Anti-*Helicobacter pylori* activity of ethoxzolamide

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**ABSTRACT**

Ethoxzolamide (EZA), acetazolamide, and methazolamide are clinically used sulphonamide drugs designed to treat non-bacteria-related illnesses (e.g. glaucoma), but they also show antimicrobial activity against the gastric pathogen *Helicobacter pylori*. EZA showed the highest activity, and was effective against clinical isolates resistant to metronidazole, clarithromycin, and/or amoxicillin, suggesting that EZA kills *H. pylori* via mechanisms different from that of these antibiotics. The frequency of single-step spontaneous resistance acquisition by *H. pylori* was less than 5 × 10⁻⁹, showing that resistance to EZA does not develop easily. Resistance was associated with mutations in three genes, including the one that encodes uncappedyl pyrophosphate synthase, a known target of sulphonamides. The data indicate that EZA impacts multiple targets in killing *H. pylori*. Our findings suggest that developing the approved anti-glaucoma drug EZA into a more effective anti-*H. pylori* agent may offer a faster and cost-effective route towards new antimicrobials with a novel mechanism of action.

**Introduction**

*Helicobacter pylori* persistently colonises the epithelium of the stomach in approximately half of the world’s population. Colonisation can lead to the development of gastric and duodenal ulcers, mucosa-associated B-cell lymphoma, and gastric adenocarcinoma. When left untreated, up to 3% of *H. pylori* infections progress to gastric cancer. Treatment of *H. pylori* infection involves complete eradication of the organism from the host. The efficacy of existing drug regimes has significantly declined over the years. In 2017, clarithromycin-resistant *H. pylori* was ranked as a high priority pathogen for antibiotic research development by the World Health Organisation (WHO), highlighting the pressing need for novel anti-*H. pylori* therapies.

New treatment strategies may target adaptation mechanisms of *H. pylori* to the acidic pH of the stomach. *H. pylori* is a neutralophile, but it is capable of maintaining its cytoplasmic pH at near-neutral levels during short-term exposure to pH as low as 1.4. This is achieved via the combined action of *H. pylori* urease and two carbonic anhydrases. Urease converts urea to NH₃ and CO₂, which have acid neutralising and buffering properties. CO₂ generated as a result of urease activity is hydrated in the periplasm and cytoplasm by two carbonic anhydrases (*HpaCA* and *HpβCA*), respectively, resulting in the production of protons (H⁺) and bicarbonate (HCO₃⁻). The protons react with NH₃ to form NH₄⁺ ions. The resultant NH₃/NH₄⁺ and CO₂/HCO₃⁻ acid-base couples buffer the cytoplasm and periplasm at pH close to neutral. Recent detection of *HpαCA* in the outer membrane vesicles produced by *H. pylori* suggested that this enzyme could have an additional, as yet unknown, role in initiating or regulating pathogenesis in the host.

*HpαCA* and *HpβCA* are strongly inhibited by primary sulphonamides RSO₂NH₂, including acetazolamide (AAZ), ethoxzolamide (EZA), and methazolamide (MZA) (Figure 1) that have been originally developed as inhibitors of human CAs and used clinically as diuretics, and antiglaucoma or antiulcer drugs known under the names Diamox (AAZ), Cardrase (EZA), and Neptazane (MZA). Analysis of the crystal structures of *HpαCA* in complex with either AAZ or MZA revealed that these sulphonamides act as active-site inhibitors that mimic the transition state of the reaction catalysed by the enzyme. Furthermore, the crystal structures of *HpαCA* in complex with a series of AAZ-related sulphonamides, including EZA, revealed that the mode of sulphonamide binding to *HpαCA* correlates well with their inhibitory activities. Cumulatively, these data have raised a question of whether the *HpαCA* inhibition by sulphonamides would result in killing *H. pylori*.

Indeed, MZA has been shown to suppress growth of *H. pylori* strains S51 and 11637 in vitro. Furthermore, the results of treatment of gastroduodenal ulcers with EZA and AAZ suggested that these drugs inhibit *H. pylori* growth in vivo. For example, administration of EZA for 3 weeks at 5–10 mg/kg body weight/day
resulted in ulcer healing in 98% of the patients.\(^\text{16}\) This could be,
in part, attributed to inhibition of human CA activity in the par-
tietal cells of the stomach which resulted in a reduced basal secre-
tion of gastric acid (antacid action). However, it has been
recognised that the EZA and AAZ treatment also likely eradicated
the \(H.\) pylori infection which caused ulcer disease in the first place,
because, two years after treatment, the ulcer recurrence rate in
patients treated with EZA (11%)\(^\text{16}\) or AAZ (6%)\(^\text{17}\) was significantly
lower than that with classical antacid drugs (34–79%) and close to
that achieved by the triple \(H.\) pylori eradication therapy.\(^\text{19}\)

Given the growing resistance of \(H.\) pylori to clinically used antibi-
otics, these findings have highlighted the potential of sulpha-
monamide inhibitors of HpxCA as lead compounds for developing novel
anti-infective agents. Moreover, since EZA, AAZ, and MZA have
been used since the 1950s as drugs to treat various human conditions,
their pharmacokinetic properties are well understood, and
repurposing these drugs as antimicrobials to treat multi-resistant
bacterial infections would be cost-effective. However, HpxCA has
not, as yet, been validated as a drug target. In this study, we have
examined the potency of the HpxCA inhibitors MZA, AAZ and EZA
against several \(H.\) pylori laboratory and clinical strains, and isolated
and characterised a mutant resistant to the most potent com-
ound, EZA, which has led to the identification of the genetic
determinants that confer resistance and to the understanding of the
aspects of the mechanism of anti-\(H.\) pylori activity of EZA.

Materials and methods

\textbf{Bacterial strains and culture conditions}

\(H.\) pylori laboratory strains P12\(^\text{20,21}\), 26695\(^\text{21}\), SS1\(^\text{22}\), and J99\(^\text{23}\), and
clinical isolates from gastric biopsies (CH425, CH426, and CH427)
were used in the study. \(H.\) pylori strains were grown on horse
blood agar (HBA) prepared using Columbia blood agar base
(Oxoid) supplemented with 5% (v/v) defibrinated horse blood
and an antibiotic cocktail comprising 10 \(\mu\)g/mL vancomycin, 5 \(\mu\)g/mL
cefsulodin, 2.5 \(\mu\)g/mL polymyxin B, 5 \(\mu\)g/mL trimethoprim, and
8 \(\mu\)g/mL amphotericin B. Plates were incubated at 37 °C for
48–72 h under microaerophilic conditions generalised using the
CampyGen (Oxoid) system. Liquid cultures were grown at 37 °C
with shaking at 120 rpm in Brucella broth (Becton Dickinson)
containing 10 \(\mu\)g/mL vancomycin and 10% (v/v) foetal bovine serum
(FBS), in microaerophilic conditions. Antibiotic solutions were pre-
pared according to Clinical and Laboratory Standards Institute
(CLSI) guidelines\(^\text{24}\). AAZ, MZA, and EZA were dissolved in dimethyl
sulfoxide (DMSO). AAZ, MZA, EZA, antibiotics, and DMSO were
purchased from Sigma-Aldrich.

\textbf{Determining minimum inhibitory concentration (MIC) and
minimum bactericidal concentration (MBC)}

MICs and MBCs of sulphonamide compounds were determined as
previously described\(^\text{25}\). Liquid cultures of \(H.\) pylori were grown to
an optical density of 0.4–0.6 at 600 nm (OD\(_{600}\)). Cells were pel-
leted, washed, resuspended in antibiotic-free medium to an OD\(_{600}\)
of 0.05, and aliquoted in 1 ml volumes supplemented with various
concentrations of sulphonamides (0–15 mM AAZ, 0–5 mM MZA, or
0–1 mM EZA), or 1% (v/v) DMSO as a control. The cultures were
incubated for 24 h, and initial and final colony forming units (CFU)
were quantified by plating out serial dilutions and counting the
colonies. Cell survival after 24 h was calculated as follows: CFU sur-
vival (\%)\(=\frac{\text{CFU}_{24\text{h}}}{\text{CFU}_{0\text{h}}}{\times100}\). \(H.\) pylori P12 had the highest sensi-
tivity to all three compounds and thus was chosen for
further studies.

MICs of antibiotics commonly used to treat \(H.\) pylori infections
(metronidazole, clarithromycin, amoxicillin, and tetracycline)
against the clinical strains were measured using E-test
(bioMérieux)\(^\text{26}\).

\textbf{Bactericidal kinetics of AAZ, MZA, and EZA}

A liquid culture of \(H.\) pylori P12 at OD\(_{600}\) of 0.05 was prepared as
above, and supplemented with AAZ, MZA, or EZA at concentra-
tions corresponding to their respective 1 × MBC or 2 × MBC.
 Cultures were grown for 48 h, and sampled at 0, 6, 12, 18, 24, 36,
and 48 h for CFU quantification.

To compare activities of AAZ, MZA, and EZA against \(H.\) pylori
P12 under neutral (pH 6.8) and acidic (pH 4.5) conditions, time-kill
curves were generated using 2 × MBC of each inhibitor in Brucella
broth/10% FBS at pH 6.8 or in the same medium adjusted to pH
4.5 using 0.2 M phosphate-citrate buffer. Liquid cultures were
grown to an OD\(_{600}\) of 0.4–0.6. The cells were then pelleted,
washed, and resuspended in liquid medium supplemented with
2 × MBC of AAZ, MZA, or EZA at either pH 6.8 or pH 4.5, to an
OD\(_{600}\) of 0.05. The cultures were grown as described above,
and sampled at 0, 3, 6, 12, 18, 24, and 36 h to quantify CFU.

\textbf{Isolation and characterisation of an \(H.\) pylori P12 mutant
resistant to EZA}

An \(H.\) pylori P12 mutant clone resistant to EZA (hereafter referred
to as MutE) was obtained by iterative selection for progressive
resistance\(^\text{27,28}\) to 0.05 mM (0.25 × MIC), 0.1 mM, and then 2 mM
EZA. The frequency of pre-existing spontaneous mutations allow-
ging growth at 2 mM EZA was estimated by single-step selection.
Plates containing the inhibitor were inoculated with \(2 \times 10^6\),
\(2 \times 10^2\), or \(2 \times 10^8\) CFU. Plates with 10 \(\mu\)g/mL rifampicin, a com-
pound with a known frequency of spontaneous resistant mutants
in \(H.\) pylori, were used as controls\(^\text{29}\). The mutation frequency
was calculated as the average CFU generated on the inhibitor-supple-
mented plate, divided by the CFU in the inoculum.

To assess the stability of the resistant phenotype, 5 colonies of
MutE were picked from plates containing the highest inhibitor
concentration, and passaged 5 times on inhibitor-free plates.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{chemical Structures.png}
\caption{The chemical structures of acetazolamide, methazolamide and ethoxzolamide.}
\end{figure}
The resulting isolates showed no significant change in MIC or MBC values relative to the starting ones.
To construct growth curves, liquid cultures of *H. pylori* P12 WT and MutE at starting OD_{600} of 0.05 were grown for 48 h in the absence of EZA. Samples were taken at 0, 3, 6, 9, 12, 18, 24, 30, 36, and 48 h, and CFU enumerated. Values were analysed in GraphPad Prism version 7.02 using two-way ANOVA with Dunnett’s multiple comparison test.

**MICs of commercial antibiotics against the EZA-resistant mutant and its WT parent**

MICs of the clinically used antibiotics against *H. pylori* P12 WT and MutE were determined by the agar dilution method. Ten μL of the starter cultures at OD_{600} 0.05 were plated onto HBA containing various concentrations of antibiotics (0.0075–0.96 μg/mL amoxicillin and clarithromycin, 0.5–16 μg/mL metronidazole, and 0.06–4 μg/mL tetracycline). CFU were determined as described above.

**Confirmation of resistance-conferring mutations by transformation**

In order to separate the mutations responsible for the resistant phenotype from all other spontaneous mutations in MutE, its genomic DNA was isolated and transformed into the WT P12 strain. WT genomic DNA and buffer were used as negative controls. Four independent transformant colonies displaying resistance to EZA (hereafter referred to as MutETF1, MutETF2, MutETF3, and MutETF4) were selected on Columbia blood agar plates containing 2 mM EZA.

**Genome sequencing and analysis**

Genomic DNA was extracted using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). Further sample preparation steps and genome sequencing were performed at the Micromon High-Throughput Sequencing Facility (Monash University). Sequencing libraries were constructed using the Illumina NexteraXT (Illumina) and sequenced using the Illumina MiSeq platform with a paired end configuration and average read length of 150 bp.

Sequence analysis was performed using CLC genomics Workbench v. 7.0.3 (Qiagen). Reads were aligned to the reference genome of *H. pylori* strain P12 (NCBI accession number NC_011498.1). Differences between the WT parental strain and MutE, identified using the Probabilistic Variant Detection and the Quality Based Variant Detection analysis tools in CLC genomics Workbench, were confirmed by Sanger sequencing at Micromon (see below).

**Sanger sequencing**

The genes of interest were PCR-amplified from genomic DNA, purified using the Wizard SV gel and PCR clean-up kit and sequenced using the conventional Sanger method. Sequences were aligned with BioEdit v 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

**Results and discussion**

**Antimicrobial activity of EZA, AAZ, and MZA against *H. pylori***

We assessed the antimicrobial activities of sulphonamide drugs AAZ, MZA, and EZA against the *H. pylori* laboratory strains P12, 26695, SS1, and J99, and against the clinical isolates resistant to metronidazole, clarithromycin, and/or amoxicillin (CH424, CH426, and CH427, Table 1). Growth inhibition assays showed that most tested strains were sensitive to high-micromolar or low-millimolar concentrations of sulphonamides (Table 2, Figure 2). EZA had the highest anti-*H. pylori* activity of all tested compounds and inhibited growth of all strains. The lowest MIC/MBC values for EZA were observed with the strains P12 and SS1 (MIC = 0.2 mM, MBC = 0.4 mM for both strains). MIC values for AAZ were approximately one order of magnitude higher than those of EZA in all tested strains (Figure 2, Table 2). Strains 26695 and J99 were the least sensitive to all CA inhibitors used, and neither showed measurable sensitivity to MZA in the tested concentrations range. Importantly, although the three clinical strains have different resistance profiles to the commercial first-line antibiotics (amoxicillin, clarithromycin, metronidazole, and tetracycline), their sensitivity to each sulphonamide was equivalent. Since *H. pylori* strain P12 showed the greatest sensitivity to all compounds tested, it was selected for the subsequent mechanistic studies.

**H. pylori growth inhibition by EZA, AAZ and MZA is time-, concentration- and pH-dependent**

To determine the duration of inhibitor treatment required to kill *H. pylori* at neutral pH (under conditions optimal for *H. pylori* growth), time-dependent killing kinetics were assessed for each sulphonamide at concentrations corresponding to their respective MIC/C2 and MBC/C2 (Figure 3(A)). For EZA, 18-h incubation with the sulphonamide had no significant effect on the growth of all strains, with the exception of strain J99, which had a slightly lower MIC/C2.

Table 1. Sensitivity profiles of *H. pylori* clinical isolate strains CH425, CH426, and CH427 to clinically used antibiotics.

| Antimicrobial       | CH425  | CH426  | CH427  |
|---------------------|--------|--------|--------|
| **MIC (μg/mL)**     |        |        |        |
| Metronidazole       | >256   | 12     | 0.047  |
| Clarithromycin      | 0.38   | >256   | 0.032  |
| Amoxicillin         | 0.064  | >256   | 0.128  |
| Tetracycline        | 0.125  | 0.125  | 0.19   |
| **Interpretation**  |        |        |        |
| Metronidazole       | Resistant | Resistant | Sensitive |
| Clarithromycin      | Intermediate | Resistant | Sensitive |
| Amoxicillin         | Sensitive | Resistant | Sensitive |
| Tetracycline        | Sensitive | Sensitive | Sensitive |

Table 2. MIC and MBC values of three sulphonamide drugs against four laboratory strains and three clinical isolates.

| Sulphonamide drugs | Ethoxzolamide | Acetazolamide | Methazolamide |
|--------------------|---------------|---------------|---------------|
| **H. pylori strains** | **MIC (mM)** | **MBC (mM)** | **MIC (mM)** | **MBC (mM)** |
| Lab strains        |               |               |               |               |
| P12                | 0.2           | 0.4           | 2             | 8             | 0.5           | 3             |
| 26695              | 0.3           | 0.5           | 8             | 15            | >5            | >5            |
| SS1                | 0.2           | 0.5           | 4             | 8             | 0.5           | 3             |
| J99                | 0.3           | 0.5           | 8             | 15            | >5            | >5            |
| Clinical strains   |               |               |               |               |
| CH425              | 0.25          | 0.4           | 2             | 8             | 1             | 4             |
| CH426              | 0.25          | 0.4           | 2             | 8             | 1             | 4             |
| CH427              | 0.15          | 0.4           | 2             | 8             | 1             | 4             |
of the compound was sufficient to kill 99.9% of cells, while a 36-h exposure was required when 1 \times \text{MBC} was used. Bactericidal kinetics for MZA were similar, except that a 24-h exposure was required to kill 99.9% of cells at 2 \times \text{MBC} of the compound (6 mM). In comparison to EZA and MZA, the bactericidal action of AAZ was slower: the time required for 1 \times \text{MBC} of the inhibitor to kill 99.9% of cells was 48 h (36 h for 2 \times \text{MBC}). This analysis has also demonstrated that anti-\text{H. pylori} activity of all three sulphonamides is concentration-dependent.

To determine the effect of low pH on the bactericidal activity of the sulphonamide inhibitors, time-dependent killing curves were also generated using the medium buffered at pH 4.5 (approximating conditions to which \text{H. pylori} is exposed during the initial colonisation) (Figure 3(B)). Firstly, the results confirm that \textit{in vitro}, \text{H. pylori} would not withstand low pH conditions for long, as even in the absence of inhibitors no bacteria survived after 12 h at pH 4.5. Secondly, 2 \times \text{MBC} of EZA, AAZ, or MZA accelerated the elimination of \text{H. pylori} at pH 4.5, which occurred after 6 h for EZA, and 9 h for MZA and AAZ (Figure 3(B)). Thus, sulphonamides exerted detectable antimicrobial activity under both neutral and low pH conditions. However, the reduced bacterial viability at low pH precluded direct quantitative comparisons.

The observation that the sulphonamide compounds display bactericidal activity at both neutral and acidic pH has not been expected, as the inhibitors of \text{H. pylori} \(\alpha\)– and \(\beta\)-carbonic anhydrases were thought to affect the cell viability only at acidic pH, when the functions of these enzymes are known to be essential. As neutral pH approximates the conditions under which \text{H. pylori} persists in the mucus layer adjacent to the gastric epithelium, we have addressed the mechanism of bacterial killing at neutral pH by isolating and characterising a spontaneous mutant resistant to the most potent compound in the series, EZA.

**Isolation and characterisation of \text{H. pylori} P12 mutant with decreased susceptibility to EZA**

Selection by serial passages of \text{H. pylori} P12 in the presence of sub-lethal concentrations of EZA enabled isolation of a mutant significantly more resistant to this compound than the parental wild type. The EZA-resistant strain MutE had an MIC >2 mM (10 \times WT MIC). The resistance phenotype was stable during growth in the absence of EZA for at least 15 days.

Estimation of the frequency of spontaneous resistant mutants using a single selection step with 2 mM EZA yielded a value of
kinetics for 2 biological replicates. The horizontal dashed line represents the limit of detection (100 cells) and the horizontal solid line corresponds to 99.9% cell acidic (pH 4.5) conditions. The horizontal dashed line represents the limit of detection (100 cells) and the horizontal solid line corresponds to 99.9% cell death. Error bars represent the standard error of the mean for three independent biological replicates.

Figure 3. Analysis of the time and dose dependency of the antimicrobial action of AAZ, MZA, and EZA on H. pylori P12. (A) Bactericidal kinetics for 1 × MBC and 2 × MBC of the respective sulphonamide, measured at neutral pH (B) Bactericidal kinetics for 2 × MBC of the respective sulphonamide under neutral (pH 6.8) and acidic (pH 4.5) conditions. The horizontal dashed line represents the limit of detection (100 cells) and the horizontal solid line corresponds to 99.9% cell death. Error bars represent the standard error of the mean for three independent biological replicates.

Figure 4. Growth curves for H. pylori P12WT and MutE measured over 36 h. Error bars represent the standard error of the mean for three independent biological replicates. Significant differences compared to wild type P12 are indicated; *p < .05, **p < .01. All other differences are not significant.

<5 × 10⁻⁹, which is significantly lower than the previously reported frequencies of spontaneous mutations leading to H. pylori resistance to rifampicin (10⁻⁶)²⁹, metronidazole, or tetracycline (10⁻⁵–10⁻⁶)³¹. This observation prompted us to determine whether the mutation(s) associated with resistance to EZA incurred a fitness cost. Indeed, MutE showed reduced growth in comparison to the parental strain (Figure 4); the doubling time of P12WT was 5 h, whereas the doubling time of MutE was 6 h.

Identification of genetic determinants linked to EZA resistance

As carbonic anhydrases were considered the likely targets for the anti-H. pylori activity of sulfonamides¹²⁻¹⁵, the genes for α- and β-carbonic anhydrases were sequenced in the parental strain and in MutE. No mutations were found, eliminating the possibility that amino acid changes in these enzymes caused the resistance phenotype.

To investigate the genetic basis for the phenotypically stable resistance to sulphonamides, we therefore determined and compared the full genome sequences of P12WT and MutE. All observed genomic differences were single nucleotide polymorphisms in 12 genes (listed in Supplementary Table 1). To separate the mutations that caused EZA resistance from unlinked random mutations, we performed natural transformation of the mutant chromosomal DNA back into a WT background, and selected four resultant EZA-resistant recombinants (MutETF1 – MutETF4, Table 3) for Sanger sequencing of the candidate genes. MutETF1, MutETF2, MutETF3, and MutETF4 retained mutations in four, four, four and five genes, respectively. Only three mutations were common to all four EZA-resistant transformants. One mutation (Glu173Lys) was in the gene HPP12_RS06100 that encodes undecaprenyl pyrophosphate synthase UppS, an enzyme essential for cell wall biosynthesis. The second mutation (Cys29Arg) was in the regulatory gene HPP12_RS07625 encoding transcription termination factor NusA. The third mutation (a frameshift) was in the gene HPP12_RS01490 encoding an inner membrane protein of unknown function (Table 3). This result suggests that acquisition of resistance by H. pylori to EZA at neutral pH was associated with mutations in these three genes, and was likely the result of a combination of different mechanisms involving modifications of cellular proteins and systems other than HprCA and Hps/ICA.

Our study allows the proposal of putative resistance mechanisms and discussion of the implications for the mode of antimicrobial action of EZA. One possible resistance mechanism is alteration of a putative sulphonamide target. We note that sulphonamides (albeit other than EZA) have been shown to inhibit H. pylori undecaprenyl pyrophosphate synthase (UppS) with micromolar IC₅₀. In our experiments, selection for resistance to EZA resulted in a mutant with the Glu173Lys substitution in UppS. The respective residue in E. coli UppS (Glu198) is proximal to the catalytic Mg²⁺ ion in the active site. We, therefore, postulate that EZA binds in the H. pylori UppS active site through coordination of Mg²⁺, in competition with the natural substrate, thus acting as a competitive inhibitor. The Glu173Lys mutation likely removes favourable interactions or introduces a steric clash with the inhibitor, conferring resistance.

Changes in cell physiology may also contribute to EZA resistance. MutE contains a mutation in a gene regulating transcription (a single amino-acid substitution Cys29Arg in the transcription termination factor NusA), which likely affects the global regulation of metabolic enzymes, aiding resistance. The third resistance-linked mutation, found in the gene HPP12_RS01490 encoding an inner membrane protein that shares no sequence similarity with any protein of a known function, may affect an entry pathway of EZA into H. pylori.
There is no cross-resistance between EZA and clinically used anti-\textit{H. pylori} antibiotics

To gain further insight into whether there were common mechanisms between the EZA-resistant mutant generated in this study and the known antibiotic resistances in \textit{H. pylori}, cross-resistance to commercial antibiotics was investigated. As described in an earlier section, clinical strains resistant to amoxicillin, clarithromycin, and/or metronidazole were sensitive to EZA. Evaluation of the bactericidal activity of these compounds against MutE showed that the reverse is also true: MIC and MBC values of the first line antibiotics for the WT strain were not significantly different from those for MutE (Figure 5), indicating that there are no shared

\begin{table}[h]
\centering
\caption{Nucleotide changes in \textit{H. pylori} P12 EZA-resistant mutants generated by transformation of WT P12 with MutE chromosomal DNA.}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Position}\textsuperscript{a} & \textbf{Type} & \textbf{Reference} & \textbf{Allele} & \textbf{Locus tag} & \textbf{Amino acid substitution} & \textbf{Gene product} \\
\hline
1275233 & SNV & C & C & HPP12\_RS06100 & Glu173Lys & UPP pyrophosphate synthase \hline
1275233 & SNV & T & C & HPP12\_RS06100 & Cys29Arg & transcription termination factor NusA \hline
294832 & Deletion & A & - & HPP12\_RS01490 & Gln113fs\textsuperscript{b} & inner membrane protein \hline
312794 & SNV & G & T & HPP12\_RS01540 & Asp56Tyr & peptide ABC transporter substrate binding protein \hline
1275233 & SNV & C & T & HPP12\_RS06100 & Glu173Lys & UPP pyrophosphate synthase \hline
1573139 & SNV & T & C & HPP12\_RS07625 & Cys29Arg & transcription termination factor NusA \hline
294832 & Deletion & A & - & HPP12\_RS01490 & Gln113fs & inner membrane protein \hline
509788 & Deletion & A & - & HPP12\_RS02505 & Lys303fs & DNA methyltransferase \hline
1275233 & SNV & C & T & HPP12\_RS06100 & Glu173Lys & UPP pyrophosphate synthase \hline
1573139 & SNV & T & C & HPP12\_RS07625 & Cys29Arg & transcription termination factor NusA \hline
294832 & Deletion & A & - & HPP12\_RS01490 & Gln113fs & membrane protein \hline
312794 & SNV & G & T & HPP12\_RS01540 & Asp56Tyr & peptide ABC transporter substrate binding protein \hline
509788 & Deletion & A & - & HPP12\_RS02505 & Lys303fs & DNA methyltransferase \hline
\end{tabular}
\textsuperscript{a}Nucleotide positions are indicated with reference to the published \textit{H. pylori} P12 genome.
\textsuperscript{b}fs: frameshift mutation.
\end{table}

![Figure 5](image-url)

\textbf{Table 3.} Nucleotide changes in \textit{H. pylori} P12 EZA-resistant mutants generated by transformation of WT P12 with MutE chromosomal DNA.

\textbf{Figure 5.} Sensitivity of \textit{H. pylori} P12 WT and its sulphonamide-resistant mutants MutE to clinically used antibiotics amoxicillin, clarithromycin, tetracycline, and metronidazole. Error bars represent the standard error of the mean for two independent biological replicates. (A) The MIC values of both the WT strain and MutE for amoxicillin were 0.0075 \(\mu\)g/ml, with the MBC value for MutE (0.015 \(\mu\)g/ml) being 2-fold lower than for the WT strain. (B) The P12 WT strain and MutE showed very close sensitivities to clarithromycin (MIC = 0.015 \(\mu\)g/ml, MBC = 0.06 \(\mu\)g/ml). (C) The sensitivity assay for tetracycline yielded the MIC and MBC values of 0.25 \(\mu\)g/ml and 1 \(\mu\)g/ml, respectively for both the WT strain and MutE. (D) The P12 WT strain and MutE showed the same sensitivity pattern for metronidazole (MIC = 0.5 \(\mu\)g/ml, MBC = 1 \(\mu\)g/ml).
resistance mechanisms. This result suggests that EZA kills \textit{H. pylori} via mechanisms that are different from the mode of action of current first-line antibiotics (amoxicillin, clarithromycin, metronidazole, and tetracycline) used to treat \textit{H. pylori} infections.

**Conclusion**

The sulphonamide drugs AAZ, MZA, and EZA displayed bactericidal activity against both laboratory strains and clinical antibiotic-resistant isolates of \textit{H. pylori}, with EZA showing the greatest activity. Importantly, the mechanism of action of EZA is different from that of conventional antibiotics used to treat \textit{H. pylori} infections, and is not restricted to impacting carbonic anhydrase function. Our findings suggest that EZA impacts multiple targets within \textit{H. pylori}, and that alterations in several functions may be required for resistance to emerge. Indeed, the frequency of spontaneous resistant mutants was found to be \( <5 \times 10^{-9} \), indicating that resistance does not develop easily. The low frequency of spontaneous resistant mutants was also in agreement with our observation that resistance to EZA incurred a significant fitness cost. EZA is an inexpensive and relatively safe drug that was used clinically since 1950s to treat various non-bacteria-related illnesses (e.g. glaucoma\(^{34}\)); its side effects are generally tolerable\(^ {15}\), and its pharmacokinetic properties are well understood. Taken together, our findings suggest that developing EZA into a more effective anti-\textit{H. pylori} agent may offer a faster and cost-effective route towards new antimicrobials with a novel mechanism of action, and that investigations of the in vivo antimicrobial action of this compound are warranted. A number of potential limitations to such approach need to be considered and addressed in follow-up studies. Activity of EZA against \textit{H. pylori} is relatively weak in comparison to existing antibiotics, and a systematic structure-activity relationship study is needed to determine if it can serve as a starting point for a drug discovery programme. In addition, it was beyond the scope of this work to examine potential antimicrobial activity of EZA against other bacteria, which leaves open the question of specificity towards \textit{H. pylori}.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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