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Helicobacter pylori Dihydroorotate Dehydrogenase: a Promising Target for Screening Potential Drug Candidates

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ABSTRACT

Background: De novo pyrimidine biosynthesis is essential for the survival of all living organisms. DHODH catalyzes the fourth step in this pathway. Inhibition of DHODH induces pyrimidine depletion and effectively eradicates microorganisms like *H. pylori*, which lacks the pyrimidine salvage pathway. Herein, we expressed rHp-DHODH, characterized its enzymatic activity, kinetics, and stability, and subsequently evaluated its inhibition by HQNO.

Methods: The gene fragment encoding the rHp-DHODH protein was synthesized, subcloned, and expressed in soluble form. The recombinant protein was purified, its identity was confirmed, and its activity was measured using a colorimetric reduction assay. Kinetic parameters and the effects of pH, temperature, and incubation time on the enzymatic activity were investigated. The inhibitory effect of HQNO on rHp-DHODH was evaluated using the DCIP reduction assay. The MIC of HQNO against *H. pylori* was determined, and its bactericidal/bacteriostatic effect was assessed.

Results: Optimal soluble expression of rHp-DHODH was achieved in *BL21(DE3)pLysS*. The enzyme exhibited a specific activity of 5.7 U/mg. Maximum activity was observed at pH 8.0 in Tris-HCl buffer at 25 °C. The K_m values were 39.75 µM for DHO and 5.37 µM for CoQ10, with a k_{cat} of 3.82 s^{-1}. The IC_{50} of HQNO against the recombinant enzyme was determined as 1.75 µM. HQNO inhibited *H. pylori* growth with an MIC of 0.5-1.0 µg/mL, displaying concentration-dependent bacteriostatic to bactericidal effects.

Conclusions: The rHp-DHODH and its optimized enzymatic assay provide a reliable platform for screening candidate inhibitors, such as HQNO, advancing drug development efforts against *H. pylori* infection.

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INTRODUCTION

Helicobacter pylori infects over half of the global population, posing a significant public health concern. It is commonly associated with several gastric pathologies, including inflammation, gastroduodenal ulcers, and gastric cancer[1,2]. Therefore, early detection and effective eradication are crucial to prevent the spread of infection and its associated complications.

The standard triple therapy regimen for *H. pylori* infection includes the combination of antibiotics, such as amoxicillin, clarithromycin, and metronidazole, along with acid-suppressing agents like proton pump inhibitors. However, the effectiveness of these antibiotics has been declining due to the rise in antibiotic

List of Abbreviations:
CoQ10: coenzyme Q10; **DHO:** dihydroorotate; **DHODH:** dihydroorotate dehydrogenase; **FMN:** flavin mononucleotide; ***H. pylori***: *Helicobacter pylori*; **HQNO:** 2-Heptyl-4-hydroxyquinoline N-oxide; **ITV:** intervenolin; **K_m:** michaelis-Menten constant; **MDR:** multidrug-resistant; **MIC:** minimum inhibitory concentration; **rHp-DHODH:** recombinant *H. pylori* DHODH

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resistance, specifically to clarithromycin and metronidazole[3,4]. As a result of rising antibiotic resistance, targeting essential bacterial metabolic pathways provides a promising approach. One such important pathway is pyrimidine biosynthesis, which is a vital biological process providing nucleotide precursors for RNA, DNA, glycoprotein, and membrane phospholipid synthesis in all living organisms[5]. These precursors are supplied through the de novo biosynthesis and salvage pathways[5]. While most organisms possess both pathways, certain human pathogens, including *H. pylori*[6,7], *Plasmodium falciparum*[6,8], and *Schistosoma mansoni*[6] lack the pyrimidine salvage pathway and rely exclusively on the de novo pyrimidine biosynthesis for growth and survival. Since the pyrimidine salvage pathway is present in humans and other bacterial populations, these organisms may exhibit greater resistance to certain treatments. Therefore, targeting the de novo pathway is less likely to adversely affect the host or its microbiome. Thus, inhibiting one or more enzyme(s) in the de novo pyrimidine biosynthesis pathway could be an outstandingly effective approach to eradicate *H. pylori* infection, without affecting the human host or its natural microbiome.

DHODH is a crucial enzyme that catalyzes the fourth step of the de novo pyrimidine biosynthesis pathway, converting DHO to orotate with the concomitant reduction of the liposoluble quinone to quinol[9] (Fig. S1). DHODH proteins are categorized into two groups based on their cellular localization, sequence similarity, and substrate preferences. Class 1 DHODH enzymes are typically found in the cytosol and have a more complex structure than class 2 DHODH enzymes, often comprising multiple domains or subunits. They are commonly found in Gram-positive bacteria such as *Streptococcus mutans*[10] and *Bacillus subtilis*[11], parasites such as *Leishmania major*[12] and *Trypanosoma spp.*[13], as well as *Saccharomyces cerevisiae*[14].

Class 2 DHODH enzymes are located in the inner membrane of eukaryotic mitochondria or prokaryotic plasma membranes[9], using respiratory quinones as electron acceptors[15]. This class of DHODH is present in various organisms, including humans[15], mammals[16], plants[17], insects[18], many fungi[19], and *H. pylori*[7]. HQNO, a natural quinone analog synthesized by *P. aeruginosa*[20], competitively inhibits ubiquinone binding to DHODH[21]. HQNO resembles ubiquinone, permitting it to bind to the quinol oxidases available in the bacterial electron transportation chain[22].

In this study, we successfully expressed the recombinant form of *H. pylori* DHODH and optimized its expression to maximize both yield and solubility. The specific activity and kinetic parameters (K_m and k_{cat}) of the enzyme were also determined. Additionally, we assessed its stability under varying conditions of pH, temperature, and storage time. Furthermore, by investigating the inhibitory effects of HQNO on both recombinant DHODH activity and *H. pylori* growth, we demonstrated a promising strategy for the selective targeting of *H. pylori* metabolism.

MATERIALS AND METHODS

Cloning and transformation

N-terminal (transmembrane region)-truncated gene, including 23 amino acids)-truncated gene encoding *H. pylori* DHODH (Uniprot accession number B5Z6I2) was synthesized and inserted into the pET-28a vector, via the NcoI and XhoI restriction sites. The construct was transformed into *E. coli* TOP10F' competent cells. The grown colonies were subsequently screened by colony PCR, using the T7 universal and DHODH-specific primer sets (F: 5'-ccatggggcatatgctttattcattagt aa-3' and R: 5'- ctcgagggtaccttaatgatgatgatgatgatgtc-3'). Colonies were also screened by restriction digestion for the presence of the DHODH gene fragment and its correct direction using NdeI/KpnI and XhoI/NcoI restriction enzymes.

Protein expression and optimization

The expression of rHp-DHODH was thoroughly evaluated under different conditions. The studied factors included the type of expression host (*SHuffle® T7*, *BL21-DE3*, and *BL21-DE3-pLysS*), the temperature of induction (16 °C, 25 °C, and 37 °C), the post-induction time (4, 5, and 16 hours), and IPTG concentration (0.25, 0.5, and 1 mM). All expression evaluations were visually assessed using SDS-PAGE and quantitatively analyzed using the ImageJ software 1.46r. In the next step, the rHp-DHODH protein was expressed in the selected host under the optimized culture condition. The culture medium was then centrifuged at 4300 ×g for 20 min, and the bacterial pellet was stored at -70 °C until further processing.

Protein purification

All protein purification steps were carried out at 4 °C unless otherwise specified. Nickel affinity chromatography was used for the purification of rHp-DHODH. The collected bacterial pellet was resuspended in 10 mL lysis buffer (500 mM NaCl, 50 mM NaH_2PO_4, and 10 mM imidazole; pH 8.0). The suspension was then disrupted by sonication (70% intensity, 20-second pulses with 10-second intervals) for 20 minutes. Insoluble cell debris was removed by

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centrifugation (15,700 ×g, at 4 °C, 30 minutes), and the supernatant was carefully collected. The supernatant was loaded onto the chromatography column, which was equilibrated with 10 mL of binding buffer (500 mM NaCl, 50 mM NaH_2PO_4, and 10 mM imidazole; pH 8.0). After washing the resin with 10 mL of wash buffer (500 mM NaCl, 50 mM NaH_2PO_4, and 20 mM imidazole; pH 8.0), the protein was eluted using the elution buffer (500 mM NaCl, 50 mM NaH_2PO_4, and 250 mM imidazole; pH 8.0). The purified rHp-DHODH was immediately buffer-exchanged against 20 mM Tris-HCl, 120 mM NaCl, and 20% (v/v) glycerol at pH 7.5, using a 10 kDa centrifugal filter unit (Amicon, Merck, Germany) to remove imidazole. The protein concentration of the elution samples was determined by bicinchoninic acid assay. Each fraction was assessed by SDS-PAGE, Western blotting using anti-histidine tag antibody (Sigma, USA), and enzymatic assay. The enzyme purity was quantified using the ImageJ program. The recombinant protein was stored at -70 °C for further analysis.

Enzyme activity assay

All assays were performed with the histidine-tagged recombinant enzyme, without cleavage of the tag. A substrate reduction assay was conducted using DCIP (Merck). Reactions were carried out in assay buffer (50 mM Tris-HCl, 150 mM KCl, 0.1% Triton X-100; pH 8.0) mixed with rHp-DHODH (0.125 µM) and dispensed into 96-well microplates. Following a 10-minute incubation to reach ambient temperature, the reactions were initiated by adding equal volumes of the freshly prepared substrate mixture (1 mM L-dihydroorotic acid (Sigma), 0.05 mM decylubiquinone, and 0.18 mM DCIP). Changes in absorbance at OD_{600} were recorded every 30 seconds, using a microplate spectrophotometer (Biotek, USA), over a period of five minutes. Samples were run in duplicates, and the mean ± SD are presented.

Specific activity calculation

Specific activity of the recombinant enzyme was calculated and expressed in units/mg, where one unit is defined as the amount of enzyme required to catalyze the reduction of 1 µmol of DCIP per minute. The specific activity was calculated using the following formula[23]:

$$\text{Specific activity}\left(\frac{U}{\text{mg}}\right) = \frac{enzyme\ activity}{amount\ of\ enzyme} = \frac{\text{Adjusted } V_{max}\left(\frac{\text{OD}}{\text{min}}\right) * (-1) * \text{well volume (L)} * 10^{12}\left(\frac{pmol}{mol}\right)}{\varepsilon\,(M^{-1}c\ m^{-1})\ x\ path\ correction\ (cm)\ x\ amount\ of\ enzyme\ (\mu g)}\ x\ 10^{-3}$$

Where the extinction coefficient (ε) for DCIP is 18,800 $M^{-1}cm^{-1}$, and the path correction is 0.57 cm.

K_m determination

Kinetic constants of the substrate were determined using the reduction assay by varying concentrations of DHO (6.25-750 µM) at a fixed concentration (50 µM) of Q10. Similarly, the K_m of the coenzyme was determined by varying concentrations of Q10 (3.125-50 µM) at a fixed concentration (1000 µM) of DHO. The initial velocity of the enzyme was measured within the first 30 seconds after the reaction commenced. The obtained data were fitted to the following equation: $v=\frac{V_{max}\,[S]}{K_m+[S]}$. Where K_m represents the substrate concentration that yields half-maximal velocity. V_{max} refers to the maximum velocity achieved when the enzyme is saturated with the substrate. The initial velocity within the first 30 seconds after the reaction has started, is denoted as v. [S] represents the substrate concentration. The turnover number (k_{cat}) was calculated using the formula: $k_{cat}=\frac{V_{max}}{[ET]}$. Where [ET] is the total enzyme concentration, based on one existing active site (monomer)[24].

pH-dependent activity

Initial velocities at saturating substrate concentrations (1 mM DHO and 0.05 mM decylubiquinone) were measured in different buffering systems (HEPES-HCl and Tris-HCl) over a pH range of 6.8 to 9.0, using the substrate reduction assay. Values in overlapping pH ranges were measured in two different buffers to exclude salt effects.

Temperature-dependent stability

To investigate the temperature-dependent stability of the rHp-DHODH, we conducted the reduction assay at two different temperatures (25 °C and 37 °C) immediately after the addition of the substrate (DHO) to the reaction mixture or after a 30-minute incubation of the recombinant enzyme-substrate mixture (without DHO) at different temperatures, before adding the substrate. The substrate mixture without enzyme was also incubated to evaluate the susceptibility of the substrate mixture to temperature.

Time-dependent stability

To assess the stability of rHp-DHODH over time, we measured its activity at 0, 1, 2, 4, 8, and 20 weeks after its purification and storage at -70 °C.

Sequence alignment

To compare the HQNO binding sites between *E. coli*[22] and *H. pylori*, we retrieved DHODH amino acid sequences from the UniProt database (https://www.uniprot.org). P0A7E1 (*E. coli-k12*), O25655 (*H. pylori*-26695), and B5Z6I2 (*H. pylori*-G27) sequences were aligned using CLUSTAL Omega (1.2.4) pairwise sequence alignment.

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Enzyme inhibition assay

The inhibitory effect of HQNO on the rHp-DHODH was evaluated using the substrate reduction assay. HQNO solution was added to the enzyme-containing mixtures at final concentrations ranging from 1.0 to 16.0 µM. Control reactions included wells with no recombinant protein or those without HQNO, where an equivalent volume of DMSO was added. After a 30-minute incubation at ambient temperature, the reactions were initiated by adding a freshly prepared substrate. The changes at 600 nm wavelength were monitored for five minutes at 30-second intervals.

MIC determination

The standard *H. pylori* 26695 strain and three clinical MDR isolates were used. The MDR strains were as follows: (1) Cla^R/LVX^R (MDR-1), (2) AMX^R/LVX^R (MDR-2), and (3) $Cla^R/AMX^R/LVX^R$ (MDR-3). These clinical strains were previously isolated and banked[25-27]. Bacterial strains were cultured on Brucella agar (Merck) plates supplemented with 10% defibrinated sheep blood, amphotericin B (8 mg/L), vancomycin (10 mg/L), and trimethoprim (5 mg/L). Plates were incubated under microaerobic conditions (10% CO_2, 5% O_2, 85% N_2) at 37 °C for 3-5 days[28,29]. The agar dilution method[30] was employed to evaluate the growth-inhibitory effect of HQNO, where HQNO was incorporated into the agar at varying concentrations. Pre-cultured *H. pylori* strains were harvested from agar plates and resuspended in sterile Brain Heart Infusion broth to an OD_{600} of 0.7 (equivalent to a 4 McFarland standard). Ten microliters of the diluted bacterial suspension were then plated. To serve as negative controls, the equivalent amounts of DMSO were also tested. After incubating the plates for 3-5 days, the MIC was designated as the lowest concentration of HQNO at which no visible bacterial growth was observed.

Bacteriostatic versus bactericidal properties

This assay was performed to evaluate the bacteriostatic/bactericidal effect of HQNO against *H. pylori*. The *H. pylori* 26695 strain was pre-cultured, and its cell density was adjusted to an OD_{600} of 0.4 in autoclaved Brucella broth medium supplemented with 0.2% sterile-filtered β-cyclodextrin, using T_{25} flasks. HQNO was tested at concentrations ranging from 0 to 4 µg/mL, while clarithromycin (0.25 µg/mL) was used as the standard bactericidal agent. Drug-free inoculated broth medium was considered as the negative control. The flasks were incubated under microaerophilic conditions at 37 °C, while shaking (120 rpm) for 72 hours. OD_{600} was measured at 24, 48, and 72 hours post-inoculation. To assess the viability and purity, 10 µL aliquots from each sample were plated at each time point. Data are presented as mean ± SD of duplicate measurements.

RESULTS

Cloning, expression and purification of recombinant DHODH protein

The presence of the rHp-DHODH expression cassette (Fig. S2) in TOP10F' colonies was verified by colony PCR using universal T7-specific primers (~1300 bp) and DHODH-specific primers (~1100 bp) (Fig. S3). Positive colonies were further confirmed by restriction digestion (data not shown). *SHuffle® T7 E. coli* cells were initially used for protein expression under different incubation times and IPTG concentrations at 16 °C. However, no significant protein expression was observed. By switching to *E. coli BL21(DE3)* host cells, protein expression at 16 °C, 25 °C, and 37 °C with 0.5 mM IPTG was tested, and no significant temperature-dependent differences were observed. rHp-DHODH expression at 37 °C for 5 hours reached 2.8% of the total protein, mostly forming insoluble inclusion bodies. To enhance the soluble expression of this potentially toxic protein, we employed the *E. coli BL21(DE3)pLysS* strain under the same conditions. Having done so, a significantly higher expression level (22%), representing 5.5% soluble protein, was achieved. These results indicate that *BL21(DE3)pLysS* is the optimal host cell for soluble expression of rHp-DHODH. The rHp-DHODH enzyme was purified from sonicated bacterial lysates using nickel affinity chromatography with an imidazole gradient. The eluted enzyme was immediately buffer-exchanged against a stabilizer buffer containing 20% (v/v) glycerol and stored at -70 °C until future use. SDS-PAGE analysis of the purified enzyme showed a single band at the expected molecular weight of approximately 40 kDa on SDS-PAGE (Fig. 1A) and immunoblotting (Fig. 1B). ImageJ software quantified the protein purity as 97.5%.

Enzyme specific activity and kinetics

The oxidation of DHO was assessed using the DCIP reduction assay with decylubiquinone (CoQ_{10}), as the electron acceptor. The rHp-DHODH displayed a specific activity of 5.7 U/mg. The kinetic analysis yielded K_m values of 39.75 µM for DHO (Fig. 2A) and 5.37 µM for Q_{10} (Fig. 2B). The calculated turnover number (k_{cat}) was 3.82 s^{-1}, while the catalytic efficiency (k_{cat}/K_m) was 0.096 $\mu M^{-1}s^{-1}$ for DHO and 0.711 $\mu M^{-1}s^{-1}$ for Q10.

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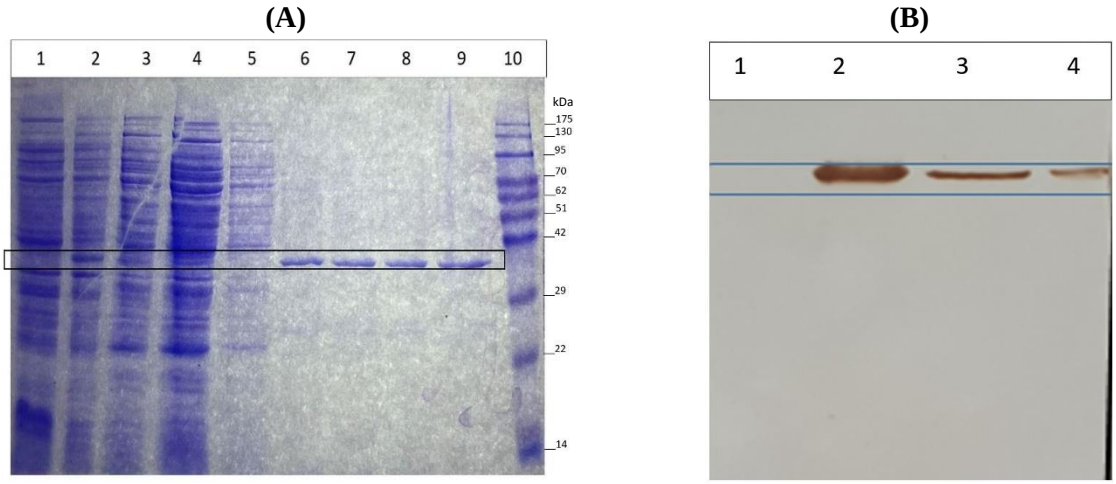

Fig. 1. Expression and purification of rHp-DHODH protein expressed in *BL21(DE3)pLysS*. (A) SDS-PAGE: lanes 1 and 2: bacterial lysates before and after induction, lane 3: initial sample for purification, lane 4: flow-through sample, lane 5: wash sample, lanes 6-9: eluted rHp-DHODH proteins, and lane 10: protein ladder; (B) Western blotting: lanes 1 and 2: bacterial cell lysates before and after induction, lane 3: initial sample for purification, and lane 4: eluted r-Hp-DHODH protein.

pH-dependent activity

As shown in Figure 3A, maximum activity of rHp-DHODH was demonstrated in Tris-HCl buffer at pH 8.0, which was higher than the obtained activity in the HEPES buffer (pH 6.8 or 8.0).

Temperature and time-dependent stability

The stability study was performed to evaluate the impact of temperature and incubation time on enzymatic activity (Fig. 3B). Immediate use of the enzyme at 25 °C represented the highest activity, while a slight decrease in activity was observed when the enzyme-substrate mixture was incubated at 25 °C for 30 minutes, before adding DHO. In contrast, incubation at 37 °C for 30 minutes significantly reduced the activity to 38%, indicating the sensitivity of the enzyme to higher temperatures. These findings suggest that rHp-DHODH is optimally active at 25 °C and prone to thermal inactivation at higher temperatures. The recombinant DHODH maintained full activity for at least eight weeks (Fig. 3C). However, after 20 weeks of storage at -70 °C, its activity decreased by approximately 50%.

Sequence alignment of *H. pylori* DHODH with its *E. coli* counterpart

According to the computational analysis performed by Horwitz et al.[22], HQNO inhibits *E. coli* DHODH activity through competitive inhibition at the ubiquinone binding site. To compare HQNO binding sites, we aligned *H. pylori*-26695 and *E. coli*-K12 DHODH amino acid sequences. Our analysis revealed that three of the four critical residues involved in HQNO binding (Pro_{15}, His_{19}, Arg_{102}, and Tyr_{318}) are conserved between *E. coli*-K12 and *H. pylori*-26695 DHODH proteins (Fig. 4), highlighting the similarity of their

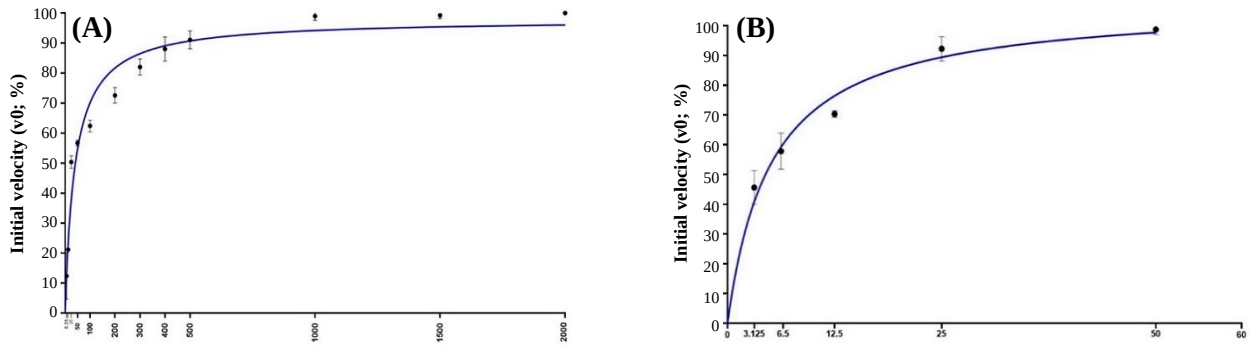

Fig. 2. Kinetic analysis of the rHp-DHODH. Initial velocities are shown as a function of varying (A) DHO concentrations, at a fixed concentration of Q10 (50 μM) and (B) Q10 concentrations at a fixed concentration of DHO (1000 μM). The curve was fitted to the Michaelis-Menten equation, $v = (V_{max} . [S])/(K_m + [S])$ using GraphPad Prism 8.

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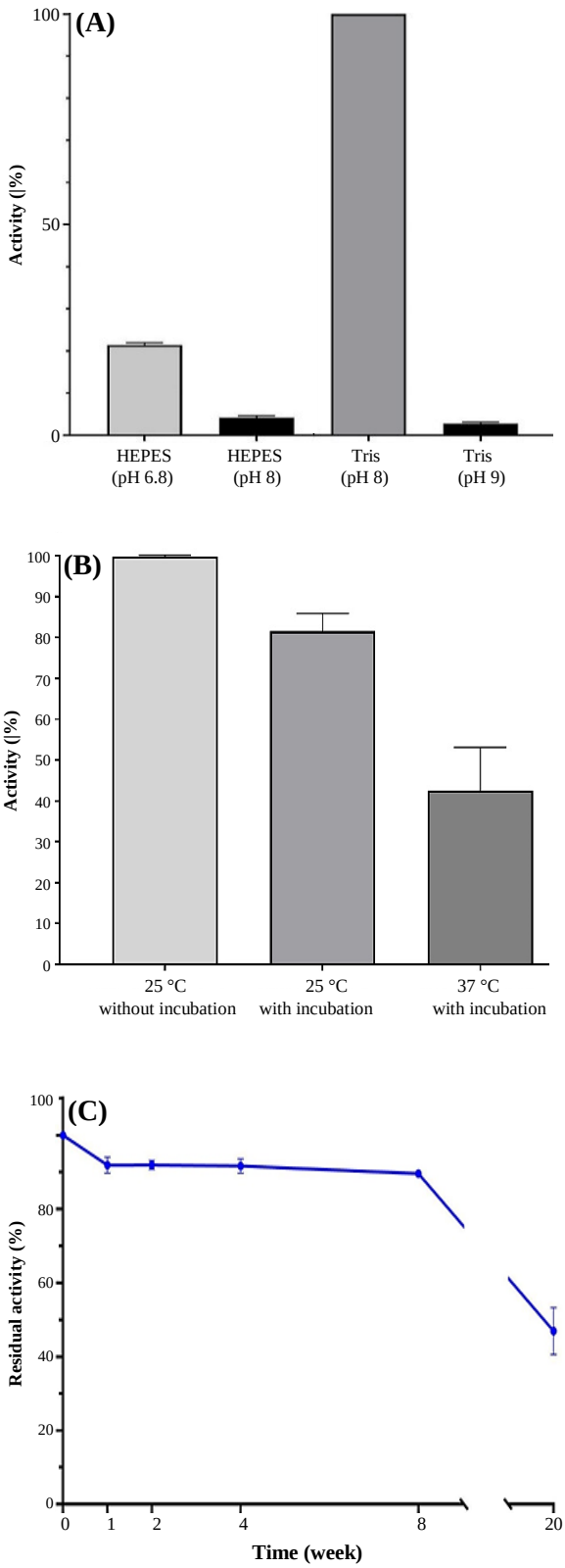

Fig. 3. (A) pH-dependent activity, (B) temperature-dependent stability, and (C) time-dependent stability of rHp-DHODH. Results are presented as mean ± SD of duplicate samples. The graphs were created using GraphPad Prism 8.

binding sites. Similar results were obtained by aligning the amino acid sequence of *E. coli*-K12 DHODH with that of *H. pylori*-G25 (data not shown).

Enzyme inhibitory effect of HQNO

We assessed the inhibitory effect of HQNO on the enzymatic activity of rHp-DHODH. As a result, HQNO inhibited rHp-DHODH activity in a dose-dependent manner, with an IC_{50} of 1.75 µM (Fig. 5A).

Growth inhibitory effect of HQNO

The antibacterial activity of HQNO was evaluated against various *H. pylori* strains. The observed MIC values ranged from 0.5 to 1.0 µg/mL among both standard and MDR strains (Table 1). To determine the bacteriostatic/bactericidal effects of HQNO, we evaluated its growth inhibitory activity at various concentrations (0.25 to 4.0 µg/mL) and time points, with clarithromycin serving as a bactericidal control (0.25 µg/mL). Bacterial growth was monitored by measuring OD_{600} nm. As illustrated in Figure 5B, at all tested concentrations, HQNO similar to clarithromycin, inhibited bacterial growth compared to the drug-free control. At each time point, samples were plated to assess the viability of *H. pylori* colonies. Notably, HQNO at lower concentrations (0.25 to 2.0 µg/mL) allowed bacterial regrowth on antibiotic-free plates. However, at 4.0 µg/mL, similar to clarithromycin, it prevented growth on culture plates entirely, indicating a dose-dependent bacteriostatic to bactericidal effect.

DISCUSSION

H. pylori, a Gram-negative, microaerophilic bacterium, is associated with the development of gastritis, gastric ulcers, and gastric cancer[1,2]. In the face of rising antibiotic resistance, the urgent need to discover new drugs to treat *H. pylori* infection has become a critical priority[31]. DHODH is an essential enzyme in the de novo pyrimidine biosynthesis, playing a crucial role in the survival and proliferation of *H. pylori*. Inhibition of DHODH effectively disrupts the growth of *H. pylori* and contributes to combating antibiotic-resistant infections. Approximately 25 years ago, Copeland et al. were the first to successfully express recombinant Hp-DHODH[7]. However, their research did not delve into the biochemical or functional properties of the enzyme in detail. Herein, we have focused on optimizing rHp-DHODH expression, activity, kinetics, and stability to create a platform for evaluating candidate drugs targeting this enzyme. To achieve optimal rHp-DHODH expression, we first evaluated its expression across various *E. coli* hosts

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CLUSTAL O (1.2.4) pairwise sequence alignment

```
E.coli-K12        MYYPFVRKALFQLDPERAHEFTFQQLRRITGTPFEALVR-----QKVPAKPVNCMGLTFK   55
H.pylori-26695    MLYSLVKKYLFSLDAEDAHEKVCKILRMLSSSPFLCNLIDSQWGYQNPKLENEILGLHFP   60
                  * * :*:* **.** * *** . : ** ::.:** . :       : *    : :** *

E.coli-K12        NPLGLAAGLDKDGECIDALGAMGFGSIEIGTVTPRPQPGNDKPRLFRLVDAEGLINRMGF   115
H.pylori-26695    NPLGLAAGFDKNASMLRALMAFGFGYLEAGTLTNEAQVGNERPRLFRHIEEESLQNAMGF   120
                  ********:**:.. : ** *:*** :* **:* . * **::***** :: *.* * ***

E.coli-K12        NNLGVDNLVENV-KKAHYDGVLGINIGKNKDTPVEQGKDDYLICMEKIYAYAGYIAINIS   174
H.pylori-26695    NNYGAILGVRSFKRFAPYKTPIGINLGKNKHIEQAHALEDYKAVLSKCLNIGDYYTFNLS   180
                  ** *.   *... : * *.  :***:****.    :. :**   :.*    ..* ::*:*

E.coli-K12        SPNTPGLRTLQYGEALDDLLTAIKNKQNDLQAMHHKYVPIAVKIAPDLSEEELIQVADSL   234
H.pylori-26695    SPNTPNLRDLQNKAFVHELFCMAKE---------MTHKPLFLKIAPDLETDDMLEIVNSA   231
                  *****.** **    :.:*:   *:          .: *: :******. :::::::.:*

E.coli-K12        VRHNIDGVIATNTTLDRSLVQGMKNCDQTGGLSGRPLQLKSTEIIRRLSLELNGRLPIIG   294
H.pylori-26695    IGAGAHGIIATNTTIDKSLVFAPK---EMGGLSGKCLTKKSREIFKELAKAFFNKSVLVS   288
                  :  . .*:******:*:*** . *   : *****: *  ** **::.*:  : .:  ::.

E.coli-K12        VGGIDSVIAAREKIAAGASLVQIYSGFIFKGPPLIKEIVTHI------------------   336
H.pylori-26695    VGGISDAKEAYERIKMGASLLQIYSAFIYNGPNLCQNILKDLVKLLQKDGFLSVKEAIGA   348
                  ****...  * *:*  ****:****.**::** * ::*:..:

E.coli-K12        ---     336
H.pylori-26695    DLR     351
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Fig. 4. Amino acid sequence alignment of *E. coli*-K12 and *H. pylori*-26695 DHODH enzymes using CLUSTAL Omega pairwise sequence alignment. Conserved residues critical for HQNO binding are highlighted in green, while a corresponding dissimilar residue in *H. pylori* is shown in pink.

considering different parameters, including post-induction time, incubation temperature, and IPTG concentrations. Given the toxic effects of DHODH overexpression[32], we chose the *BL21(DE3)pLysS* strain, as it is capable of expressing soluble toxic proteins[33]. Optimal expression was obtained with 0.5 mM IPTG at 37 °C. To minimize protein aggregation, we limited the incubation time to five hours. Extending the incubation period led to growth termination, possibly due to the cytotoxicity associated with DHODH overexpression[32]. Additionally, we performed protein expression without supplementing exogenous FMN, as *E. coli* endogenously synthesizes sufficient levels of FMN to support the recombinant production of DHODH enzymes[7,34]. We then purified rHp-DHODH using one-step nickel affinity chromatography. To minimize loss of enzyme activity, we avoided vigorous washing[35]. Having taken this precaution, the purity of our eluted rHp-DHODH reached 97.5%. Immediately following purification, the protein was buffer-exchanged with a stabilizing solution, comprising 20 mM Tris-HCl, 120 mM NaCl, and 20% (v/v) glycerol at pH 7.5 for storage at -70 °C. In contrast, Copeland et al.[7] purified rHp-DHODH

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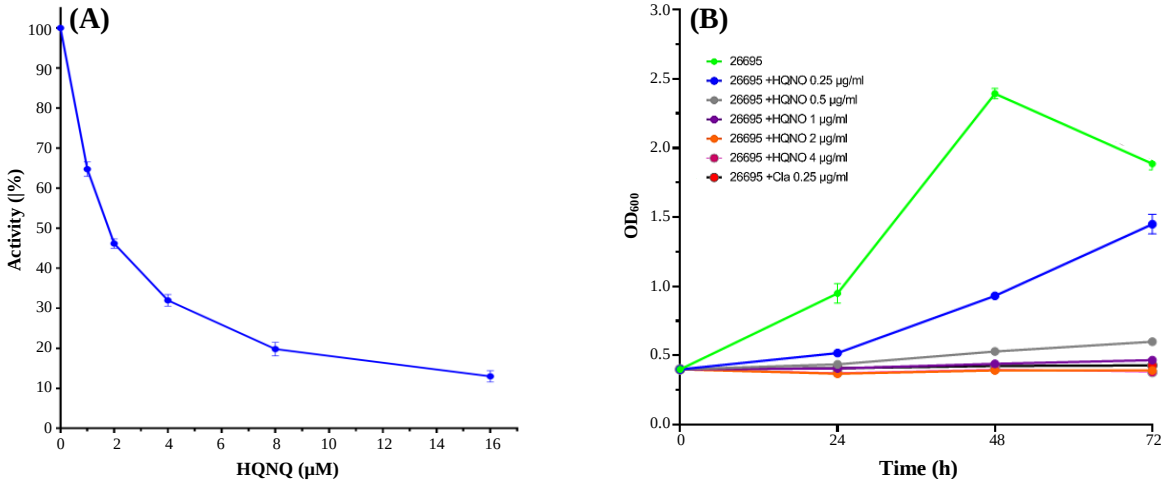

Fig. 5. The dose-dependent inhibitory effect of HQNO on (A) rHp-DHODH enzyme activity compared to the control and (B) on *H. pylori* growth, in comparison with clarithromycin (0.25 µg/mL). Data are presented as mean ± SD of duplicate samples.

using a combination of nickel affinity chromatography and S-200 gel filtration chromatography, achieving a comparable purity of over 90%. Their earlier work with recombinant human DHODH involved Matrex Green (an affinity chromatography) and ion-exchange chromatography (Q Sepharose HP column) for purification[34]. However, they observed reduced specific activity of the human enzyme when using this method, attributing this to dye leaching, which inhibited enzyme activity but could be mitigated through dialysis[34].

As the next step, we evaluated the enzyme activity of rHp-DHODH across a range of pH values using different buffer systems, as well as time and temperature. The recombinant enzyme displayed its highest activity in Tris-HCl buffer at pH 8.0, at an optimal temperature of 25 °C. These findings suggest that the specific ionic composition of the buffers plays a critical role in modulating the performance of the enzyme. Although rHp-DHODH showed peak activity at 25 °C, lower than *H. pylori*'s physiological temperature, this discrepancy can be attributed to in vitro assay limitations and the effects of recombinant expression in *E. coli*. Prior studies have also used 25 °C for DHODH enzymatic assays, supporting the validity of this experimental condition[7,34]. Accordingly, Copeland et al.[7] performed their assays at 25 °C in Tris buffer at pH 7.5. In their earlier work with recombinant human DHODH, they identified two key transition pKa values of 7.41 and 9.36[34]. These observations align with our findings, further supporting the conclusion that Tris-HCl buffer at pH 8.0 is optimal for rHp-DHODH enzymatic activity. In their study, Copeland et al.[7] noted that the His-tag on rHp-DHODH does not influence its kinetic constants, but the specific activity was not reported. We also retained the His-tag and determined the specific activity of the produced rHp-DHODH to be 5.7 U/mg, with K_m value of 39.75 µM for the substrate (DHO). Class 2 DHODH enzymes use a membrane-soluble quinone as their electron acceptor[15]. The specific quinone used under physiological conditions is largely dictated by its availability at the membrane. Most organisms typically produce only one type of quinone[36]. In our study, the obtained K_m value for the CoQ10 was 5.37 µM. However, Copeland et al.[7] provided K_m values for several other coenzymes such as Q6, Q0, menaquinone, and menadione, with values of 19, 5.3, 8.7, and 5.9 µM, respectively. Furthermore, we determined the turnover number (k_{cat}) of rHp-DHODH to be 3.82 s^{-1}. The k_{cat} represents the number of substrate molecules converted to product, per active site, per second, serving as a crucial metric for assessing the

Table 1. The MIC of HQNO on reference strain (26695) and three clinical MDR strains of *H. pylori*

H. pylori strains	MIC (µg/mL)			
	HQNO	Carithromycin[1]	Amoxicillin[2]	Levofloxacin[3]
26695	≤1.0	≤0.25	≤0.25	≤1.0
MDR-1	≤0.5	>16.0	≤1	>16.0
MDR-2	≤0.5	≤0.25	>2.0	>16.0
MDR-3	≤0.5	>16.0	>2.0	>16.0

R: resistance; [1]R > 0.5; [2]R > 0.125; [3]R > 1.0[30]

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efficiency of the enzyme in catalyzing reactions[37]. This value provides valuable insight into the catalytic efficiency of rHp-DHODH.

To evaluate the robustness of rHp-DHODH, we assessed its stability over time. The recombinant enzyme retained its activity for at least eight weeks, when stored at -70 °C in a stabilizing buffer. However, at 20 weeks post-production, its activity declined by up to 50%. This stability is comparable to that of the commercial recombinant human DHODH produced by R&D Systems[23], which reportedly remains stable for approximately 12 weeks when stored at -20 °C or -70 °C. This evaluation is essential for assessing the long-term applicability of rHp-DHODH in diverse contexts, including biochemical assays and drug evaluation studies.

DHODH class 2 inhibitors typically act by interfering with the interaction between CoQ and DHODH, thereby disrupting the electron transfer process essential for the catalytic activity of the enzyme[9]. Human DHODH inhibitors, such as Brequinar, Leflunomide, and Teriflunomide, demonstrate how disrupting the CoQ-DHODH interaction is a potent strategy for inhibiting pyrimidine synthesis[38-42]. In the case of *H. pylori* DHODH, two major classes of inhibitors have been identified, which are quinolone-based[7,43] and pyrazole-based compounds[44]. Both compound classes act as CoQ competitors, effectively blocking electron transfer by occupying the ubiquinone-binding site of the enzyme[7,43,44].

Agarwal and colleagues[45] analyzed the crystal structure of rHp-DHODH bound to FMN in order to confirm the inhibitory effect of compounds such as pyrazoles and found that rHp-DHODH binds ubiquinone through a hydrophobic channel. Key residues in the FMN binding pocket were found to be crucial for enzymatic function, which present potential targets for inhibitors mimicking FMN or ubiquinone[46]. HQNO, a natural quinone analogue, represents inhibitory effects on DHODH enzymes of multiple organisms[46-49]. Furthermore, in silico studies performed by Horwitz et al.[22], demonstrated that HQNO inhibits *E. coli* DHODH activity through competitive inhibition at the ubiquinone binding site. Accordingly, we conducted a sequence alignment of *H. pylori* and *E. coli* DHODH enzymes. This alignment revealed that three of the four key residues for coenzyme binding (His_{19}, Arg_{102}, and Tyr_{318}) identified by Horwitz et al.[22] are conserved in both organisms. To validate this observation on *H. pylori*, we evaluated its inhibitory effect on rHp-DHODH enzyme activity, which yielded an IC_{50} value of 1.75 µM. This finding is in accordance with HQNO inhibiting rDHODH of *S. aureus* with an IC_{50} of 0.68 ± 0.05 µM[46] and that of *P. falciparum* with an IC_{50} of 17.7-18 µM[48]. In future studies, molecular docking analyses should be performed to validate the predicted interaction between HQNO and the active site of rHp-DHODH. This approach would provide structural insights into the binding mode and affinity of HQNO, thereby supporting the hypothesis generated from our sequence alignment results. We next determined the inhibitory effect of HQNO on *H. pylori* growth, which indicated an MIC of 0.5 to 1.0 µg/mL. We also assessed whether olorofim exerted bactericidal or bacteriostatic effects, revealing a concentration-dependent activity. In other words, HQNO acted as a bacteriostatic agent at lower concentrations (0.25 to 2.0 µg/mL), while at 4 µg/mL, it exhibited bactericidal effects against *H. pylori*. This finding is in line with the study by Wang et al.[49], who found this compound to reduce *H. pylori*-induced pathogenicity, particularly CagA translocation.

In 2000, Copeland et al.[7] recognized a pyrazole-based compound through biochemical screening that effectively inhibited rHp-DHODH, exhibiting average K_i values of 26 ± 0.12 nM in the presence of Q6 (with detergent) and 10.2 ± 0.2 nM in the presence of Q0 (without detergent). Additionally, they introduced another structural analog that inhibited rHp-DHODH with a K_i value of 50 nM in the presence of Q6. Subsequent modifications to the side chains of these compounds by Haque et al.[44] enhanced their specificity, achieving K_i values below 10 nM.

In 2018, Ohishi et al.[43] introduced ITV, a specific natural product of the quinolone family, as an inhibitor of *H. pylori* DHODH. This natural quinolone, isolated from the culture broth of *Nocardia* sp. ML96-86F2, was previously recognized for its inhibitory effects on human cancer cell lines[50]. To evaluate its efficacy, ITV and its derivatives (AS-1934 and AS-1664) were tested for DHODH inhibition in *H. pylori* membrane fractions, yielding IC_{50} values of 11.11 ± 2.44 µM, 4.55 ± 0.49 µM, and 0.06 ± 0.02 µM for ITV, AS-1934, and AS-1664, respectively. Furthermore, these compounds also inhibited DHODH activity in *E. coli* membrane fractions overexpressing *H. pylori* DHODH, with IC_{50} values of 6 ± 0.26 µM, 3.01 ± 0.23 µM, and 0.22 ± 0.01 µM, respectively. As a result, AS-1934 was declared as a candidate drug for monotherapy of *H. pylori* infection[43]. In comparison, our obtained IC_{50} for HQNO on rHp-DHODH (1.75 µM) seems quite efficient.

CONCLUSION

In this study, we successfully developed a recombinant form of *H. pylori* DHODH, and focused on

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optimizing protein expression to achieve maximum yield and solubility by employing various bacterial strains and experimental conditions. After determining the optimal expression conditions, we assessed the specific activity of the recombinant enzyme to evaluate its efficiency and functionality. Additionally, we conducted a detailed analysis of the kinetic properties of the enzyme, including the K_m and turnover number (k_{cat}), to gain insights into its catalytic performance. Stability assessments were performed over time, under different temperatures and pH conditions, showing the robustness and potential suitability of the enzyme for a wide range of applications. Ultimately, we used this platform to evaluate a potential drug candidate, HQNO, and found it to be effective in inhibiting both the enzymatic activity of recombinant *H. pylori* DHODH and bacterial growth. However, to further establish HQNO as a potential therapeutic agent against *H. pylori*, it is essential to conduct general cytotoxicity assays using mammalian cell lines, particularly gastric epithelial cells. Assessing the selectivity and potential cytotoxic effects of the compound will be critical for evaluating its safety profile and determining its suitability for clinical development.

DECLARATIONS

Acknowledgments

AI tools have been used to improve the English language in certain texts.

Ethical approval

All experimental procedures in this study were approved by the Research Ethics Committee of Pasteur Institute of Iran, Tehran, Iran (ethical code: IR.PII.REC.1401.022).

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

SGh: designed the methodology; performed the experiments, analyzed the data, and wrote the first draft of the manuscript; ME: performed the experiments; FA: performed the experiments and wrote the first draft of the manuscript; YT: designed the methodology; MM: wrote the first draft of the manuscript, conceived the study, supervised the project, approved the final manuscript, and secured the funding. All authors contributed to revisions.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version contains supplementary material.

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