted by using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction and stored at −20°C until usage.

Construction of Recombinant Plasmid, Protein Expression, and Purification
Primer pairs with the BamHI and XhoI restriction sites were designed based on the full-length open reading frame (ORF) of BmLDH obtained from the GenBank database under the accession number MN102392 (Table 1). The PCR thermo-cycling was done in a 50 µl reaction volume including 50 ng gDNA, 0.2 µM primers, 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 10 µl 5 × TransStart® KD Plus Buffer, and 1 U TransStart® KD Plus DNA Polymerase (TransGen Biotech, Beijing, China). The PCR reaction was performed at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 68°C for 1 min, and finally at 68°C for 10 min. The resulting PCR product was purified by using EasyPure® PCR Purification Kit (TransGEN, Beijing, China), and then cloned into a PET-28a expression vector. The plasmid construct was confirmed by DNA sequencing.

The sequence-correct recombinant plasmid was transformed into the Escherichia coli BL21 expressing host cells. The transformed cells were cultured at 37°C in LB medium containing 0.1 mg/ml (1:1000 dilution) kanamycin for 3 h. When the culture density reached an optical density of 0.6 to 0.8 at 600 nm (OD600), induction was performed with 0.8 mM IPTG (Biosharp, Anhui, China), and the growth of cells continued for additional 12 h at 28°C before harvesting.

For protein purification, the induced cells were harvested by centrifugation at 7,000 rpm for 10 min in a high-speed refrigerated centrifuge (Hitachi, Tokyo, Japan), followed by suspending with His binding buffer (300 mM NaCl, 10 mM Tris-
base, 50 mM NaH₂PO₄·2H₂O, 10 mM imidazole, pH7.5) and lysis by passage through an high-pressure homogenizer at 1,000 bar. After centrifugation at 10,000 rpm/min for 10 min, the supernatant was filtered through a 0.45-um-pore-size filter and loaded onto Ni Sepharose™ High Performance affinity matrix (GE Healthcare, Uppsala, Sweden). The proteins were eluted gradiently with elution buffer (20–400 mM imidazole) and stored at –80°C. His tag on the N-terminus was not cleaved.

**Western Blot Analysis**

According to the standard method, the purified rBmLDH and *B. microti* lysates were separately subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto a nitrocellulose (NC) membrane. Next, the Western blot membranes were blocked with 0.05% Tween 20 in TBS (TBST) plus 1% BSA for 8 h at 4°C. After five ilk twice with TBST, the NC membranes were separately probed by using *B. microti*-infected positive serum, anti-BmLDH monoclonal antibody (McAb), and specific pathogen-free (SPF) mouse serum (1:200, diluted with TBST) at 37°C for 1 h. Then, the NC membranes were washed five times with TBST, followed by incubation at 37°C for 1 h with horseradish peroxidase (HRP) labeled goat anti-mouse IgG secondary antibody diluted with TBST, the NC membranes were separately probed by using HRP-labeled goat anti-mouse IgG secondary antibody diluted with TBST (1:1,000). After washing five times, the NC membranes were visualized using the ECL method.

**Enzyme Inhibition Analysis**

The enzyme activity of rBmLDH was analyzed using Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, Shanghai, China) according to the manufacturer’s instruction. Briefly, the enzymatic activity of BmLDH was detected by catalyzing the conversion of lactate to pyruvate, with concomitant reduction of enzymatic activity of BmLDH was detected by catalyzing the coupled reaction was followed by conversion of lactate to pyruvate, with concomitant reduction of enzymatic activity of BmLDH and stored at –80°C. His tag on the N-terminus was not cleaved.

**TABLE 2** | In vitro evaluation of three naphthalene-based compounds against *Babesia microti* at the asexual blood stage, cytotoxicity in Vero cells, and selectivity index (SI).

| Structure | Compound ID | BmLDH IC₅₀ (mM) | PfLDH IC₅₀ (mM) | HmLDH IC₅₀ (mM) | PRA-99 IC₅₀ (mM) | CC₅₀ Vero (mM) | SI |
|-----------|-------------|-----------------|-----------------|-----------------|-----------------|----------------|----|
| ![Structure](image) 2,6-Naphthalenedicarboxylic acid (NDCA) | > 0.5 | 5.1* | 1.4* | – | – | – | – |
| ![Structure](image) 1,6-Dibromo-2-hydroxynaphthalene 3-carboxylic acid (DBHCA) | 0.054 | 0.31* | 5.9 | 0.085 | 0.217 | 2.6 |
| ![Structure](image) 3,5-Dihydroxy 2-napthoic acid (DHNA) | 0.030 | 1.7* | 150 | 0.086 | 1.9 | 22.1 |

*These values were acquired from R. Conners et al., 2005. BmLDH, Babesia microti lactate dehydrogenase; PfLDH, Plasmodium falciparum lactate dehydrogenase; HmLDH, human lactate dehydrogenase; PRA-99, *B. microti* ATCC PRA-99™ strain; IC₅₀, half-maximal inhibitory concentration; CC₅₀, half-maximal cytotoxic concentration.

were purchased from Sigma-Aldrich (Shanghai, China) with 99% purity for NDCA and 97% purity for DBHCA and DHNA. In the inhibitory analysis, three naphthalene-based compounds were prepared as 100 mM stock solution in 100% dimethylsulfoxide (DMSO) (Table 2). The percentage inhibition of BmLDH was determined separately at various concentrations (50–500 μM). As a positive control, the phenolic aldehyde gossypol at 0.7 µM was used to confirm the validity of the experimental results (Vudrîko et al., 2014). The concentrations of the two compounds that inhibited NADH production by 50% (IC₅₀ values) were calculated separately using GraphPad prism5. The optimal additive amount of rBmLDH was 100 ng. The final concentrations of DMSO did not influence the BmLDH activity as determined in a preliminary experiment and all the experiments were repeated three times.

**Surface Plasmon Resonance Assays**

The binding properties of the rBmLDH against DBHCA and DHNA were measured separately by SPR experiment with a Biacore T200 system (GE Healthcare, Uppsala, Sweden). Briefly, a sensor chip CM5 was put into a Biacore T200 system and washed with the running buffer (PBST+0.5% DMSO, pH7.5). The CM5 chip was activated by injecting the mixture of 0.1 M N-hydroxsuccinimide ( NHS) and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (1:1, v/v) at a flow rate of 10 μl·min⁻¹ for 20 min. Then, the rBmLDH diluted to 10 μg·ml⁻¹ with 10 mM sodium acetate buffer (pH 4.0, 4.5, 5.0, and 5.5) was covalently immobilized on the CM5 chip at a flow rate of 10 μl·min⁻¹ until the immobilization level of BmLDH reached 2,100 resonance units (RU). Ethanolamine solution was injected to block the remaining unreacted esters. Flow cell 1 was activated and blocked as the reference surface, while flow cell 2 served as the experimental surface. The 100 mM stocks of the two small molecule compounds were further diluted with PBST into 500, 250, 125, 62.5, 31.25, and 15.625 μM. The surface of CM5 sensor chip was fully regenerated using 10 mM glycine pH2.5 at a flow rate of 30 μl min⁻¹ for 30 s. The SPR kinetic experiments were
performed three times and the values were presented as the mean ± SD of three independent experiments.

**Inhibitory Effect of Two Naphthalene-Based Compounds Against the Growth of Babesia microti In Vitro**

Three BALB/c mice were intraperitoneally injected with 1 x 10⁷ parasites (100 µl fresh blood), and their blood was collected from the tail when parasitemia reached ~60% at day 7 post-infection. The growth inhibition assay of *B. microti in vitro* was performed by incubating freshly isolated infected mouse red blood cells (RBCs) with varying concentrations of the drugs. Briefly, 25 µl infected mouse RBCs was diluted with 5 µl non-infected mouse RBCs and 10 µl non-infected human RBCs to achieve ~3% parasitemia in a total of 40 µl and suspended in 110 µl of a growth medium (20% bovine serum and 80% medium HL20) containing the indicated concentrations of drugs (50, 100, and 250 µM). The cultures were maintained at 37°C for 72 h in a gas mixture of 2% O₂, 5% CO₂, and 93% N₂. Two naphthalene-based compounds DBHCA and DHNA were prepared as 100 mM stock solutions in DMSO and further diluted with growth medium. Each concentration of the compounds was tested in triplicate, while three wells received a drug-free culture medium as a control. 10 µM diminazene aceturate (DA) was selected as a positive control. Parasitemia was determined at 72 h post-treatment by enumerating a total of at least 1,000 RBCs in a Giemsa-stained thin smear. Differences in the percent parasitemia were statistically analyzed using one-way ANOVA analysis, and the IC₅₀ values were calculated manually for obtaining more reliable data (Käber method). The hemolysis of DBHCA and DHNA against *B. microti*-free erythrocytes was monitored by cell counting chamber at 72 h post-treatment. All the experiments were separately repeated three times.

**Cytotoxicity Test by the 3-((4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Method**

Mycoplasma-free Vero cell line (ATCC-CCL-81, immortalized cells) was preserved in liquid nitrogen with the additive of DMSO in the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, China. The cytotoxicities of DBHCA and DHNA were determined in the kidney cells of the African green monkey (Vero cells) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method according to a previous report (Pobsuk et al., 2019). Briefly, Vero cells (100 µl, 5,000 cells/well) were cultured in Dulbecco’s modified Eagle medium (DMEM) medium containing 10% fetal bovine serum (FBS) and 100 U/ml Penicillin-Streptomycin at 37°C with 5% CO₂ for 24 h and then with different concentrations of DBHCA or DHNA for 72 h. After adding 10 µl MTT (5 mg/ml in medium) into each test well, the plate was further incubated for another 2 h, followed by removing the culture from the incubator and discarding the medium. The resulting formazan crystals were dissolved by adding 100 µl of MTT solubilization solution (10% Triton X-100 plus and 0.1 N HCl in anhydrous isopropanol). The plate was gently mixed on a gyratory shaker for 10 min and the absorbances were measured at a wavelength of 570 nm using a microplate reader (BioTek, Vermont, USA). The background absorbances of the multi-well plate at 690 nm were subtracted from the 570 nm measurement. The DBHCA and DHNA compounds were prepared as 100 mM and 1 M of the stock solutions in 100% DMSO. In these tests, 3 µM of doxorubicin and 0.5% DMSO were used as the positive control and negative control, respectively. The CC₅₀ values were calculated using GraphPad Prism 5 (San Diego, CA, USA), and all the cytotoxicity tests were performed three times.

**RESULTS**

**Molecular Models of the Compounds 2,6-Naphthalenedicarboxylic Acid, 1,6-Dibromo-2-Hydroxynaphthalene 3-Carboxylic Acid, and 3,5-Dihydroxy 2-Napthoic Acid With Babesia microti Lactate Dehydrogenase**

Our previous study based on the crystal structures of BmLDH indicated that the mutation of residue Arg99 to Ala significantly reduced the enzyme activity of BmLDH (up to 86%), but had no impact on Human LDH-A (Yu et al., 2019). For testing the probability of BmLDH as a selective drug target, the potent inhibitors interacting with the residue Arg99 were screened via a molecular docking based on the crystal structure of BmLDH from a series of gossypol derivatives or structural analogs. In CDOCKER analysis, these compounds were located by DS software for simulating the binding of ligands, and three ligands NDCA, DBHCA, and DHNA interacting with the Arg99 of BmLDH while inhibiting human LDH with a high IC₅₀ value were selected for subsequent tests (Figures 1A–C).

**Cloning Babesia microti Lactate Dehydrogenase Gene and Expression of the Recombinant Babesia microti Lactate Dehydrogenase**

The open reading frame (ORF) sequence of BmLDH obtained from *B. microti* gDNA by PCR had a full length 999 bp (Figure 2). The intact ORF encoding BmLDH was expressed in *E. coli* BL21 (DE3) and the size of recombinant BmLDH (rBmLDH) was ~37 kDa (Figure 3, lane 3). The rBmLDH with the N-terminal His-tag was purified by Ni sepharose for subsequent enzyme activity inhibitory analysis (Figure 3, lane 4).

**Recombinant Babesia microti Lactate Dehydrogenase Against Babesia microti Infected Serum and Mouse Negative Serum**

To verify that the rBmLDH expressed in *E. coli* BL21 was precisely encoded by the ORF of BmLDH, an immunoblotting analysis was performed in this study. The Western blot analysis result indicated that the rBmLDH differentiated between *B. microti* positive and
negative sera from BALB/c mouse. A ~37 kDa band agreeing with the rBmLDH size was revealed by using *B. microti* infected mouse positive serum, and no signal appeared with SPF serum as negative control (Figure 4A). Furthermore, the monoclonal antibody
against BmLDH recognized the native BmLDH (~37 kDa) in extracts from B. microti-infected mouse RBCs, but not in the control (uninfected mouse erythrocytes) (Figure 4B).

Inhibition of Babesia microti Lactate Dehydrogenase Activity

For finding novel BmLDH inhibitors, three naphthalene-based compounds (NDCA, DBHCA, and DHNA) were used to explore their inhibitory effects against BmLDH. The results revealed that the rBmLDH catalyzed the conversion of lactate and pyruvate, and the catalysis was moderately inhibited by the two naphthalene-based compounds at micromolar concentrations. The positive control 0.7 mM gossypol ~100% inhibited the catalytic activity of BmLDH (Figures 5A–C). The IC_{50} values of DBHCA and DHNA were determined as 53.89 ± 13.28 and 30.19 ± 8.49 μM, respectively (Figures 5B, C). However, the NDCA showed no inhibitory effect on the BmLDH activity, even at the high concentration of 500 μM (Figure 5A).

Binding Kinetics of Babesia microti Lactate Dehydrogenase

For binding kinetic assays, standard solutions of two naphthalene-based compounds (DBHCA and DHNA) at different concentrations were tested and the data were fitted.
with a 1:1 binding kinetic model. The SPR kinetic sensorgrams were presented in Figure 6 and the binding kinetic values of DBHCA and DHNA against BmLDH were shown in Table 3. Comparatively, DHNA showed a lower $K_D$ value to BmLDH ($3.766 \times 10^{-5} \text{M}$), but DBHCA exhibited a higher value with BmLDH ($3.988 \times 10^{-8} \text{M}$). A comparison of kinetic parameters ($k_a$ and $k_d$) indicated that DBHCA bound the target faster than the DHNA, while the complex of DHNA with the target dissociated slower than that of DBHCA.

### Two Lactate Dehydrogenase Inhibitors Interfere With the Growth of Babesia microti In Vitro

We further tested the effect of DBHCA and DHNA on the in vitro cultivation of B. microti. Parasitemia was counted at 72 h post-treatment by microscopy. The positive control, 10 $\mu$M DA inhibited the growth of the parasite by ~75.54%. Compared to the control group, the two inhibitors exhibited anti-B. microti activity at a low micromolar concentration (Figures 7A, C). The IC$_{50}$ values of DBHCA and DHNA were calculated as $84.83 \pm 6.96$ and $85.65 \pm 7.23 \mu$M, respectively. Both DBHCA and DHNA showed no cellular hemolysis at drug levels up to 250 $\mu$M (Figures 7B, D).

### Mammalian Cytotoxicity of the Two Naphthalene-Based Compounds

The above results showed that the compounds DBHCA and DHNA could inhibit both rBmLDH catalysis and the growth of B. microti in vitro, but their cytotoxic risk has not yet been evaluated. In this study, the cytotoxicity of DBHCA and DHNA was assessed in Vero cells using the MTT based method. The cytotoxicity test results indicated that the compound DBHCA with a selectivity indexes (SI) of 2.6 had a stronger cytotoxic activity against Vero cells, and its half-maximal cytotoxic concentration (CC$_{50}$) value (50% cytotoxicity concentration) was $216.5 \pm 18.03 \mu$M (Figure 8A), while the compound DHNA with a SI of 22.1 had a lower cytotoxic effect on Vero

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**FIGURE 6** | Surface plasmon resonance (SPR) kinetic sensorgrams. Two naphthoic acid compounds 1,6-dibromo-2-hydroxynapthalene 3-carboxylic acid (DBHCA) (A) and 3,5-dihydroxy 2-napthoic acid (DHNA) (B) bind to Babesia microti lactate dehydrogenase (BmLDH) at different concentrations (500, 250, 125, 62.5, 31.25, and 15.625 $\mu$M), respectively. The responses were reference subtracted and blank deducted.

**TABLE 3** | Binding kinetic characterization of the two naphthoic acids.

| Ligand | Compounds | $k_a$ (1/Ms) | $k_d$ (1/s) | $K_D$ (M) |
|-------|-----------|-------------|-------------|-----------|
| BmLDH | 1,6-Dibromo-2-hydroxynapthalene 3-carboxylic acid (DBHCA) | $3.766 \times 10^4 \pm 8.57 \times 10^2$ | $1.502 \times 10^{-3} \pm 1.76 \times 10^{-5}$ | $3.988 \times 10^{-8} \pm 1.42 \times 10^{-9}$ |
| BmLDH | 3,5-Dihydroxy 2-napthoic acid (DHNA) | $20.49 \pm 4.18 \times 10^{-1}$ | $7.718 \times 10^{-4} \pm 6.55 \times 10^{-6}$ | $3.766 \times 10^{-5} \pm 1.96 \times 10^{-6}$ |

**Yu et al. Activity Evaluation of Naphthalene-Based Compounds**
cells, and its CC50 value was calculated as 1.9 ± 0.1 mM (Figure 8B). The positive control doxorubicin inhibited the growth of Vero cells by ~95% at 3 µM while the 0.5% DMSO as the negative control revealed no cytotoxicity against Vero cells.

**DISCUSSION**

Previous studies have shown that gossypol and its derivatives are non-selective drugs and interact with a great range of oxidoreductases, imposing restrictions on the usage of these drugs as effective medicaments for treatment of different parasitications by apicomplexan (Montamat et al., 1982; Deck et al., 1998; Razakantoanina et al., 2000). Gossypol and its derivatives and analogs have been reported to compete with co-factors in the nicotinamide binding site of PfLDH and display powerful effects on the enzyme, but the core of the gossypol structure exhibits weak inhibition (Conners et al., 2005). Herein, three naphthalene-based compounds (NDCA, DBHCA, and DHNA), the core of the gossypol structure, were used for exploring new BmLDH inhibitors and therapeutic drugs for babesiosis. Interestingly, rBmLDH activity was 100% inhibited by DHNA at ~60 µM, and the DHNA displayed ~5,000-fold selectivity over human LDH and ~57-fold selectivity over PfLDH (Table 2). Cytotoxicity tests demonstrated that the compounds DBHCA and DHNA had SI of 2.6 and 22.1 between *B. microti* and Vero cells. The results suggest that the DHNA could be a better candidate of lead compound for developing new anti-*Babesia* drug with high affinity and selectivity than that of the DBHCA.

In this study, we characterized the binding properties of DBHCA and DHNA against BmLDH by SPR experiments. DBHCA was identified as a chemical compound with a feature of rapid combination and dissociation, whose association ($k_a$) and dissociation ($k_d$) rates were $3.766 \times 10^4$ (1/Ms) and $1.502 \times 10^{-3}$ (1/s), respectively. As previously reported, an ideal medicine is supposed to bind its target with fast association and slow dissociation, which means the rapid dissociation of drug represents a safer drug selection for human or animal babesiosis, while a too fast dissociation rate could be deleterious to the medical treatment (Jiao et al., 2018). For this
reason, we suggest that the compound DBHCA could be improved and optimized by suitably reducing its dissociation rate. Conversely, the DHNA compound revealed a feature of slow association and dissociation and its $k_a$ and $k_d$ values are 20.49 (1/Ms) and $7.718 \times 10^{-4}$ (1/s). As slow association could delay the onset of the drug and frequently cause the deterioration of disease, an effort should be made to improve the association rate ($k_a$) of DHNA. Therefore, the renewed structure-based development of DBHCA and NDNA might enable them to serve as new anti- Babesia drugs, especially the low cytotoxic compound NDNA. Available data show that the compound FX11, a derivative of gossypol, selectively inhibit the human LDH-A ($K_i = 8$ μM), but not the human LDH-B and glyceraldehyde-3-phosphate dehydrogenase, even at high concentrations (Deck et al., 1998; Rellinger et al., 2017). Currently, FX11 exhibits a preclinical efficacy for the treatment of cancers, including adult lymphoma cancer, pancreatic cancer and prostate cancer (Le et al., 2010).

In malaria parasites, the crystal structures of PfLDH complexed with a series naphthalene-based compounds, such as 2,6-naphthalenedicarboxylic acid, 2,6-naphthalene disulphonic acid, and 3,7-dihydroxy naphthalene-2-carboxylic acid have been resolved at high resolutions, and the complex structures revealed that the PfLDH could form the binding site.
for gossypol and its derivatives in two binding modes: one overlapping the substrate site but not the co-factor site, and the other bridging the binding sites for the co-factor and the substrate (Conners et al., 2005). Therefore, understanding the complex structures of BmLDH with naphthalene-based compounds could lay a structural basis for the design and development of novel babesial pharmaceuticals. To this end, we explored the complex crystal structures of BmLDH with DBHCA or DHNA in this study. Despite the success in obtaining the crystal of BmLDH apo form and solving the structure of BmLDH at a resolution of 2.79 Å (Hampton, California, USA), we failed to prepare the crystals complexed with DBHCA or DHNA using the soaking method.

It is worth noting that the naphthoic acid compounds, especially structure-based derivatives, are easily and economically accessible for artificial synthesis (Lu et al., 2017). The research on the naphthalene-based compounds or nucleophilic groups interacting with catalytic residues of the BmLDH would contribute to the discovery of new anti-Babesia drugs with higher efficiency and lower cost. At present, with the development of computer technology, the drug design has ushered in the era of virtual screening. Potential drugs can be predicted by computer virtual screening, molecular docking, and molecular dynamics simulation on the interactions between drug candidates and their target calculated affinities (Saxena et al., 2018; Brandao et al., 2018). Our subsequent study will focus on the virtual screening of drugs based on the pharmacophore of these compounds to discover new LDH inhibitors and specific medicines.

As the inhibitory effects of both DBHCA and DHNA are not very strong, a renewed structure-based development needs to be performed. Future study will focus on the renewed structure-based on DHNA to improve the affinity of DHNA to BmLDH. The preliminary structural modification for the compound DHNA has been finished, and a compound library will be tested by SPR experiment to verify the modification effect. On the other hand, since both DBHCA and DHNA have the same target, they may have a synergistic interaction and the combination therapy based on both compounds could be another future scope.

CONCLUSION

In conclusion, two naphthalene-based compounds DBHCA and DHNA were identified to target BmLDH and inhibit both the enzyme activity of BmLDH and the growth of B. microti in vitro. SPR analysis offered more novel insights into the binding properties (association and dissociation) between BmLDH and the two compounds. Additionally, cytotoxicity tests of DBHCA and DHNA in Vero cell line further demonstrated that DHNA has a higher selectivity index than DBHCA between B. microti and Vero cells. These findings provide some theoretical basis for renewed structure-based development of the two naphthalene-based compounds as novel anti-Babesia agents for treatment of human babesiosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of the article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

LY, LH, and JZ designed the study and wrote the draft of the manuscript. LY, XZ, QL, YS, ML, YZ, XA, and YT performed the experiments and analyzed the results. All authors have read and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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