Pyrazolopyrimidinediones Are Selective Agents for *Helicobacter pylori* That Suppress Growth through Inhibition of Glutamate Racemase (MurI)[▽]

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Pyrazolopyrimidinediones are a novel series of compounds that inhibit growth of *Helicobacter pylori* specifically. Using a variety of methods, advanced analogues were shown to suppress the growth of *H. pylori* through the inhibition of glutamate racemase, an essential enzyme in peptidoglycan biosynthesis. The high degree of selectivity of the series for *H. pylori* makes these compounds attractive candidates for novel *H. pylori*-selective therapy.

Helicobacter pylori is a gram-negative pathogen whose colonization of the human gastric mucosa can cause gastritis that can lead to peptic ulceration (23). The organism is also implicated as a causative agent for certain types of gastric cancer (20, 23, 24, 27), and therefore, eradication of the organism is advised for people with ulcer disease (5). Recommended therapies consist of a proton pump inhibitor in combination with broad-spectrum antibacterials, but emerging resistance and poor patient compliance compromise the effectiveness of these treatments (23). Thus, alternative therapies without these issues are needed for continued successful eradication of *H. pylori* in patients.

The non-life-threatening nature and unique disease manifestation of *H. pylori* infections allow highly selective therapy directed only against the specific organism. The advantage of such a selective therapy would be to limit adverse effects caused by disturbances in the microbial gut flora, thereby improving patient compliance and reducing selection for resistance in other species.

A target-based research program that integrated genetic, biochemical, biophysical, and structural characterization of targets was undertaken to identify selective targets for therapy directed against *H. pylori* (17). Glutamate racemase (MurI), an essential enzyme in peptidoglycan biosynthesis (11, 12, 25) (Fig. 1), was identified through these efforts as a potentially selective target for therapy directed against *H. pylori*. A high-throughput screen was carried out with the enzyme, and pyrazolopyrimidinediones were identified as a class of selective *H. pylori* MurI inhibitors that also showed whole-cell activity (17). This study describes the microbiological characterization of this class of inhibitors.

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science Conference on Antimicrobial Agents and Chemotherapy, Washington, DC [10a].)

MATERIALS AND METHODS

Bacterial strains and susceptibility testing. The bacterial strains, plasmids, and primers used in this study are listed in Table 1. Susceptibilities for *H. pylori*, *Campylobacter coli*, and *Campylobacter jejuni* were determined as described previously (16). MICs for anaerobic species were determined according to CLSI broth microdilution guidelines for *Bacteroides fragilis* (9). MICs for all other species were determined according to CLSI guidelines (10). Compounds were dissolved in dimethyl sulfoxide, and the final concentration of this solvent in all MIC assays was 2%, a concentration that was used as control.

Frequency of spontaneous resistance development. Spontaneous frequencies of resistance were determined in triplicate with *H. pylori* strains SS1 and ARHp80 by plating $>10^9$ cells (collected from blood agar plates) on blood agar plates containing serial twofold dilutions of compound D (Fig. 2) at 1, 2, 4, and 8× MIC. The plates were incubated for 7 days under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂) at 37°C. The frequency of resistance was expressed as the average number of mutants able to grow on compound-containing plates divided by the total cell inoculum. Colonies from different agar plates were randomly selected, and MICs were measured to confirm decreased susceptibility against compound D.

Peptidoglycan precursor analysis. Peptidoglycan precursors were extracted and analyzed according to published procedures (13). *H. pylori* J99 was grown at 37°C in brucella broth (Difco) supplemented with 5% fetal calf serum (Biowhitaker) under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂). Exponentially grown cells were exposed to the compound at 2× MIC for about 3 h, which equals about one generation of uninhibited growth. The cells were rapidly cooled in an ice bath and harvested by centrifugation. The pellet was extracted with 5% cold trichloroacetic acid. After removal of the trichloroacetic acid through ether extraction, the supernatant was concentrated by lyophilization and salts were removed by size exclusion chromatography (Superdex peptide HR10/30 column; Pharmacia). UDP containing fractions were pooled and concentrated by lyophilization. The concentrates were analyzed using reversed-phase high-performance liquid chromatography on a 3-μm C₁₈ ODS Hypersil column (Keystone Scientific). Chromatography was performed with 250 mM ammonium formate (pH 4.0) at 30°C for the first 25 min with a 0.5-ml/min flow rate and at 60°C with a flow rate of 1 ml/min for the remainder of the time. Peaks were identified based on the retention times of known standards, and the identity was confirmed by tandem mass spectrometry (28).

General DNA manipulations. Standard molecular biology protocols were used for PCR, DNA cloning, agarose gel electrophoresis, and sequencing (21). The relevant oligodeoxynucleotide primers used in this study are listed in Table 1. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). The Rapid DNA Ligation Kit (Roche Diagnostics Corp., Indianapolis, IN) was used for DNA cloning. High Fidelity PCR Supermix (Invitrogen) was used to amplify DNA fragments for cloning and sequence analysis. All PCR-generated clones and selected PCR-generated DNA fragments were sequenced (both

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FIG. 1. Cytoplasmic steps of the peptidoglycan biosynthetic pathway. Amino acids are sequentially added to UDP-*N*-acetyl muramic acid (UDP-Mur) by MurC to -F to form the UDP-MurNac-pentapeptide, the final cytoplasmic peptidoglycan precursor (25). MurI provides D-glutamate. Inhibition of MurI results in the inhibition of peptidoglycan biosynthesis at the D-glutamate addition stage (MurD). As a result, inhibition of MurI will result in the depletion of MurD to -F products and accumulation of UDP-*N*-acetyl muramic acid-alanine (MurD substrate). Dap, diaminopimelic acid.

strands) using an ABI Prism 3100 Genetic Analyzer after preparing ABI Prism BigDye Terminator Cycle Sequencing v.2.0 Ready Reactions (PE Biosystems, Foster City, CA). The resulting DNA sequence chromatographs were assembled and analyzed using Sequencher software v.4.0.5 (Gene Codes Corporation, Ann Arbor, MI). The oligodeoxynucleotide primers used for PCR and sequencing were synthesized by Invitrogen (Table 1). DNA was extracted from agarose gels with the QIAEX II kit (Qiagen Inc., Valencia, CA).

Plasmid constructions. A suicide protein expression vector was constructed to facilitate efficient site-specific integration of cloned DNA into the *H. pylori* chromosome, followed by overexpression. The *flaA* locus (jhp0548), encoding the flagellin A subunit, was used as the target site for integration into the *H. pylori* chromosome so that the *flaA* promoter would drive protein expression for selected genes, such as *murI*. This pUC19-based vector contains the ColE1 origin of replication and therefore does not replicate outside of the *Enterobacteriaceae*. The final vector was assembled as described below using cloning vectors, intermediate plasmids, oligonucleotide primers, and DNA templates listed in Table 1 and described below. Primers *flaA1* and *flaA2* (containing engineered AatII and NdeI sites, respectively) were used to amplify a 586-bp DNA fragment of the J99 chromosome corresponding to the *flaA* promoter region. This fragment was cloned into pGEM-T to make pSM104. Primers *flaA3* and *flaA4* (containing engineered PstI and SphI sites, respectively) were used to amplify a 438-bp DNA fragment corresponding to the 3' region downstream of the *flaA* gene (3' *flaA*). This fragment was cloned into pGEM-T to make pSM103. The 5' *flaA* DNA fragment was excised from pSM104 (AatII-NdeI fragment) and cloned into pUC19 to make pSM105. The 3' *flaA* DNA fragment was excised from pSM103 (PstI-SphI fragment) and cloned into pSM105 to make pSM107. Next, an 830-bp DNA fragment containing the chloramphenicol acetyltransferase (CAT) gene was excised from pRY109 using PstI and ligated into pSM107 to make pSM109. The J99 *murI* gene (jhp0496) was excised from pET23b/*murI* as an 810-bp NdeI-Sall DNA fragment and ligated into pSM109 to make pSM116.

Transformation of *H. pylori*. *H. pylori* cells were transformed with DNA as previously described (26). Briefly, *H. pylori* was grown to an optical density at 600 nm (OD₆₀₀) of 0.5 to 0.8. The cells were then concentrated by centrifugation and suspended in brucella broth to a concentration of 100 OD₆₀₀ units, 20 μ l of which ($\sim 10^8$ cells) was spotted onto blood agar plates (Remel) and incubated for 2 h at 37°C under microaerophilic conditions. Next, approximately 1 μ g DNA was applied directly onto the spotted *H. pylori* cells (sterile water was used as a negative control) and incubated overnight. The spotted cells were suspended in 1 ml of brucella broth and spread directly onto blood agar plates containing 10 μ g/ml chloramphenicol for the selection of transformants. Colonies generally appeared within 3 to 5 days. Transformants were characterized by PCR analysis and Western blot analysis.

Western blot analysis. Exponentially growing *H. pylori* cells were concentrated and suspended in sodium dodecyl sulfate protein-loading buffer to a final concentration of 1 OD₆₀₀ unit/100 μ l and boiled for 5 min. Ten microliters of each sample was then run on Novex precast 4 to 20% Tris-glycine polyacrylamide gradient gels (Invitrogen; no. EC60252BOX), and the proteins were transferred onto a nitrocellulose membrane (Novex; no. LC2000) under the conditions described by the manufacturer (XCell SureLock Mini-Cell; Invitrogen). *H. pylori* FlaA-specific mouse monoclonal antibody 220a was obtained from T. J. Trust and used as described previously (15). Rabbit polyclonal anti-MurI antiserum was produced and characterized using standard protocols following the immunization of rabbits with purified *H. pylori* MurI (15, 17). Secondary alkaline phosphate (AP) conjugates (goat anti-mouse immunoglobulin G-AP and goat anti-rabbit immunoglobulin G-AP) were used according to the manufacturer's instructions (Bio-Rad; no. 170-6520 and 170-6518, respectively). The blots were developed using a premixed color development solution (Bio-Rad; no. 170-6432).

Morphology alterations. Cells were grown at 37°C in brucella broth (Difco) supplemented with 5% fetal calf serum (Biowhittaker) under microaerophilic

conditions (5% O₂, 10% CO₂, and 85% N₂). Changes in morphology upon compound exposure were observed through phase-contrast microscopy using $\times 100$ magnification.

RESULTS AND DISCUSSION

Activities of pyrazolopyrimidinediones. Pyrazolopyrimidinediones were discovered through high-throughput screening (HTS) with *H. pylori* MurI (17). The original HTS hit (Fig. 2) showed selective growth inhibition of *H. pylori* (Table 2), and this selectivity was retained throughout the program, as shown for representative analogues A to E. The MIC₉₀ of compound D against an additional 24 *H. pylori* clinical isolates that originated from different geographic regions and patients with various disease states and that exhibited different resistance profiles was 2 μ g/ml. Compound D was also further profiled against a broader range of species, including commensals of the gut flora and species closely related to *H. pylori*, such as *C. coli* and *C. jejuni*. No activity was seen against any of these species (Table 2). The selective whole-cell activity of the pyrazolopyrimidinediones is in agreement with the observed selective inhibition of *H. pylori* MurI by these compounds, which can be explained by the unique biochemical and structural features of the enzyme (17).

The activities of pyrazolopyrimidinediones against *H. pylori* could be compromised by efflux. Some compounds (e.g., B and C) showed much lower MICs against a mutant that lacked a component (*hefC*) (16) of a multidrug efflux pump system (Table 2). However, efflux appeared not to be a trait of the series in general, as other analogues were less severely impacted (e.g., compounds D and E). It is possible that reduced compound permeability and/or increased efflux in other species also plays a role in the high selectivity of the pyrazolopyrimidinediones for *H. pylori*, together with the reported selective enzymatic inhibition (17).

Spontaneous resistant mutants could be obtained only on plates containing 2 $\times$ and 4 $\times$ MIC with frequencies of 10⁻⁷ and 10⁻⁸, respectively. At 8 $\times$ MIC, no mutants could be isolated (frequency < 10⁻⁹). Decreased susceptibility of isolated mutants to compound D was confirmed, and no cross-resistance to other, unrelated antibiotics was detected. These susceptibility data are consistent with the findings that the mutations causing this phenotype were mapped to *murI* (17).

Peptidoglycan precursor pool analyses. Peptidoglycan precursor analyses were carried out to determine if growth suppression of *H. pylori* by the pyrazolopyrimidinediones was due to the inhibition of MurI. Inhibitors of MurI should inhibit the biosynthesis of peptidoglycan precursors at the stage where D-glutamate is linked to UDP-MurNac-Ala (Fig. 1). Thus, it

TABLE 1. Strains and vectors used in this study

Strain, vector (clone), or primer	Relevant genotype, phenotype, or sequence	Source or reference
Strains		
<i>H. pylori</i>		
ARHp80 (AH244)	Wild type	1
Hp80.1	FlaA KO	This study
Hp80.2	FlaA KO; overexpressing Hp MurI	This study
J99	Wild type	2
<i>E. coli</i>		
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15 endA1 recA1</i>	14; Invitrogen
<i>Streptococcus pneumoniae</i>	D39; NCTC7466	This study
<i>Enterococcus faecium</i>	ATCC 19434	ATCC
<i>Haemophilus influenzae</i>	ATCC 51907	ATCC
<i>Moraxella catarrhalis</i>	ATCC 43617	ATCC
<i>B. fragilis</i>	ATCC 25285	ATCC
<i>Clostridium difficile</i>	Clinical isolate	This study
<i>Lactobacillus jensenii</i>	Clinical isolate	This study
<i>Fusobacterium mortiferum</i>	Clinical isolate	This study
<i>Bacteroides thetaiotaomicron</i>	Clinical isolate	This study
<i>Fusobacterium necrophorum</i>	Clinical isolate	This study
<i>Prevotella melaninogenicus</i>	Clinical isolate	This study
<i>C. coli</i>	ATCC 33559	ATCC
<i>C. jejuni</i>	ATCC 33560	ATCC
Vectors (clones)		
pUC19	Cloning vector; Ap ^r	29
pRY109	pUC18 containing <i>cat</i> cassette; Ap ^r Cm ^r	30
PGEM-T	TA cloning vector; Ap ^r	Promega
pET23b/J99 <i>murI</i>	pET23b containing <i>H. pylori</i> strain J99 <i>murI</i> as an 810-bp NdeI-SalI fragment for overexpression	17
pSM103	468-bp J99 3' <i>flaA</i> in pGEM-T	This study
pSM104	607-bp J99 5' <i>flaA</i> in pGEM-T	This study
pSM105	607-bp AatII-NdeI (from pSM104) 5' <i>flaA</i> fragment in pUC19	This study
pSM107	468-bp PstI-SphI (from pSM103) 3' <i>flaA</i> fragment in pSM105	This study
pSM109	854-bp PstI-PstI (from pRY109) <i>cat</i> gene in pSM107	This study
pSM116	810-bp NdeI-SalI (from pETb/J99 <i>murI</i>) <i>murI</i> -containing fragment in pSM109	This study
Primers		
FlaA-1 (<i>AatII</i>)	TACTGACGTCATTTAGGGGGCGGATGTGCAGTCCG	This study
FlaA-2 (<i>NdeI</i>)	AAGCCATATGTGTAACTCCTTGTTATAAAAAACCCAAAGG	This study
FlaA-3 (<i>PstI</i>)	GCGCCTGCAGGCAGCTATGCGATGAGTCAAGCC	This study
FlaA-4 (<i>SphI</i>)	ATAAAGCATGCTAGGGCGGACACACTCTGCAAGC	This study

can be expected that MurI inhibition leads to a reduction in the UDP-MurNAc-dipeptide, -tripeptide, and -pentapeptide products and an accumulation of UDP-MurNAc-Ala substrate (Fig. 1).

Analysis of the pool of cytoplasmic peptidoglycan precursors of untreated *H. pylori* showed a significant amount of UDP-MurNAc-pentapeptide (Fig. 3, top), similar to what was observed in *Escherichia coli* (18). When growth of *H. pylori* was

suppressed by the original HTS hit, an accumulation of UDP-MurNAc-Ala (peak B) was observed (Fig. 3, middle), which was expected for inhibition of MurI within the cell. However UDP-MurNAc-pentapeptide (peak E), the final product of the cytoplasmic peptidoglycan precursor biosynthetic pathway, was still present in substantial amounts (Fig. 3, middle), calling into question the extent of inhibition of MurI and peptidoglycan biosynthesis by this compound. The presence of UDP-MurNAc-

FIG. 2. Pyrazolopyrimidinediones described in this study.

Ala-pentapeptide was not seen with a compound that showed improved activity (compound D) (Fig. 3, bottom), indicating complete inhibition of MurI in *H. pylori* by this compound.

Susceptibility of a MurI overexpression strain. The peptidoglycan precursor results were confirmed in a mode-of-action study that measured MICs for a MurI-overexpressing strain. Recombinant strains Hp80.1 and Hp80.2 were constructed in the wild-type background of strain ARHp80 (Table 1) and confirmed by PCR and sequence analysis (data not shown). Hp80.1 is a *flaA* mutant, constructed using pSM109, in which the FlaA open reading frame is replaced by the CAT gene. Hp80.2 is a *flaA* mutant, constructed using pSM116, in which

FIG. 3. High-performance liquid chromatography analysis of peptidoglycan precursors of *H. pylori* J99 in the absence (top) and presence of pyrazolopyrimidinediones (HTS hit [middle] and compound D [bottom]). Elution times of *N*-acetyl-muramic acid (A), *N*-acetyl-muramic acid-alanine (B), *N*-acetyl-muramic acid-alanine-glutamate (C), *N*-acetyl-muramic acid-alanine-glutamate diaminopimelic acid (D), and *N*-acetyl-muramic acid-alanine-glutamate diaminopimelic acid-alanine (E) peptidoglycan precursors (Fig. 1) are indicated.

the FlaA open reading frame is displaced by *H. pylori murI* and the CAT gene. The overexpression of MurI in Hp80.2 was evaluated relative to ARHp80 and Hp80.1 by Western blot analysis (Fig. 4). ARHp80 produced a detectable band corresponding to FlaA (a high-abundance protein), but not to MurI (a low-abundance protein). The *flaA* knockout strains, Hp80.1 and Hp80.2, did not produce detectable FlaA, while the MurI overexpression strain, Hp80.2, produced a significant protein band corresponding to MurI (Fig. 4).

The rationale for using a strain that overexpresses MurI is that it requires additional compound to inhibit the additional copies of the MurI target in the cell, thus resulting in an elevated MIC compared to the isogenic parental strain. The usefulness of such strains has been demonstrated for several targets (8, 19, 31). MIC determinations with the MurI-overexpressing strain HP80.2 indeed showed an elevated MIC for

TABLE 2. Activities of pyrazolopyrimidinediones^a

Compound	MIC (μg/ml)					<i>E. coli</i>	<i>S. aureus</i>	Other
	<i>H. pylori</i>							
	SS1	ARHp80	Hp80.2	ARHp80 HefC ^{−b}				
HTS hit	4	4	8	1	>64	>64	>64 ^c	
A	2	1	>64	0.5	>64	>64	ND	
B	16	8	16	0.25	>64	>64	>64 ^c	
C	16	16	ND	0.25	>64	>64	>32 ^c	
D	1	0.5	4	0.25	>64	>64	>64 ^d	
E	2	1	8	0.25	>64	>64	ND ^e	
Tetracycline	0.25	0.13	0.13	0.015	ND	ND	ND	

^a Structures are shown in Fig. 2.

^b Efflux mutant (16).

^c Panel included *S. pneumoniae*, *E. faecium*, *H. influenzae*, and *M. catarrhalis*.

^d Panel included *S. pneumoniae*, *E. faecium*, *H. influenzae*, and *M. catarrhalis* plus *B. fragilis*, *C. difficile*, *L. jensenii*, *F. mortiferum*, *B. thetaiotaomicron*, *F. necrophorum*, *P. melaninogenicus*, *C. coli*, and *C. jejuni*.

^e ND, not determined.

FIG. 4. Overexpression of *H. pylori* MurI. An additional copy of *H. pylori* MurI was inserted in the genome of *H. pylori* behind the *fla* promoter (replacing the nonessential *flaA*). Overexpression of MurI and subsequent loss of FlaA expression were monitored by Western blotting using antibodies raised against *H. pylori* MurI (left blot) and FlaA (right blot). ARHp80 represents the wild-type strain, Hp80.1 represents ARHp80 transformed with the empty *flaA* allelic replacement construct pSM109, and Hp80.2 represents ARHp80 transformed with the *murI* expression construct pSM116.

compound D, conclusively showing that growth suppression by this compound was due to the inhibition of MurI (Table 2). In contrast, the original HTS hit did not show elevated MICs for the MurI-overexpressing strain (Table 2), showing that although this compound inhibits MurI in the cell (Fig. 3, middle), growth suppression must be mediated via a different target. Identification of the off-target activity of the HTS hit was not pursued, as more optimized compounds all showed the desired mode of action.

These results illustrate the usefulness of different mode-of-action assays in the different stages of drug discovery. In the earlier stages, when weaker compounds are profiled, less definitive assays, such as the peptidoglycan precursor analysis assay, are very useful, as they can demonstrate that compounds enter the cell and interfere with the intended target (4), even though growth suppression may still occur through a different mechanism. As such, they provide confidence that growth suppression through the intended target can be achieved within the series upon compound optimization. This important information will be missed if only more definitive mode-of-action assays, such as the MurI-overexpressing strains, are used, with the risk that valuable compounds would be discarded outright as promiscuous growth inhibitors. However, as compounds are optimized, the more definitive higher-throughput assays gain value.

***H. pylori* morphology changes upon pyrazolopyrimidinedione exposure.** Inhibition of MurI leads to disturbance of peptidoglycan biosynthesis, and since peptidoglycan is a determinant of the cell shape (7), alterations in cell morphology are expected for compounds that suppress growth through MurI inhibition. When *H. pylori* was exposed to compound D, changes in its morphology were seen (Fig. 5). The cells lost their characteristic spiral shape and turned into transparent

FIG. 5. Morphology alterations of *H. pylori* following exposure at 2× MIC to pyrazolopyrimidinedione D starting at zero minutes.

cocci. Such alterations are also seen when *H. pylori* enters stationary phase (22), except the change here was rapid upon compound exposure. The rapid response was typical of the pyrazolopyrimidinediones and was not seen when cell growth was inhibited with tetracycline for the same time (data not shown). When cells were exposed to amoxicillin, a β -lactam that inhibits peptidoglycan biosynthesis, similar rapid morphology changes were noted (6). Thus, the morphological changes seen with the pyrazolopyrimidinediones are in agreement with inhibition of peptidoglycan biosynthesis through MurI.

In conclusion. Pyrazolopyrimidinediones are newly discovered agents that suppress *H. pylori* growth through inhibition of MurI. The high selectivity of these compounds for *H. pylori* combined with their low propensity for resistance development make these analogues attractive candidates for therapy aimed specifically at *H. pylori*. Oral bioavailability is another important feature of therapy specifically directed against *H. pylori*, and improvements in that area could be achieved through prodrug approaches (3). However, the most potent compounds showed low solubility and high protein binding, and in order for this series to progress to compounds of clinical utility, improvements in these areas will be needed.

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