Helicobacter pylori-selective Antibacterials Based on Inhibition of Pyrimidine Biosynthesis*

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We report the discovery of a class of pyrazole-based compounds that are potent inhibitors of the dihydroorotate dehydrogenase of Helicobacter pylori but that do not inhibit the cognate enzymes from Gram-positive bacteria or humans. In culture these compounds inhibit the growth of H. pylori selectively, showing no effect on other Gram-negative or Gram-positive bacteria or human cell lines. These compounds represent the first examples of H. pylori-specific antibacterial agents. Cellular activity within this structural class appears to be due to dihydroorotate dehydrogenase inhibition. Minor structural changes that abrogate in vitro inhibition of the enzyme likewise eliminate cellular activity. Furthermore, the minimum inhibitory concentrations of these compounds increase upon addition of orotate to the culture medium in a concentration-dependent manner, consistent with dihydroorotate dehydrogenase inhibition as the mechanism of cellular inhibition. The data presented here suggest that targeted inhibition of de novo pyrimidine biosynthesis may be a valuable mechanism for the development of antimicrobial agents selective for H. pylori.

Helicobacter pylori is the causative agent associated with gastritis and gastric ulcers in humans and has also been associated with some types of gastric cancers (1). In the U.S. alone some 80 million people are infected with H. pylori, and a significant portion of those infected will go on to present clinical symptoms of gastric diseases. Worldwide, the rate of H. pylori infection varies from 25 to >75% of the population, depending on sanitation levels and other socioeconomic factors (2). Treatment of gastric ulcers was largely based simply on the amelioration of symptoms until the recognition of the causal relationship between H. pylori infection and disease. Eradication of the infection is now the standard of care for these diseases, and a battery of antibacterial agents, in combination with symptom-relieving drugs, has been used to treat H. pylori infections. The antibacterials in current clinical use are far from ideal; drawbacks to their use include side effects associated with eradication of beneficial gastrointestinal flora. The widespread use of these antibacterials for treatment of general bacterial infections has generated resistant strains of H. pylori in patient populations. The phenomenon of genetic exchange between H. pylori and other prokaryotes has also been observed, providing an additional potential route to antibacterial resistance (3). Many of these difficulties could be minimized by using agents that were highly selective against H. pylori.

The complete genomes of two strains of H. pylori have recently been reported (4, 5). Metabolic pathways for purine and pyrimidine biosynthesis and for purine salvage are clearly identifiable in these genomes. In striking contrasts, however, is the apparent absence in these genomes of key enzymes of the pyrimidine salvage pathway, common to both prokaryotic and eukaryotic organisms (Table I). The absence of these enzymes would make H. pylori critically dependent on de novo pyrimidine biosynthesis for both growth and survival. Indeed biochemical data support the suggestion that de novo synthesis is the only route to pyrimidine accumulation for this organism (6). These data suggest that inhibition of one or more of the enzymes of the de novo pyrimidine pathway might be a particularly effective means of treating H. pylori infections. Owing to the presence of intact pyrimidine salvage pathways, other bacteria might be refractory to such treatments, thus making inhibitors of pyrimidine de novo biosynthesis selective for H. pylori.

We have exploited this unique metabolic feature of H. pylori to identify small molecules that selectively kill this bacterium without effect on human cells or other bacterial species. In this paper we report the first examples of H. pylori-selective antibacterial agents that function by inhibition of a key enzyme of the de novo pyrimidine biosynthetic pathway, dihydroorotate dehydrogenase (DHODe) (7).

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—H. pylori and human DHODe were expressed as His₆-tagged proteins (on the N and C termini of the protein, respectively) in a DHODe-deficient strain of Escherichia coli (7). The recombinant proteins were purified by a combination of nickel affinity and S-200 gel filtration chromatography, similar to the conditions reported previously for the enzyme from E. coli (8). The final enzyme samples in each case were >90% pure as judged by SDS-polyacrylamide gel electrophoresis with Coomassie Blue staining. Both enzymes were also produced without the His₆ tag and displayed similar kinetics and inhibition as that reported here for the His-tagged ver.

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1 The abbreviations used are: DHODe, dihydroorotate dehydrogenase; DCIP, 2, 6-dichloroindophenol; DHO, L-dihydroorotate; FMN, flavin mononucleotide; menadione, vitamin K₁; (2-methyl-1,4-naphthoquinone); menaquinone, vitamin K₂ (2-methyl-3-all-trans-polypropenyl-1,4-naphthoquinone); MIF, minimal inhibitory concentration; Q₀, coenzyme Q₀ (2,3-dimethoxy-5-methyl-1,4-benzoquinone); Q₁, coenzyme Q₁ (2,3-dimethoxy-5-methyl-6-[farnesyrlfarnesyl]-1,4-benzoquinone).
**Gene**

| De novo synthesis |
|-------------------|
| pyrAu and pyrAb (2 genes) |
| pyrBl |
| pyrC (2 genes) |
| pyrD |
| pyrE |
| pyrF |
| pyrH |
| ndk |
| pyrG |

| Salvage |
|--------|
| cdd |
| cdaA |
| cdaB |
| tdk |
| deaA |
| upp |
| udh |
| udp |

**Function**

- Carbamoyl-phosphate synthetase
- Aspartate transcarbamoylase
- Dihyroorotate
- Orotate phosphoribosyltransferase
- Orotidine-5'-phosphate decarboxylase
- Uridine 5'-monophosphatase kinase
- Nucleoside diphosphate kinase
- CTP synthetase
- Cytidine deaminase
- Cytosine deaminase
- Cytosine permease
- Thymidine kinase
- Thymidine phosphorylase
- Uracil phosphoribosyltransferase
- Uridine kinase
- Uridine phosphorylase

**Presence in *H. pylori* genomes**

A positive symbol (+) indicates the presence of the indicated gene in both reported *H. pylori* genomes (4, 5). Gene presence is determined by homology with the corresponding genes of other Gram-negative bacteria where enzymatic function has been identified with the specific gene product. A negative symbol (−) indicates the absence of an identifiable gene for the indicated function in either *H. pylori* genome.

### Enzymatic Activity Assay—Enzyme activity was measured with the DCIP assay described previously (7, 9). Assay conditions were as follows, except where indicated in the text and figure captions: 0.1 M Tris, pH 7.5, 0.1 mM 2,6-dichloroindophenol (DCIP), 0.1% Triton X-100. The final enzyme concentration in the assay was between 12 and 25 mM, depending on the enzyme source (i.e., human, *E. coli*, etc.). In some experiments with the *H. pylori* enzyme, the water-soluble quinone coenzyme Q6 (0.03 mM) was used in place of coenzyme Q6, and the Triton X-100 detergent was eliminated from the assay buffer. Assays were run at 25 °C over a 5-min time window, where linear progress curves were observed. The reaction was followed at 600 nm using an extinction coefficient of 20,000 M⁻¹ cm⁻¹ for DCIP. Initial velocities were determined as the slopes of the progress curves at each condition (9, 10).

### Inhibition Studies—The fractional activity of the enzymes in the presence of varying concentrations of inhibitors was measured relative to enzyme activity in the absence of inhibitor. Compounds were added to assay mixtures from concentrated stocks dissolved in dimethyl sulfoxide (MeSO). The final MeSO concentration in all assays (including control samples lacking inhibitor) was 1% (v/v). Four replicates were performed for each concentration of inhibitor, and these were averaged to define the fractional activity at each concentration. The entire inhibitor titration experiment was performed at least twice for each inhibitor described here. Plots of fractional activity as a function of inhibitor concentration were constructed, and the data fit to the equation for a standard Langmuir isotherm (12) or to Morrison’s equation for tight binding inhibition (12, 13). In either case, the apparent $K_I$ (IC₅₀) was converted to the true $K_I$ by assuming competitive inhibition with coenzyme Q₆ (see below) and using the equation $K_I = IC₀/[1 + (S/K_m)]$ (12).

Inhibitor modality was determined by measuring the effects of inhibitor concentration on the enzymatic velocity as a function of substrate concentration. In experiments where dihydroorotate (DHO) was the varied substrate, the coenzyme Q₆ concentration was fixed at 100 μM, whereas the DHO concentration was held constant at 100 μM when coenzyme Q₆ was the varied substrate. For each experiment a total of 20 combinations of substrate and inhibitor concentrations were fit globally to the appropriate equations for competitive (Equation 1), noncompetitive (Equation 2), and uncompetitive (Equation 3) inhibitor modalities (11, 12) using the computational method of CLELAND (11). The choice of appropriate equation was made based on comparison of the variance, sigma values, and the magnitude of the standard errors associated with the fitted parameters for each equation, as described by CLELAND (11).

\[ V = \frac{V_{max}[S]}{[S] + K_m (1 + \frac{I}{K_I})} \]  
(Eq. 1)

\[ V = \frac{V_{max}[S]}{[S] (1 + \frac{I}{K')} + K_m (1 + \frac{I}{K'})} \]  
(Eq. 2)

\[ V = \frac{V_{max}[S]}{[S] (1 + \frac{I}{K'}) + K_m} \]  
(Eq. 3)

where $[S]$ and $K_m$ are the concentration and Michaelis constant of the varied substrate, respectively; $K_I$ is the dissociation constant for the inhibitor-enzyme complex, and $K'$ is the dissociation constant for the inhibitor-enzyme-substrate complex (12). The global fitting used here makes use of all of the data at each combination of substrate and inhibitor to fit the entire data set to a single equation for velocity as a function of substrate and inhibitor concentrations. For such fitting, the confidence interval of the fitted parameters depends on the degrees of freedom for the fitting, which is directly related to the number of substrate/inhibitor concentrations used (14). Hence to maximize the degrees of freedom in a reasonable number of measurements, we ran 20 combinations of substrate/inhibitor concentrations with a single measurement for each concentration. The entire experiment was performed a second time, and the two data sets were averaged and fit to the appropriate kinetic models (Equations 1–3). The best fit model was selected from among Equations 1–3 by comparing the goodness of fit as described above.

For visual presentation the results of this fitting are displayed as double-reciprocal plots (Fig. 4, A and B). The lines drawn through the data in the double-reciprocal plots were constructed using the reciprocal velocity equation with the apparent $K_m$ and $V_{max}$ values, at each inhibitor concentration, obtained from the global fits of the untransformed data (12).

Photocross-linking of the enzyme from *H. pylori* with compound 2 (see below) was performed by incubating the enzyme (500 nM) with 10 μM compound 2 and the indicated concentration of coenzyme Q₆ at room temperature for 1 h. Samples were then dialyzed against 100 volumes of inhibitor-free buffer over night. Residual activity was measured after diluting the enzyme sample (final enzyme concentration 25 nM) into the assay mixture. The percent inhibition reported here is relative to a control sample that was treated as above but without inhibitor present.

All of the inhibitors discussed here were synthesized by the Medicinal Chemistry group at DuPont Pharmaceuticals Company. Compound purity and identity were confirmed by a combination of mass spectral and NMR analysis, as will be detailed elsewhere. All other reagents were obtained from the sources described above.

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**Gene Function Presence in *H. pylori* genomes**

| Gene | Function |
|------|----------|
| pyrAb | Carbamoyl-phosphate synthetase + |
| pyrBl | Aspartate transcarbamoylase + |
| pyrC | Dihydroorotate + |
| pyrD | Orotate phosphoribosyltransferase + |
| pyrE | Orotidine-5'-phosphate decarboxylase + |
| pyrF | Uridine 5'-monophosphatase kinase + |
| pyrG | Nucleoside diphosphate kinase + |
| pyrH | CTP synthetase + |
| cdd | Cytidine deaminase - |
| cdaA | Cytosine deaminase - |
| cdaB | Cytosine permease + |
| tdk | Thymidine kinase + |
| deaA | Thymidine phosphorylase - |
| upp | Uracil phosphoribosyltransferase - |
| udh | Uridine kinase - |
| udp | Uridine phosphorylase - |

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H. pylori DHODase Inhibitors

| Table I | Genes of pyrimidine biosynthesis and salvage pathways |
|--------|------------------------------------------------------|
| De novo synthesis | Salvage |
| pyrAu and pyrAb (2 genes) | cdd |
| pyrBl | cdaA |
| pyrC (2 genes) | cdaB |
| pyrD | tdk |
| pyrE | deaA |
| pyrF | upp |
| pyrH | udh |
| ndk | udp |

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*Note:* A positive symbol (+) indicates the presence of the indicated gene in both reported *H. pylori* genomes (4, 5). Gene presence is determined by homology with the corresponding genes of other Gram-negative bacteria where enzymatic function has been identified with the specific gene product. A negative symbol (−) indicates the absence of an identifiable gene for the indicated function in either *H. pylori* genome.
were the highest quality commercially available. All solutions were prepared with deionized, distilled water.

**Cellular Studies**—MIC measurements for all bacterial species except *H. pylori* were performed in liquid media according to the National Committee of Clinical Laboratory Standards (NCCLS) Document M7-A4 using a 2-fold serial dilution of compound from 64 to 0.03 μM, in duplicate. MIC determinations for *H. pylori* were performed in liquid medium (*H. pylori* broth (HP broth): brain/heart infusion broth, 0.25% yeast extract, 10% horse serum) with incubation at 37 °C, 10% CO2 in a humid chamber for 72 h. Minimal medium used for orotate supplementation studies was as defined by Reynolds (15). For all MIC measurements the turbidity of each sample was determined by visual inspection, and the MIC was defined as the lowest concentration of inhibitor resulting in an optically clear bacterial culture. Bacterial strains were obtained from the American Type Culture Collection (ATCC), except where indicated. Clinical isolates of *H. pylori* were obtained from the Vanderbilt University *H. pylori* strain collection (16).

Human mixed lymphocyte proliferation and human Jurkat cell proliferation assays were performed as described previously in Refs. 17 and 18, respectively.

**RESULTS AND DISCUSSION**

**Characterization of *H. pylori* DHODase**—We have focused our attention on the enzyme dihydroorotate dehydrogenase (DHODase), which catalyzes the fourth step in de novo pyrimidine biosynthesis, the oxidation of dihydroorotate to orotate (7). The enzyme from *H. pylori* was expressed in an *E. coli* host deleted for the endogenous *pyrD* gene (encoding DHODase) (7), and the enzyme was purified to homogeneity. The amino acid sequence of the enzyme displays most similarity to *H. pylori* (Fig. 1), further distinguished from that of the mammalian enzymes on the basis of inhibition studies. Brequinar sodium and the active metabolite A771726 of leflunomide (A771726) are both potent inhibitors of *H. pylori* DHODase; circles, brequinar sodium inhibition of *H. pylori* DHODase; hexagons, A771726 inhibition of *H. pylori* DHODase. The curves drawn through the human enzyme data represent the nonlinear least squares best fits to a standard Langmuir isotherm (12).

We have found that the *H. pylori* enzyme will donate electrons not only to coenzyme Q6 (at saturating DHO) but also to the more water-soluble analog coenzyme Q6 (Km = 5.3 ± 0.7 μM). In contrast, the human enzyme showed minimal activity (~10%) that seen with Q6 in the presence of Q6. Furthermore, the *H. pylori* enzyme will also donate electrons to the bulkier bicyclic naphthoquinones menaquinone (vitamin K2; Km = 8.7 ± 1.3 μM) and medainodide (vitamin K3; Km = 5.9 ± 1.4 μM), whereas the human enzyme utilizes these cofactors very poorly (only 5–7% residual activity was observed with these cofactors; this residual activity is most likely due to the quinone-independent, O2 reducing activity of the human enzyme as reported by Lakaschus and Loeffler (24)).

This quinone specificity may reflect differences in the physiological environments that the mammalian and bacterial enzymes experience. The mammalian forms of DHODase are localized to the inner mitochondrial membrane. Here Q6 is the only electron acceptor available to the enzyme. Electron donation to Q6 by mammalian DHODase has the added advantage that these electrons can be directly utilized by the mitochondrial respiratory pathway to fuel oxidative phosphorylation. In contrast, chromatographic analysis of *H. pylori* cells and those of related *Helicobacter* species demonstrated that these organisms lack Q6 and instead contain menaquinone (menaquinone-6 and -4) as their major isoprenoid quinone (25, 26).

The coenzyme Q binding pocket of the *H. pylori* enzyme is further distinguished from that of the mammalian enzymes on the basis of inhibition studies. Brequinar sodium and the active metabolite of leflunomide (A771726) are both potent inhibitors of mammalian DHODase, displaying K values for the human enzyme of ~6 and 179 nM, respectively (7, 27). Both compounds are competitive inhibitors with respect to coenzyme Q6, and have been shown through crystallographic studies to share a common binding site within the coenzyme Q pocket of the human enzyme (23). We have tested both brequinar sodium and A771726 as inhibitors of the *H. pylori* and *E. coli* enzymes (Fig. 2). In striking contrast to their effects on mammalian DHODases, neither compound demonstrated any inhibition of the Gram-negative bacterial enzymes at concentrations as high as 100 μM. The data shown for brequinar sodium and A771726 in Fig. 2 are typical titrations for these inhibitors; similar data were obtained in more than three separate titration experiments.

These results suggest that there are significant structural differences between the *H. pylori* and mammalian DHODases.

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**TABLE 1.** Concentration-dependent inhibition of *H. pylori* and human DHODase by brequinar sodium and by the leflunomide metabolite A771726. Triangles, brequinar sodium inhibition of human DHODase; circles, brequinar sodium inhibition of *H. pylori* DHODase; diamonds, A771726 inhibition of human DHODase; hexagons, A771726 inhibition of *H. pylori* DHODase. The curves drawn through the human enzyme data represent the nonlinear least squares best fits to a standard Langmuir isotherm (12).

| Compound        | Human | *H. pylori* |
|-----------------|-------|-------------|
| Brequinar sodium| 0.7 mM| 5.3 μM      |
| A771726         | 0.7 mM| 5.3 μM      |

**FIG. 1.** Amino acid sequence alignment of the DHODase of *H. pylori* (H) and *E. coli* (E). Sequence data for *E. coli* taken from Ref. 22.
differences between the coenzyme Q binding pockets of the Gram-negative bacterial and mammalian forms of DHODase. These differences are reflected in the selectivity of the enzymes for ligands that bind at the coenzyme Q site. Hence this binding pocket may provide a target for the development of selective inhibitors against the bacterial enzymes.

**Inhibitor Screening**—We have utilized the *H. pylori* enzyme to screen a chemical library of >150,000 compounds for potential inhibitors. In this way several distinct chemical classes of compounds were identified that showed >1000-fold selectivity for the *H. pylori* enzyme over the human enzyme. One class of compounds identified through this screening effort was the pyrazole-based compounds exemplified by compound 1 (Table II). This compound and several structural analogs inhibited the DHODase of *H. pylori* with nanomolar affinity while displaying no ability to inhibit the cognate enzymes from the Gram-positive bacterium *E. faecalis* (9, 10) or from human (7) (Table II and Fig. 3). The inhibitor titration shown in Fig. 3 represents a typical experimental data set. This experiment was repeated 8 times, yielding an average IC_{50} of 167 ± 75 nM. This value was converted to a *K_i* value by assuming competitive inhibition with respect to Q_6 (see next section). The average *K_i* value determined by this method was 26 ± 12 nM under assay conditions identical to those used for the human enzyme. *K_i* values for the related compounds 2-4 were determined in a similar fashion and are summarized in Table II. Since the *H. pylori* enzyme can readily donate electrons to the more water-soluble cofactor Q_0, we also determine the *K_i* of compound 1 in the absence of Triton X-100 detergent. In the absence of detergent the average (n = 8) *K_i* value was reduced to 10.2 ± 0.2 nM. This result most likely reflects some partitioning of the compound into detergent micelles, where it is unavailable for inhibiting the enzyme; the modest activity of the human enzyme using Q_0 (in the absence of detergent) was not inhibited by compound 1 at concentrations as high as 100 μM.

**Inhibitor Modality**—Kinetic analysis for compound 1 (Fig. 4) indicates that the pyrazoles are competitive inhibitors with respect to coenzyme Q and uncompetitive with respect to the substrate dihydroorotate (12). The untransformed data sets used to construct Fig. 4, A and B, were each fit to Equations 1 and 2 for competitive inhibition gave the best global fit with the following fitting parameters: *K_m* = 19.0 ± 3.9 μM; *V_{max} = (7.2 ± 0.3) × 10^{-6}-μM/s; *K_i* = 43.1 ± 8.7 nM; δ = 4.0 × 10^{-3}; variance = 2.9 × 10^{-4}. Alternatively fitting these data to Equation 2 for noncompetitive inhibition yielded the following fitting parameters: *K_m* = 21.2 ± 3.9 μM; *V_{max} = (7.4 ± 0.4) × 10^{-5}-μM/s; *K_i* = 51.9 ± 16.1 nM; α *K_i* = 1626 ± 2162 nM; δ = 4.1 × 10^{-3}; variance = 2.9 × 10^{-4}. Addition of the α *K_i* term for noncompetitive inhibition did not improve the data fitting relative to the simpler competitive model. Furthermore, the best fit of these data to a noncompetitive model gave a value for α *K_i* that was ~100-fold higher than the corresponding *K_i* value and was associated with significant error. Hence, we reject the noncompetitive model for this data set and conclude that compound 1 is best described as competitive with respect to coenzyme Q.

The data collected at varying DHO concentrations yielded a series of intersecting lines when plotted as a double-reciprocal plot (Fig. 4A), thus eliminating uncompetitive type inhibition as a reasonable model. Fitting of these data to Equation 1 for competitive inhibition gave the best global fit with the following fitting parameters: *K_m* = 36.6 ± 3.7 μM; *V_{max} = (1.9 ± 0.3) × 10^{-6}-μM/s; α *K_i* = 118.1 ± 8.2 nM; δ = 7.1 × 10^{-3}; variance = 5.0 × 10^{-4}. Alternatively fitting these data to Equation 2 for noncompetitive inhibition gave large error for the additional fitting term α *K_i* and higher variance. The fitting parameters were as follows: *K_m* = 32.2 ± 3.8 μM; *V_{max} = (1.9 ± 0.1) × 10^{-7}-μM/s; *K_i* = 337.2 ± 201.0 nM; α *K_i* = 136.1 ± 13.8 μM; δ = 6.5 × 10^{-3}; variance = 8.4 × 10^{-1}. Thus the addition of the α *K_i* term did not improve the fitting for the data in Fig. 4B. We therefore conclude that compound 1 is best described as uncompetitive with respect to DHO, although noncompetitive inhibition with re-
also inhibited \( \text{H. pylori} \) at 10 \( \mu \text{M} \); open inverted triangles prior to photolysis reduced this level of inactivation to 60\%.

Addition of \( \text{coenzyme Q} \) at concentrations up to 100 \( \mu \text{M} \) led to 70\% inhibition of the enzyme activity. Addition of open circles had no protective effect against photoinduced inactivation of the enzyme. The enzyme could be protected with 10 \( \mu \text{M} \) of \( \text{coenzyme Q} \) and to 30\% at 100 \( \mu \text{M} \). Incubation of 500 \( \mu \text{M} \) enzyme with 10 \( \mu \text{M} \) compound 1 followed by photolysis led to irreversible inactivation of the enzyme. The enzyme could be protected against this irreversible inactivation by the presence of the water-soluble quinone coenzyme \( \text{Q} \).

Confirmation that these compounds bind to the enzyme in a mutually exclusive fashion with coenzyme \( \text{Q} \) was obtained by synthesis of compound 2 that contained a benzophenone moiety that could be photolysed to irreversibly cross-link to the enzyme (28). Kinetic analysis in the absence of photolysis confirmed that compound 2 was also a coenzyme \( \text{Q} \) competitive inhibitor with a \( K_i \) of 150 ± 21 \( \mu \text{M} \). Incubation of 500 \( \mu \text{M} \) enzyme with 10 \( \mu \text{M} \) compound 2 followed by photolysis led to irreversible inactivation of the enzyme. The enzyme could be protected against this irreversible inactivation by the presence of the water-soluble quinone coenzyme \( \text{Q} \). In the absence of \( \text{Q} \), photolysis led to 70\% inhibition of the enzyme activity. Addition of \( \text{Q} \) prior to photolysis reduced this level of inactivation to 60\% at 10 \( \mu \text{M} \) \( \text{Q} \), and to 30\% at 100 \( \mu \text{M} \) \( \text{Q} \). In contrast, addition of the other substrate, dihydroorotate (at concentrations up to 10× \( K_m \)) during incubation of the enzyme with compound 2 had no protective effect against photoinduced inactivation of the enzyme.

**Cellular Activity**—Compound 1 and its structural analogs also inhibited \( \text{H. pylori} \) growth in vitro. Compound 1 displayed an MIC of 1–16 \( \mu \text{g/ml} \) in HP broth and as low as 0.25 \( \mu \text{g/ml} \) in minimal medium. Related compounds that were more potent inhibitors of the \( \text{H. pylori} \) DHODase displayed lower MICs, and there was a general relationship between in vitro inhibition of DHODase and cellular inhibition of \( \text{H. pylori} \), as detailed elsewhere. ^2^ Minor structural changes in these compounds that inhibit enzyme activity also eliminate cellular activity. For example, substitution of an ortho-hydroxyphenyl for the benzyl group at \( K_i \) (compound 3; see Table II) was well tolerated, with retention of both DHODase inhibition (\( K_i = 50 \pm 4 \text{ nM} \)) and cellular activity against \( \text{H. pylori} \) (MIC ~16 \( \mu \text{g/ml} \)). In contrast, however, changing the position of the phenylhydroxyl group from ortho (compound 3) to para (compound 4) diminishes the in vitro DHODase inhibition by ~500-fold and likewise abolishes cellular inhibition of \( \text{H. pylori} \) (MIC >64 \( \mu \text{g/ml} \)).

As summarized in Table III, compound 1 and its structural analogs were selective inhibitors of \( \text{H. pylori} \) in culture, with no activity on other Gram-negative bacteria, on Gram-positive bacteria, or human cells. The lack of effect on Gram-positive bacteria and mammalian cells can be explained at the molecular level, as compound 1 and its analogs did not inhibit the DHODases from these organisms. In contrast, the DHODase from \( E. coli \) was inhibited by 1 in vitro with about the same potency as the enzyme from \( \text{H. pylori} \) (data not shown). Hence, the lack of inhibition of other Gram-negative bacteria is based on factors other than inherent affinity for the molecular target. Possible explanations for this observation include the following: 1) differences in cell membrane permeability between \( \text{H. pylori} \) and other bacteria; 2) differences between these organisms in export pumps capable of eliminating the compounds; and 3) resistance to cellular inhibition due to the presence of an active pyrimidine salvage pathway in bacteria other than \( \text{H. pylori} \).

The good correlation between \( \text{H. pylori} \) DHODase \( K_i \) and the MIC for these compounds suggests that the cellular mechanism of inhibition is primarily through binding to and inhibiting the catalytic activity of DHODase. If this hypothesis is correct, a reduction of the effects of these compounds might be expected if the cells were provided with a supply of the metabolic product of DHODase activity, orotate. Indeed, human lymphocytes can be rescued from inhibition of cell proliferation by the mammalian DHODase inhibitors brequinar sodium (29) and leflunomide (30) by addition of uridine to cell culture medium. Menz and co-workers (6) have previously shown that uridine is not imported well into \( \text{H. pylori} \) cells from culture medium, but

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**TABLE III**

**Cellular activity of compound 1**

| Cell/bacterium (Gram +/−) | MIC (µg/ml) |
|---------------------------|------------|
| \( \text{H. pylori ATCC} 43629 \) (−) | 2–8 |
| \( \text{H. pylori clinical isolates} \) (−) | 1–16 |
| \( \text{Moraxella catarrhalis} \) ATCC 25238 (−) | >64 |
| \( \text{Bacteroides fragilis} \) ATCC 25285 (anaerobic −) | >64 |
| \( \text{E. coli} \) ATCC 25922 (−) | >64 |
| \( \text{E. coli tolC mutant} \) (−) | >64 |
| \( \text{Pseudomonas aeruginosa} \) ATCC 27853 (−) | >64 |
| \( \text{Haemophilus influenzae} \) ATCC 49766 (−) | >64 |
| \( \text{Enterococcus faecalis} \) ATCC 29212 (+) | >64 |
| \( \text{Staphylococcus aureus} \) ATCC 13709 (+) | >64 |
| \( \text{Streptococcus pyogenes} \) ATCC 12962 (+) | >64 |
| Human lymphocytes | >50 |
| Human Jurkat cells | >50 |

* MIC values measured as described under “Experimental Procedures.” ^3^ Strains of \( \text{H. pylori} \) isolated from human patients, representing a diverse geographical distribution were studied. These strains (identification numbers 87–81, 95–58, 95–95, 96–16, 96–26, 96–68, and 97–645) were obtained from the Vanderbilt University \( \text{H. pylori} \) strain collection (16). ^4^ Represents a strain of \( E. coli \) in which the channel for a multidrug resistance pump has been eliminated (31). ^5^ MIC was >64 \( \mu \text{g/ml} \) under both aerobic and anaerobic growth conditions. ^6^ Measured in a human mixed lymphocyte proliferation assay as described (17). ^7^ Measured in an assay of cultured Jurkat cell proliferation as described (18).

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**FIG. 4.** Kinetic analysis of \( \text{H. pylori} \) DHODase by compound 1. A, double-reciprocal plot of velocity data as a function of coenzyme \( \text{Q} \) concentration at varying concentrations of compound 1 (closed circles, 0 \( \mu \text{M} \); open inverted triangles, 60 \( \mu \text{M} \); closed inverted triangles, 100 \( \mu \text{M} \); open circles, 200 \( \mu \text{M} \)). B, double-reciprocal plot of the velocity data as a function of dihydroorotate concentration at varying concentrations of compound 1 (closed circles, 0 \( \mu \text{M} \); open inverted triangles, 60 \( \mu \text{M} \); closed inverted triangles, 100 \( \mu \text{M} \); open circles, 200 \( \mu \text{M} \)).
The cellular selectivity demonstrated by these compounds results from a combination of differential affinity for the target enzymes from different organisms (Gram-positive bacteria and mammalian cells) and possibly from differences among prokaryotes in transport mechanisms and alternative metabolic routes to pyrimidine supply (Gram-negative bacteria). Pharmacological optimization (in terms of bioavailability, pharmacokinetics, etc.) of compounds with these in vitro characteristics could provide a clear path for the development of clinically useful agents for the treatment of *H. pylori* infections in humans. Compounds that selectively inhibit *H. pylori* cell growth and survival have the potential for providing significant clinical benefits to patients infected with this organism, particularly in terms of ameliorating the adverse effects often associated with the use of broad spectrum antibacterials.

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**FIG. 5. Effect of orotate concentration, in minimal media, on the MIC for inhibition of in vitro *H. pylori* cell growth by compound 1.** Each individual MIC determination is shown for each orotate concentration. The duplicate MIC values at 0, 10, 50, and 150 μg/ml were identical to each other. The vertical lines connecting the data points for orotate concentrations of 300 and 500 μg/ml serve merely as visual aids to highlight the range of these values. The dashed line likewise serves as a visual aid, connecting the average values of the duplicate MIC values at each orotate concentration. The controls rifampin, amoxicillin, and lansoprazole displayed MIC values between 1 and 2 μg/ml which did not change with increasing orotate concentration (data not shown).

[^14]Orotate is imported modestly. Therefore, we grew *H. pylori* in minimal medium containing a gradient of orotate concentrations and determined the MIC for compound 1 under these conditions (Fig. 5). As summarized in Fig. 5, the MIC for compound 1 showed a clear trend of increasing value with increasing orotate concentration in the medium, consistent with a primary cellular effect through inhibition of DHODase. As controls, rifampin, amoxicillin, and lansoprazole, which all act on *H. pylori* by mechanisms other than DHODase inhibition, showed no changes in MIC with increasing orotate concentration in the medium (data not shown).

**Summary**—The pyrazole-based compounds exemplified by compounds 1, 2, and 3 are potent inhibitors of *H. pylori* DHODase and represent the first examples of selective antibacterials for this organism. In vitro these compounds are selective, coenzyme Q-competitive inhibitors of the enzymes from Gram-negative bacteria, including *H. pylori*. The selectivity of the pyrazoles for Gram-negative bacterial DHODases most likely reflects their binding to the coenzyme Q binding pocket of the enzyme. The Gram-positive bacterial DHODases are all family 1 enzymes, lacking a coenzyme Q-binding site. Hence the inability of coenzyme Q-competitive inhibitors (including brequi-nar sodium, A771726, and the pyrazole compounds described here) to affect enzymes from this family is understandable. The substrate and inhibitor specificity of the coenzyme Q binding pocket has been shown to vary considerably between the mammalian and Gram-negative bacterial forms of the enzyme. Although we have focused attention on the pyrazoles in this paper, we note that every chemical class of compounds identified through our screening efforts as selective, reversible inhibitors of *H. pylori* DHODase were competitive with respect to coenzyme Q. Hence, the structural divergence between the mammalian and bacterial enzymes at this ligand-binding site may provide a rationale for the development of selective inhibitors. This concept is exemplified both by the previously described selective inhibitors of mammalian DHODases (brequinar sodium and A771726; see above) and by the selective pyrazole inhibitors of the Gram-negative bacterial enzymes described in this work.