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Article

Novel Drug-like HsrA Inhibitors Exhibit Potent Narrow-Spectrum Antimicrobial Activities against *Helicobacter pylori*

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Abstract: *Helicobacter pylori* infection constitutes a silent pandemic of global concern. In the last decades, the alarming increase of multidrug resistance evolved by this pathogen has led to a marked drop in the eradication rates of traditional therapies worldwide, encouraging scientific community to discover and develop new antimicrobial strategies. In the present work, we identified a battery of novel drug-like HsrA inhibitors with MIC values ranging from 0.031 to 4 mg/L against antibiotic-resistant strains of *H. pylori*. Most of these novel antimicrobials exhibited minor effects against both Gram-negative and Gram-positive species of human microbiota. The most potent anti-*H. pylori* candidate demonstrated a high therapeutic index, additive effect in combination with metronidazole and clarithromycin, as well as a strong antimicrobial action against *Campylobacter jejuni*, another clinically relevant pathogen of phylum *Campylobacterota*. Transcriptomic analysis suggested that *in vivo* inhibition of HsrA triggered lethal global disturbances in *H. pylori* physiology, including the arrest of protein biosynthesis, malfunction of respiratory chain, detriment in ATP generation, and oxidative stress. The novel drug-like HsrA inhibitors described here constitute valuable candidates to a new family of narrow-spectrum antibiotics that allow overcoming the current resistome, protecting from dysbiosis, and increasing therapeutic options for novel personalized treatments against *H. pylori*.

Keywords: *Helicobacter pylori*; antimicrobial resistance; narrow-spectrum antibiotic; antibacterial target; response regulator; HsrA; *Campylobacter jejuni*

1. Introduction

Helicobacter pylori, a pathogenic member of phylum *Campylobacterota* (formerly *Epsilonproteobacteria*) [1], is recognized as the leading cause of several human gastric pathologies, including acute and chronic gastritis, peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [2,3]. In addition, this microaerophilic Gram-

negative bacterium has been associated to some extragastric disorders such as idiopathic thrombocytopenic purpura, idiopathic iron deficiency anaemia, vitamin B₁₂ deficiency, cardiovascular and neurodegenerative diseases, among others [4]. *H. pylori* is presumably colonizing the gastric mucosa of more than half of the world population [5], causing chronic long-lasting inflammation that may progress to gastric cancer in about 1-3% of untreated or mistreated cases [6,7]. Despite *H. pylori* constitutes the only bacterial pathogen recognized as class I carcinogen by the International Agency for Research on Cancer [8], neither prophylactic nor therapeutic vaccines are currently available.

Effective treatment of *H. pylori* infection remains a challenge for clinicians and sanitary authorities worldwide [9–11]. Only a handful of antibiotics are currently used in clinical practice for *H. pylori* eradication, including metronidazole, clarithromycin, amoxicillin, tetracycline, and levofloxacin [12,13]. Due to the poor *in vivo* efficacy of antimicrobial monotherapies against this pathogen [14], current antibiotics are administrated as part of several aggressive and prolonged combinatory therapies which are commonly prescribed empirically, resulting frequently in *H. pylori* eradication failures, emergence of secondary antibiotic resistances, and refractory infections [15–18]. In the last two decades, the alarming increase of the antibiotic resistance levels to first-line and even “rescue” antibiotics, especially clarithromycin, metronidazole and levofloxacin [19–21], has led to a marked decrease of the eradication rates of traditional therapies [22,23]. This fact prompted the World Health Organization (WHO) to define *H. pylori* as a high priority pathogen in the current global efforts to R&D of novel antimicrobials [24].

On the other hand, even when current antimicrobial therapies against *H. pylori* infections are generally safe, the prolonged administration of high doses of broad-spectrum antibiotics into combinatory therapies frequently result in dysbiosis [25–28]. In some cases, *H. pylori* eradication regimens might cause major disruption of normal gut microbiome, promoting severe complications such as pseudomembranous colitis due to *Clostridioides difficile* proliferation [29–32].

The discovery and development of narrow-spectrum antibiotics, also known as precision antimicrobials, exemplify a current accurate strategy for reducing or slowing down the appearance and propagation of antimicrobial resistance as well as minimizing undesirable side effects on normal microbiome [33–37]. Ideally, a precision antimicrobial exerts its action on molecular targets shared by only a reduced number of closely related pathogenic species, but not expressed by most representative commensal microbes [38,39].

In previous works, we have validated the essential protein HsrA as an effective therapeutic target for *H. pylori* infection [40–42]. HsrA is an OmpR/PhoB-type orphan response regulator [43,44], unique and highly conserved in members of phylum *Campylobacterota* [45], which appears involved in a variety of crucial physiological processes including transcription, translation, redox homeostasis, chemotaxis and energy metabolism [46–49], and thereby resulting indispensable for cell viability [50,51]. In the present study, we have identified several novel low-molecular weight inhibitors of HsrA, which fit the Lipinski’s rule-of-five for oral drug-likeness and exhibited potent bactericidal activities against *H. pylori*, with minor antimicrobial effects on both Gram-negative and Gram-positive species of commensal bacteria. At least one of these novel precision antimicrobials exerted also a strong antimicrobial action against *Campylobacter jejuni*, another clinically relevant pathogen of phylum *Campylobacterota*.

2. Results

2.1. High-Throughput Screening of the Maybridge HitFinder™ Chemical Library Identified Novel HsrA Ligands

The Maybridge HitFinder™ chemical collection, comprising 12,000 compounds that fit the Lipinski’s rule-of-five for oral drug-likeness [52,53], was screened for the identification of low-molecular weight ligands of the *H. pylori* essential response regulator HsrA. The high-throughput screening (HTS) of the chemical library was carried out by using a previously described fluorescent thermal shift-based method [40,54]. With this approach, those compounds from the library that

induced a thermostabilization of the protein in $\geq 5^{\circ}\text{C}$ compared to reference controls with dimethyl sulfoxide (DMSO, vehicle) were considered as HsrA ligands (Figure 1). According to this selection criterion, our HTS led to identify 31 drug-like small molecules from the Maybridge collection than acted as ligands of HsrA (Supplementary Table S1). For practical purposes, the HsrA ligands were identified by consecutive Roman numerals according to the magnitude of the melting temperature (T_m) upshifts that they induced after binding to the native state of the protein. Almost a third part of the HsrA ligands identified by the HTS induced T_m upshifts above 10°C . Notably, two HsrA-ligand complexes increased protein thermal stability in 20°C or more. Chemical structures and some physicochemical properties of most relevant HsrA ligands identified and evaluated in this work are showed in Table 1.

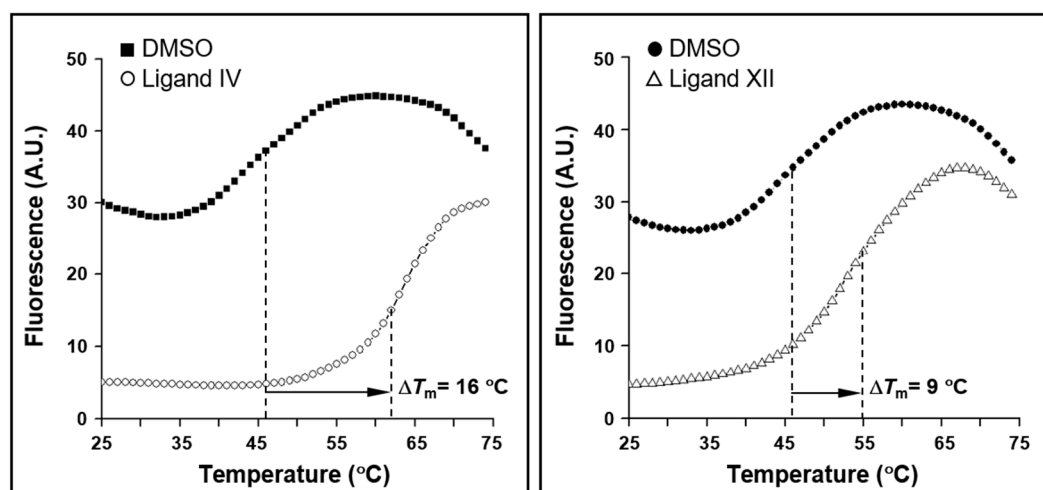

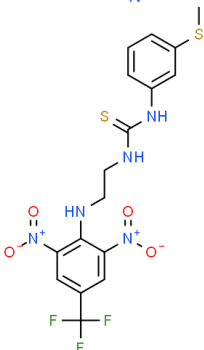
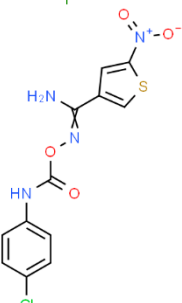
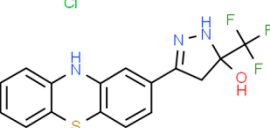


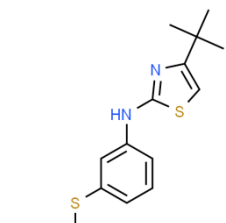
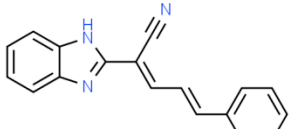
Figure 1. Upshifts in the thermal unfolding curve of response regulator HsrA triggered by ligands IV and XII. The high-throughput screening (HTS) of the Maybridge HitFinder™ chemical library for HsrA ligands was assessed by a fluorescence-based thermal shift assay. Mixtures of DMSO (vehicle) instead of compounds were used as reference controls. Any compound that preferentially binds to the native state of HsrA would increase protein stability and cause an increase of the midpoint temperature of unfolding (T_m). Those compounds that clearly increased the thermal stability of HsrA observed in reference controls ($T_m \geq 5^{\circ}\text{C}$) were considered as HsrA ligands.

2.2. Several Drug-like Ligands of HsrA Inhibited Its DNA Binding Activity In Vitro

The inhibitory capabilities of all HsrA ligands identified by the HTS on the *in vitro* DNA binding activity of the response regulator were evaluated by electrophoretic mobility shift assays (EMSA). Previous works have demonstrated an *in vitro* high affinity of HsrA for its target promoter $P_{porGDAB}$ in a concentration-dependent manner [40,41]. In the present work, titration of the HsrA activity confirmed that 120 ng of target DNA were completely and specifically complexed with at least $6\ \mu\text{M}$ of freshly purified recombinant protein under the experimental conditions used in EMSA (data not shown). Accordingly, mixtures of these amounts of protein and target DNA were incubated in the presence of increasing concentrations of each HsrA ligands, from $100\ \mu\text{M}$ to $2\ \text{mM}$. Twelve of the 31 low-molecular weight ligands of HsrA caused a noticeable inhibition of the *in vitro* DNA binding activity of this essential response regulator according to EMSA (Figure 2). Despite $2\ \text{mM}$ of any of these compounds was sufficient to completely inhibit the *in vitro* biological activity of $6\ \mu\text{M}$ of recombinant HsrA protein, the *in vitro* inhibitory effects of some ligands like IV and VIII appeared appreciably stronger than those exerted by other ligands such as XI and XXVI.

Table 1. Chemical structure and physicochemical properties of relevant HsrA ligands identified by a fluorescent thermal shift-based HTS of the Maybridge HitFinder™ chemical library.

Ligand	Chemical structure	Formula	Molecular Weight (Da)	log P^1	H-bond donors	H-bond acceptors	Lipinski violations	TPSA (Å) ²	Rotatable bonds	Log D^3
I		C ₁₁ H ₆ F ₃ N ₅ O ₂	297.19	1.56	1	7	0	113.45	3	2.26
IV		C ₁₇ H ₁₆ F ₃ N ₅ O ₄ S ₂	475.47	3.08	3	7	0	185.12	11	4.97
V		C ₁₂ H ₉ ClN ₄ O ₄ S	340.74	1.99	2	5	0	150.77	6	2.92
VIII		C ₁₆ H ₁₂ F ₃ N ₃ OS	351.35	3.50	3	5	0	81.95	2	3.60

XI		<chem>C14H18N2S2</chem>	278.44	4.18	1	1	0	78.46	4	4.77
XII		<chem>C18H13N3</chem>	271.32	3.53	1	2	0	52.47	3	3.79

Molecular properties obtained from the SwissADME web tool (<http://www.swissadme.ch/>) and the ChemSpider database (www.chemspider.com). ¹Log *P* values correspond to consensus Log *P*_{o/w} from SwissADME. ²TPSA, Topological Polar Surface Area. ³Log *D* values correspond to ACD/LogD (pH 7.4) from ChemSpider. .

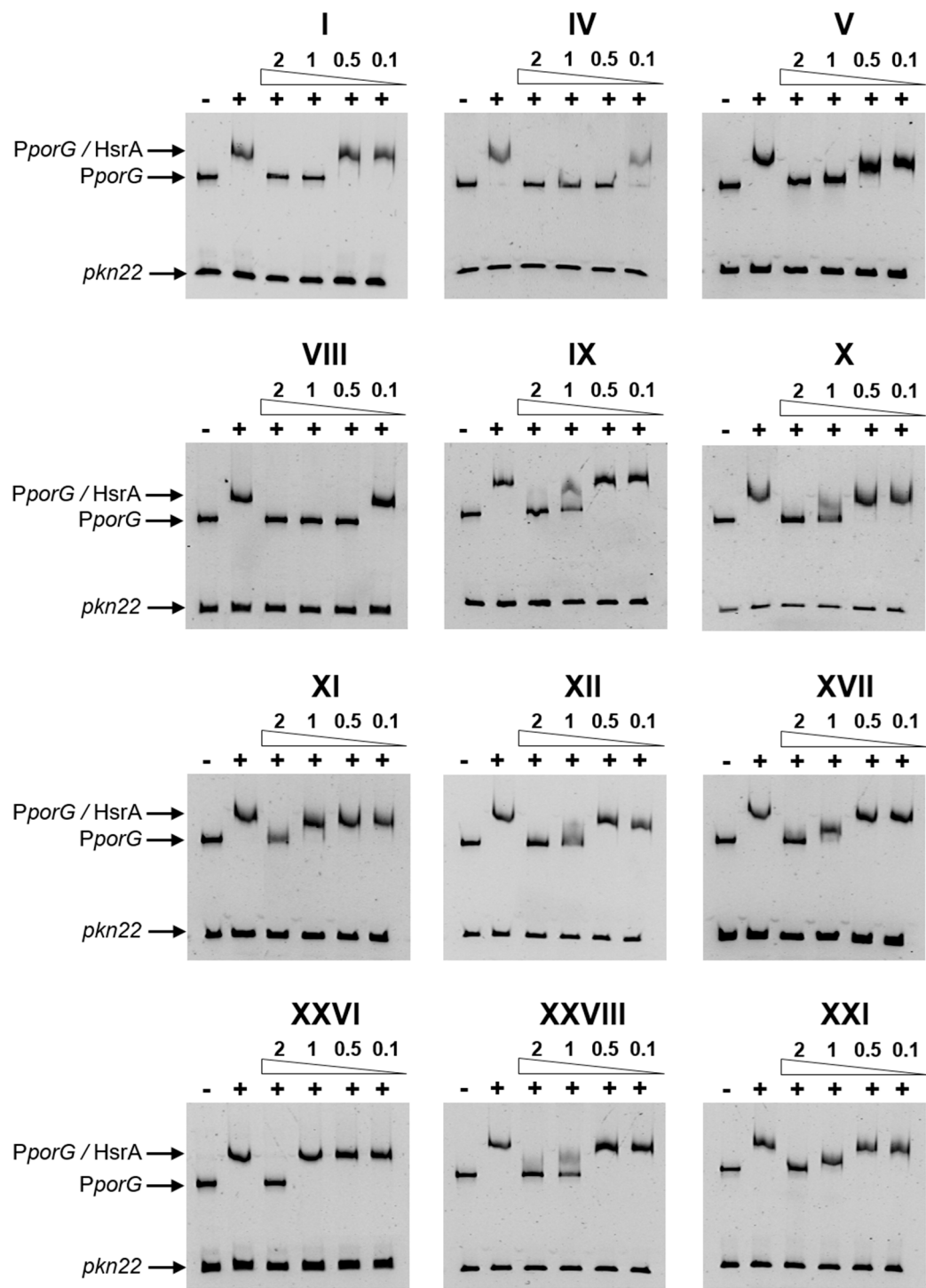


Figure 2. Specific *in vitro* inhibition of the DNA binding activity of response regulator HsrA by selected ligands, according to EMSA. Recombinant HsrA was mixed with its target promoter *PporG* in the presence of 2, 1, 0.5, and 0.1 mM of ligands. DMSO instead of inhibitors was included as vehicle controls, while an internal sequence of the *Anabaena* sp. *pkn22* gene was used as non-specific competitor in all assays. Addition of protein in the reaction mixtures are indicate by +. Protein-DNA interactions were analysed by 6% non-denaturing electrophoresis.

2.3. Novel HsrA Inhibitors Exhibited Potent Bactericidal Effects against *H. pylori*

The new twelve drug-like HsrA inhibitors identified by HTS and EMSA were evaluated for their antimicrobial properties against four different strains of *H. pylori*, including strains resistant to

clarithromycin (ATCC 700684), metronidazole (ATCC 43504), and levofloxacin (strain Donostia 2). As shown in Table 2, at least six HsrA inhibitors (I, IV, V, VIII, XI, and XII) exhibited strong bactericidal activities against all the *H. pylori* strains tested, showing minimal inhibitory concentration (MIC) values ranging from 0.031 to 4 mg/L. Notably, the HsrA ligand V appeared specially effective against *H. pylori* viability, exhibiting a bactericidal potency even stronger than those demonstrated by first-line antibiotics like metronidazole and levofloxacin against the same *H. pylori* strains. Other HsrA inhibitors, such as IX, X, XVII, and XXVI demonstrated moderate anti-*H. pylori* action (MIC = 8 - 16 mg/L), while inhibitors XXVIII and XXXI appeared poorly effective as antimicrobials (MIC ≥ 32 mg/L). No relevant differences were observed in the antimicrobial activities of these molecules with respect to the antibiotic-resistance pattern of the *H. pylori* strains used in the assays.

Table 2. Minimal inhibitory and bactericidal concentrations of HsrA inhibitors against different strains of *H. pylori*.

Inhibitor or drug	MIC (MBC), mg/L			
	<i>H. pylori</i> ATCC 700392 (26695)	<i>H. pylori</i> ATCC 43504 (MTZ-R)	<i>H. pylori</i> ATCC 700684 (CLR-R)	<i>H. pylori</i> Donostia 2 (LVX-R)
I	4 (4)	1 (1)	4 (4)	2 (2)
IV	2 (4)	2 (4)	4 (4)	4 (4)
V	0.063 (0.125)	0.125 (0.125)	0.031 (0.031)	0.125 (0.125)
VIII	1 (2)	1 (2)	1 (2)	2 (2)
IX	8 (16)	4 (8)	4 (8)	8 (16)
X	8 (16)	8 (8)	8 (8)	8 (16)
XI	4 (4)	2 (4)	2 (4)	2 (4)
XII	2 (2)	0.5 (0.5)	0.5 (0.5)	1 (1)
XVII	16 (16)	4 (8)	8 (8)	8 (8)
XXVI	16 (16)	8 (8)	8 (8)	8 (16)
XXVIII	32 (64)	16 (16)	32 (32)	32 (32)
XXXI	> 64 (> 64)	64 (64)	> 64 (> 64)	> 64 (> 64)
MTZ	1 (2)	64 (128)	1 (2)	8 (8)
CLR	< 0.03 (< 0.03)	< 0.03 (< 0.03)	16 (32)	< 0.03 (< 0.03)
LVX	0.12 (0.12)	0.5 (0.5)	0.12 (0.12)	16 (32)

MTZ-R, metronidazole-resistant strain. CLR-R, clarithromycin-resistant strain. LVX-R, levofloxacin-resistant strain.

To further characterize the bactericidal activities of most effective HsrA inhibitors, time-kill kinetic assays were carried out by exposing *H. pylori* strain ATCC 700392 to 4 × MIC of compounds I, IV, V, VIII, XI, and XII (Figure 3). Despite all compounds resulted completely lethal after 8 h of exposure, significant differences ($p < 0.05$) were noticed in the decline of bacterial counts produced by each HsrA inhibitor from 2 h of treatment. Thus, at the concentration used in this experiment, compounds V and VIII completely killed 10⁴ *H. pylori* cells after 2 h of exposure. The same total bactericidal effect was observed after 4 h for compounds IV, XI, and XII. No live bacteria could be detected after 8 h of exposure with compound I.

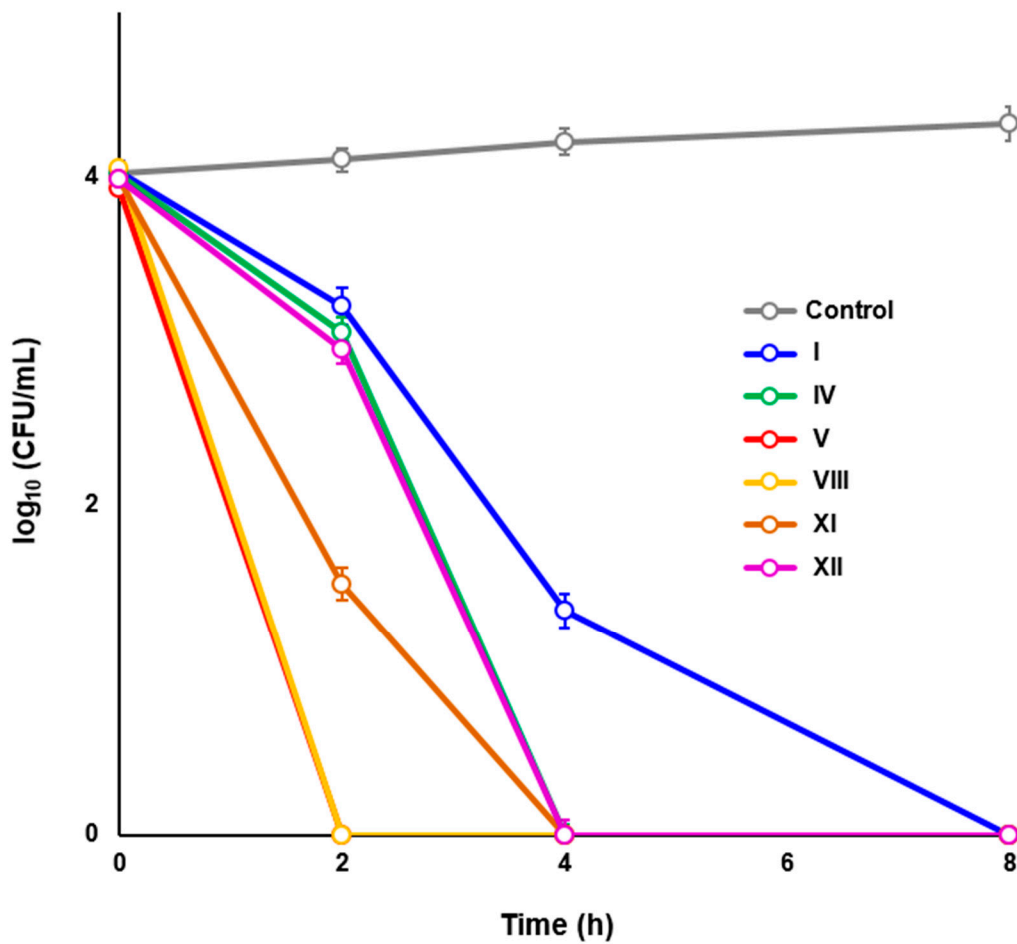


Figure 3. Time-kill kinetics of selected HsrA inhibitors against *Helicobacter pylori* strain ATCC 700392. Fresh grown cells of *H. pylori* (10^4 CFU/mL) were exposed to $4 \times$ MIC of each compound. Mixtures of bacteria with DMSO (vehicle) instead of inhibitors were used as controls. CFU were determined at time zero and after 2, 4, 8, and 24 h of exposure. Values are the means of three independent determinations; standard deviations are represented by vertical bars.

Putative combinatory effects in the antimicrobial action of these bactericidal HsrA inhibitors with first-line antibiotics including clarithromycin, metronidazole and levofloxacin against *H. pylori* were assessed by checkerboard assays. As described in Table 3, only compound XII exhibited synergistic effect in combination with metronidazole, while compound IV did not demonstrate interaction with any of the antibiotics analysed. The highly bactericidal compounds V, VIII, and XI interacted additively with both clarithromycin and metronidazole; however, all HsrA inhibitors resulted neutral in combination with levofloxacin.

Table 3. Interaction of selected bactericidal HsrA inhibitors with first-line antibiotics against *H. pylori* ATCC 700392.

Antibiotic ¹	HsrA inhibitor	FIC _{antibiotic}	FIC _{inhibitor}	FICI ²	Interaction ³
CLR	I	1	1	2	neutral
	IV	1	1	2	neutral
	V	0.5	0.06	0.56	additive
	VIII	0.5	0.03	0.53	additive
	XI	0.25	0.5	0.75	additive
	XII	0.25	0.5	0.75	additive
MTZ	I	0.125	0.5	0.625	additive

	IV	1	1	2	neutral
	V	0.125	0.5	0.625	additive
	VIII	0.5	0.125	0.625	additive
	XI	0.5	0.063	0.563	additive
	XII	0.25	0.25	0.5	synergism
LVX	I	1	1	2	neutral
	IV	1	1	2	neutral
	V	1	1	2	neutral
	VIII	1	1	2	neutral
	XI	1	1	2	neutral
	XII	0.5	0.5	1	neutral

¹CLR, clarithromycin. MTZ, metronidazole. LVX, levofloxacin. ²Fractional inhibitory concentration index (FICI) is calculated as: FIC_A (MIC_A in the presence of B/MIC_A alone) + FIC_B (MIC_B in the presence of A/MIC_B alone). ³According to the FICI value, the interaction between two compounds against a particular bacterial strain can be classified as synergism (FICI ≤ 0.5), additive (FICI > 0.5 to ≤1), no interaction or neutral (FICI > 1 to ≤4), and antagonism (FICI > 4).

2.4. An HsrA Bactericidal Inhibitor Bound the HsrA Orthologue Protein CosR and Exhibited Strong Bactericidal Effect against *C. jejuni*

Since HsrA is an essential response regulator unique, but also highly conserved among members of phylum *Campylobacterota*, those HsrA inhibitors that previously demonstrated strong bactericidal activities against *H. pylori* (MIC ≤ 4 mg/L) were additionally evaluated for their antimicrobial properties against other *Campylobacterota* pathogenic species, *C. jejuni*. Notably, the most effective HsrA inhibitor against *H. pylori*, ligand V, exhibited also a strong antimicrobial activity against *C. jejuni*, with MIC and MBC values ≤ 0.5 mg/L (Table 4). However, the antimicrobial effect of other HsrA ligands against *C. jejuni* appeared moderate (16 – 32 mg/L) or quite low (≥ 64 mg/L) for the rest of compounds.

Table 4. Antimicrobial activities of relevant HsrA inhibitors against *C. jejuni* and several representative members of normal human microbiota.

Inhibitor or drug	MIC (MBC), mg/L						
	<i>C. jejuni</i> ATCC 33560	<i>E. coli</i> ATCC 25922	<i>K. pneumoniae</i> ATCC 700603	<i>E. faecalis</i> ATCC 29212	<i>S. aureus</i> ATCC 29213	<i>S. epidermidis</i> ATCC 12228	<i>S. agalactiae</i> ATCC 12386
I	32 (32)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	32 (64)
IV	64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)
V	0.25 (0.5)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)
VIII	16 (32)	>64 (>64)	>64 (>64)	64 (64)	16 (32)	4 (4)	16 (32)
XI	>64 (>64)	>64 (>64)	>64 (>64)	64 (64)	16 (32)	16 (64)	8 (8)
XII	64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)
LVX	< 0.12 (< 0.12)	N.D.	0.5 (0.5)	N.D.	N.D.	N.D.	N.D.
AMP	N.D.	4 (4)	N.D.	4 (4)	2 (4)	4 (4)	0.25 (0.5)

LVX, levofloxacin. AMP, ampicillin.

Considering that the essential response regulator CosR constitutes the orthologue of HsrA in *C. jejuni* [44,45], we analysed the effect of ligand V on the *in vitro* DNA binding activity of CosR by EMSA. As depicted in Figure 4, the HsrA inhibitor V also caused a noticeable inhibition of the *in vitro* affinity of CosR by its target promoter *PsodB*, suggesting that this low-molecular weight compound acts as an inhibitory ligand of both orthologues, HsrA and CosR.

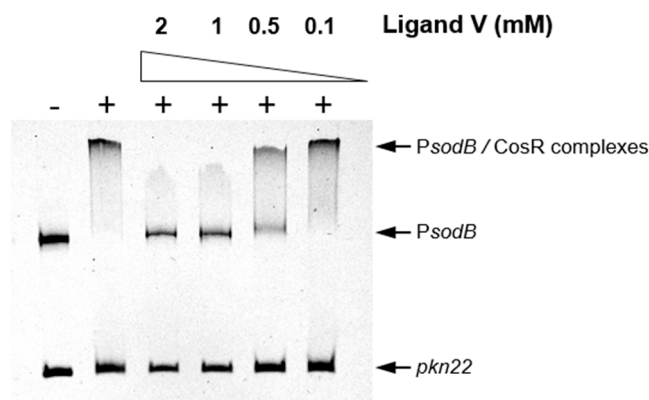


Figure 4. The HsrA ligand V noticeably inhibited the *in vitro* DNA binding activity of the *C. jejuni* response regulator CosR, an orthologue protein of HsrA. Recombinant CosR was mixed with its target promoter *PsodB* in the presence of 2, 1, 0.5, and 0.1 mM of ligand V. DMSO instead of inhibitors was included as vehicle controls, while an internal sequence of the *Anabaena* sp. *pkn22* gene was used as non-specific competitor in all assays. Addition of protein in the reaction mixtures are indicate by +. Protein-DNA interactions were analysed by 6% non-denaturing electrophoresis.

2.5. HsrA Bactericidal Inhibitors Exhibited Low Antimicrobial Actions against Gram-Positive and Gram-Negative Species of Human Microbiota

Furthermore, we evaluated the antimicrobial activities of most potent bactericidal anti-*H. pylori* inhibitors against several Gram-negative and Gram-positive representative species of the human normal microbiota. MIC and MBC values of HsrA ligands I, IV, V, VIII, XI, and XII against control strains of *E. coli*, *K. pneumoniae*, *E. faecalis*, *S. aureus*, *S. epidermidis*, and *S. agalactiae* were determined according to the EUCAST guidelines. As shown in Table 4, most of the HsrA inhibitors exhibited a narrow spectrum of action against *H. pylori*, and did not exerted relevant antimicrobial effects against all the microbiota species tested. Unexpectedly, compounds VIII and XI appeared particularly effective against *Staphylococcus* sp. and *Streptococcus* sp. strains, which could suggest cross-inhibition of specific molecular targets expressed by these bacterial species.

2.6. Cytotoxicity and Therapeutic Index

As a preliminary study of toxicity, cytotoxicity effects of highly bactericidal anti-*H. pylori* inhibitors I, IV, V, VIII, XI, and XII were evaluated on HeLa cells. Mammalian cells were exposed during 24 h to increasing concentrations of each compound, from 0.125 to 128 mg/L, and cell viability was measured by the PrestoBlue™ method. None of the six HsrA inhibitors resulted cytotoxic for HeLa cells under 2 mg/L, while compounds I, IV, V, and XI seemed completely safe even at 8 mg/L (Figure 5A). Compound VIII proved to be the most cytotoxic among the highly bactericidal HsrA ligands, causing 68% of cell death at 8 mg/L. Notably, the 50% cytotoxic concentrations (CC₅₀) of compounds I, IV and V were 30 times or higher their values of MIC (Figure 5B), which supposes wide therapeutic windows of these novel anti-*H. pylori* antimicrobial candidates. Surprisingly, the most potent bactericidal HsrA ligand was also the safest candidate.

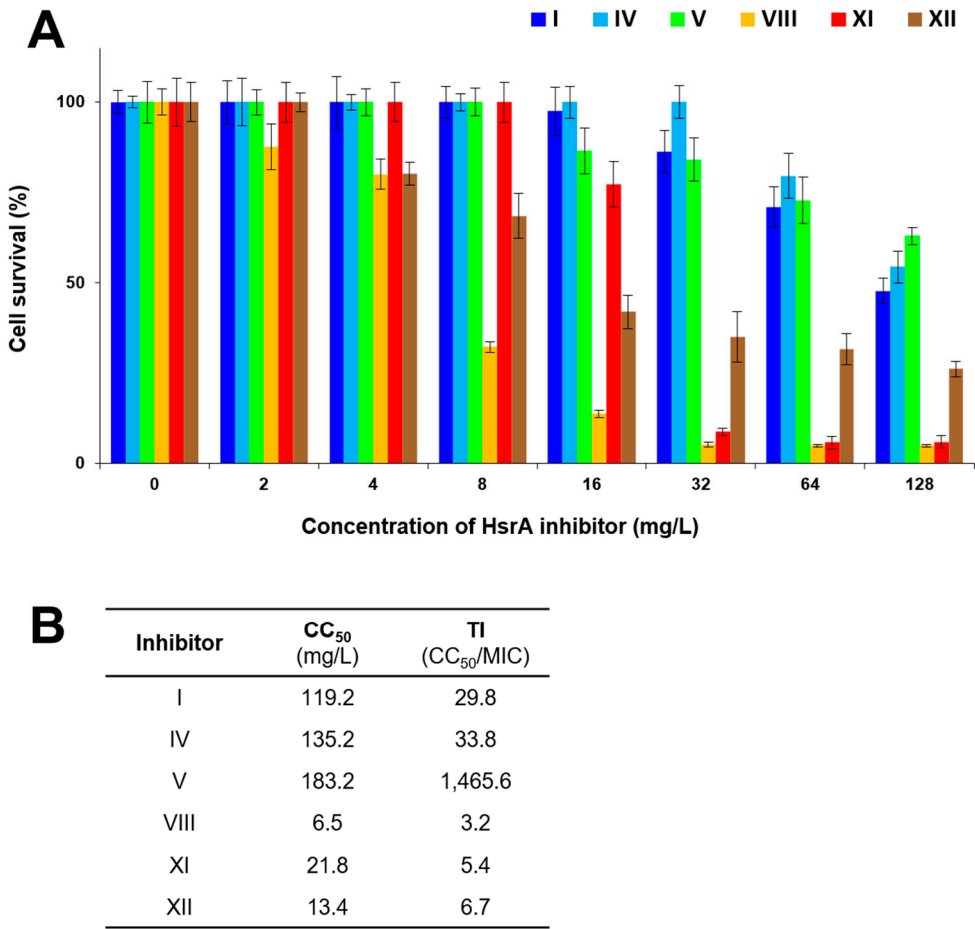


Figure 5. Cytotoxicity and therapeutic index of selected highly bactericidal HsrA inhibitors. **(A)** Cytotoxicity of HsrA inhibitors I, IV, V, VIII, XI, and XII toward HeLa cells was assessed at 24 h of exposure through the PrestoBlue™ method. Experiments were performed twice in triplicate, vertical bars represent standard deviations. **(B)** The 50% cytotoxic concentration (CC₅₀) was defined as the ligand concentration that reduced the viability of DMSO (vehicle) - treated cell cultures by 50%. Therapeutic index (TI) of each compound was calculated as the ratio between CC₅₀ and MIC.

2.7. Thermodynamics and Predicted Models of HsrA-Inhibitor Interactions

Isothermal titration calorimetry (ITC) experiments and molecular docking analyses were combined in order to achieve a better understanding of thermodynamic, stoichiometry and putative structures of HsrA-inhibitor complexes. As shown in Table 5, all highly bactericidal inhibitors of HsrA appeared to interact with amino acid residues involved in the helix-turn-helix (HTH) DNA binding motif of the regulator and their immediate environments, following a 1:1 stoichiometry. Thus, each HsrA monomer binds one molecule of inhibitor with dissociation constants in the micromolar range. Ligands XI and XII exhibited significant lower binding affinities to HsrA according to their *K_d* values (Table 5, Supplementary Figure S1). ITC experiments also confirmed the molecular interaction of highly bactericidal ligand V with the orthologue of HsrA in *C. jejuni*, the orphan response regulator CosR (Supplementary Figure S2). This low-molecular weight inhibitor of HsrA bond to CosR with moderate affinity, showing a dissociation constant in the micromolar range, and following a 1:1 stoichiometry.

Table 5. Thermodynamic parameters and interacting amino acid residues of selected HsrA-inhibitor complexes, according to ITC and molecular docking analyses.

Inhibitor	ITC ¹			Molecular Docking ²	
	n	K _d (μM)	ΔH (kcal/mol)	ΔG (kcal/mol)	Interacting Residues
I	1.0	13	−1.2	−6.7	I121, I123, L126, I135, L152, L155, A156, R159, M195, L199 , T203
IV	0.9	3.9	−1.6	−7.4	I135, V142, V144, K145, G146, P148, L152, K194, P198
V	1.0	13	−1.9	−6.7	L126, I135, L152, K194, M195, P198, L199 , T203
VIII	0.9	10	0.8	−6.8	V144, K145, G146, F149, L152, I191, K194, M195
XI	1.1	49	−3.1	−5.9	I123, I128, L152, R159, D160, M195, L199 , T203, C215, Y216
XII	1.0	43	0.8	−6.0	I123, L126, I128, I135, L152, M195, P198, L199 , T203

¹Relative error in K_d is 15%, absolute error in ΔH is 0.4 kcal/mol, absolute error in ΔG is 0.1 kcal/mol. ²Amino acid residues directly involved in forming the helix-turn-helix (HTH) DNA binding motif of HsrA are highlighted in bold fonts.

As previously observed with other HsrA low-molecular weight ligands [42], the novel drug-like inhibitors described here presumably interact with the C-terminal DNA binding domain of HsrA at a putative binding pocket, predominantly shaped by the nonpolar residues I121, I123, I126, I135, V142, V144, G146, P148, F149, L152, M195, P198, and L199, with few polar amino acids such as Y137, K145, and K194. Inside this predicted binding pocket, the best-ranked pose of the most effective HsrA bactericidal inhibitor against *H. pylori*, ligand V, appears to establish several non-covalent interactions with neighbouring amino acids that boosts the stabilization of the ligand-protein complex (Figure 6). Thus, the phenyl ring of ligand V establishes CH/π interactions with residues Leu126, Thr203, Leu199 and Leu152 (Figure 6C). In addition, the hydroxyl group in the side chain of Thr203 interacts through hydrogen bonding with the carbonyl group of ligand V, and through halogen bonding with the chloride substituent located in *para*-position of the ligand phenyl group. A series of hydrophobic interactions are formed between the heterocyclic thiophene group of ligand V and residues Lys194, Met195, Leu152, and Ileu135. Furthermore, the NH₂ group of ligand V establishes a hydrogen bond with carbonyl group of Lys194, while the aromatic ring of Pro198 could interact with thiophene through perpendicular π/π stacking at 4.5 Å of distance. In the best ranked-pose of ligand V into the pocket of the C-terminal DNA binding domain of HsrA, the nitro group at C2 position in the thiophene of the ligand appears located very outside the binding pocket and therefore too far away from the amino groups of Leu152 and Lys194 to may establish any interaction (Figure 6A,C).

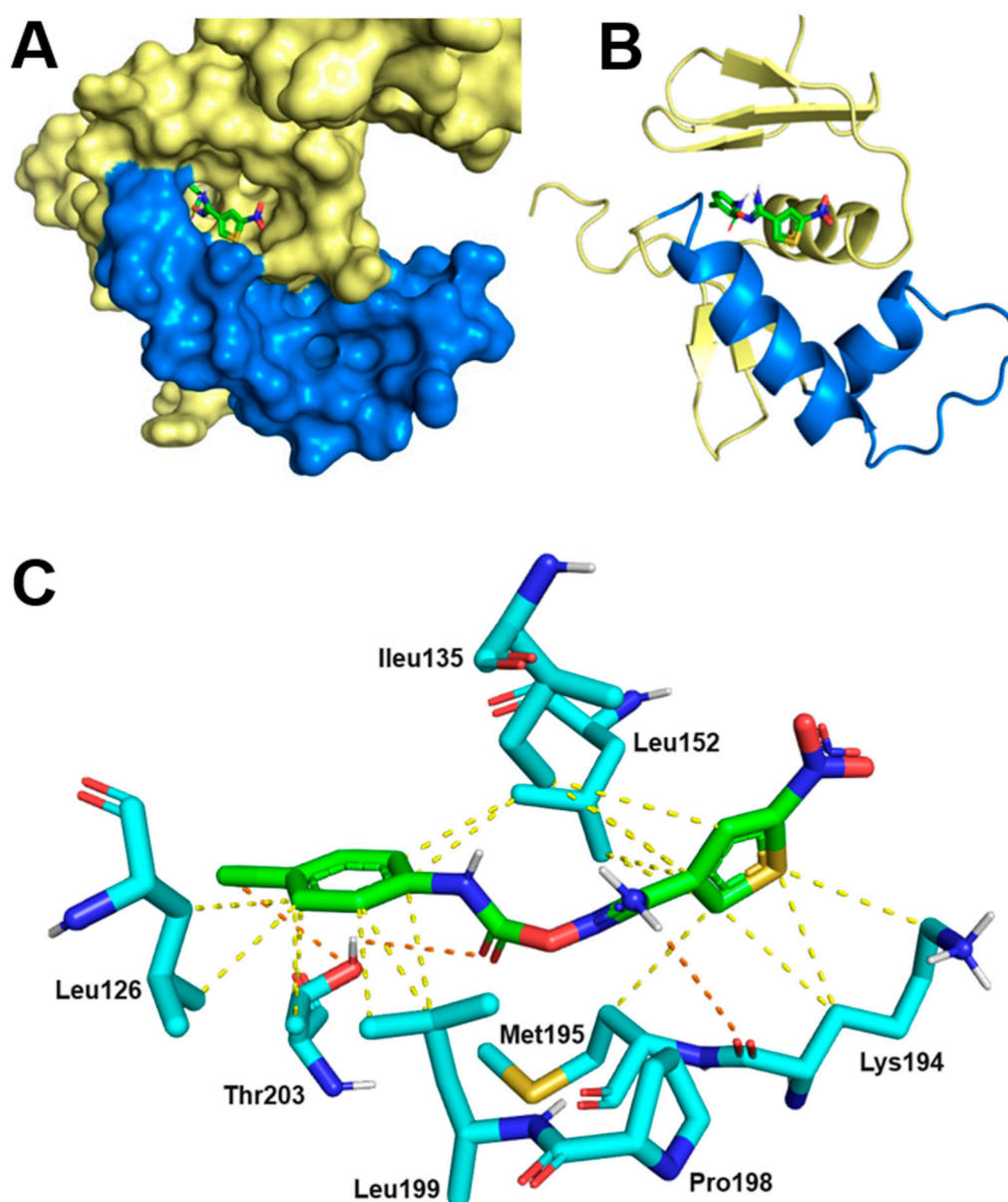


Figure 6. Predicted model of molecular interaction between HsrA and ligand V. (A) 3D view (molecular surface) and (B) ribbon diagram of the HsrA effector domain and the best-ranked docking pose of ligand V. The helix-turn-helix (HTH) DNA binding motif has been highlighted in blue. (C) Detailed view of the amino acid residues directly involved in the interaction of HsrA with ligand V. Hydrophobic interactions are indicated with yellow dashed lines, H-bonds and halogen bonds are indicated with orange dashed lines.

2.8. *In Vivo* Inhibition of HsrA Uncovered New Insights into the Essential Role of this Orphan Response Regulator

The inhibitory effect of the highly bactericidal inhibitor V on the essential transcriptional regulatory role of HsrA *in vivo* was assessed by RNA-seq. For this purpose, $\sim 3 \times 10^7$ CFU/mL freshly grown *H. pylori* 26695 cells were exposed to lethal concentrations of compound V ($4 \times \text{MIC}$) until \log_{10} CFU/mL resulted diminished in one unit (~ 2 h of exposure). As depicted in Figure 7, the transcriptomic analysis suggested that the *in vivo* inhibition of HsrA induced a global misregulation of *H. pylori* gene expression, which appears conducive to cell death as a result of several serious and possibly synergistic physiological alterations.

Treatment with the bactericidal HsrA inhibitor V significantly changed the transcript levels of 367 ORF (absolute \log_2 fold change > 1 , p -value < 0.05), of which 212 genes appeared upregulated

and 155 genes resulted in downregulation, as compared with control samples (Figure 7A,B, Supplementary Table S2). Thus, *in vivo* HsrA inhibition seems to influence, directly or indirectly, the expression of 23% of ORFs encoded by the *H. pylori* 26696 genome [55,56]. Among the 268 differentially expressed genes (DEGs) with defined functions, two functional categories were highly enriched with downregulated genes involved in essential physiological processes: (1) ribosome biogenesis, and (2) electron transfer and oxidative phosphorylation (Figure 7C, Supplementary Table S3).

At least 29 ribosomal structural proteins and 7 enzymes involved in ribosome biogenesis resulted strongly downregulated upon exposure of *H. pylori* cells to lethal concentrations of HsrA inhibitor V. In fact, some of these proteins corresponded with those DEGs with major decrease in transcript abundance, including the 50S ribosomal proteins L21, L35, and L27, but also the 30S ribosomal protein S10, all of which exhibited > 5.8-fold depletion in gene expression (Figure 7B,C, Supplementary Table S3). Notably, depletion in ribosomal protein abundances was accompanied by a noticeable increase in ribosomal RNAs transcription (Figure 7B,C, Supplementary Table S3). Taking into account that rRNA genes have been traditionally used as housekeeping controls in *H. pylori* due to their relatively constant rates of expression under very different environmental conditions, we hypothesize that the marked increase in rRNAs observed here could be a pleiotropic adaptive response of cell in an attempt to overcome the lethal disruption of ribosome biogenesis. In addition, the arrest of translation as a consequence of ribosome deficit could also explain the significant increase in tRNAs biosynthesis as well as in a variety of enzymes involved in amino acids metabolism. However, these hypotheses do not rule the possibility that some of these DEGs actually constitute unrecognized or little studied direct targets of HsrA transcriptional regulation [49].

In vivo inhibition of HsrA led also to decrease of transcript amounts of its previously recognized target *cyt553* [49]. Moreover, other components of the respiratory chain and coupled oxidative phosphorylation, including subunit NuoN of NADH-quinone oxidoreductase, cytochrome b subunit of fumarate reductase (FrdC), and subunits A, B, B', and D of ATP synthase also diminished their transcription (Supplementary Table S3).

Exposure of *H. pylori* to the bactericidal HsrA inhibitor V induced the expression of antioxidant enzymes and chaperones including *katA*, *sodB*, *ccp*, *dnaK*, *groEL*, *clpB*, *grpE*, and *hspG*, among other oxidative stress response proteins (Supplementary Table S3). Notably, the *H. pylori* nonhaem iron-containing ferritin (*pfr*, *hp0653*) and the ferritin-like iron-binding protein NapA appeared 4.7- and 2.3-fold upregulated, respectively, while the high-affinity ferrous iron transporter FeoB [57] was ~2.5-fold downregulated. Ferritin is required to sequester cytoplasmic free Fe²⁺ in order to minimize the formation of highly reactive hydroxyl radicals by the Fenton reaction, playing a major role in protection against oxidative stress in *Campylobacterota* [58,59]. A similar free-iron removal role has been described for the neutrophil-activating protein NapA in *H. pylori* [60,61].

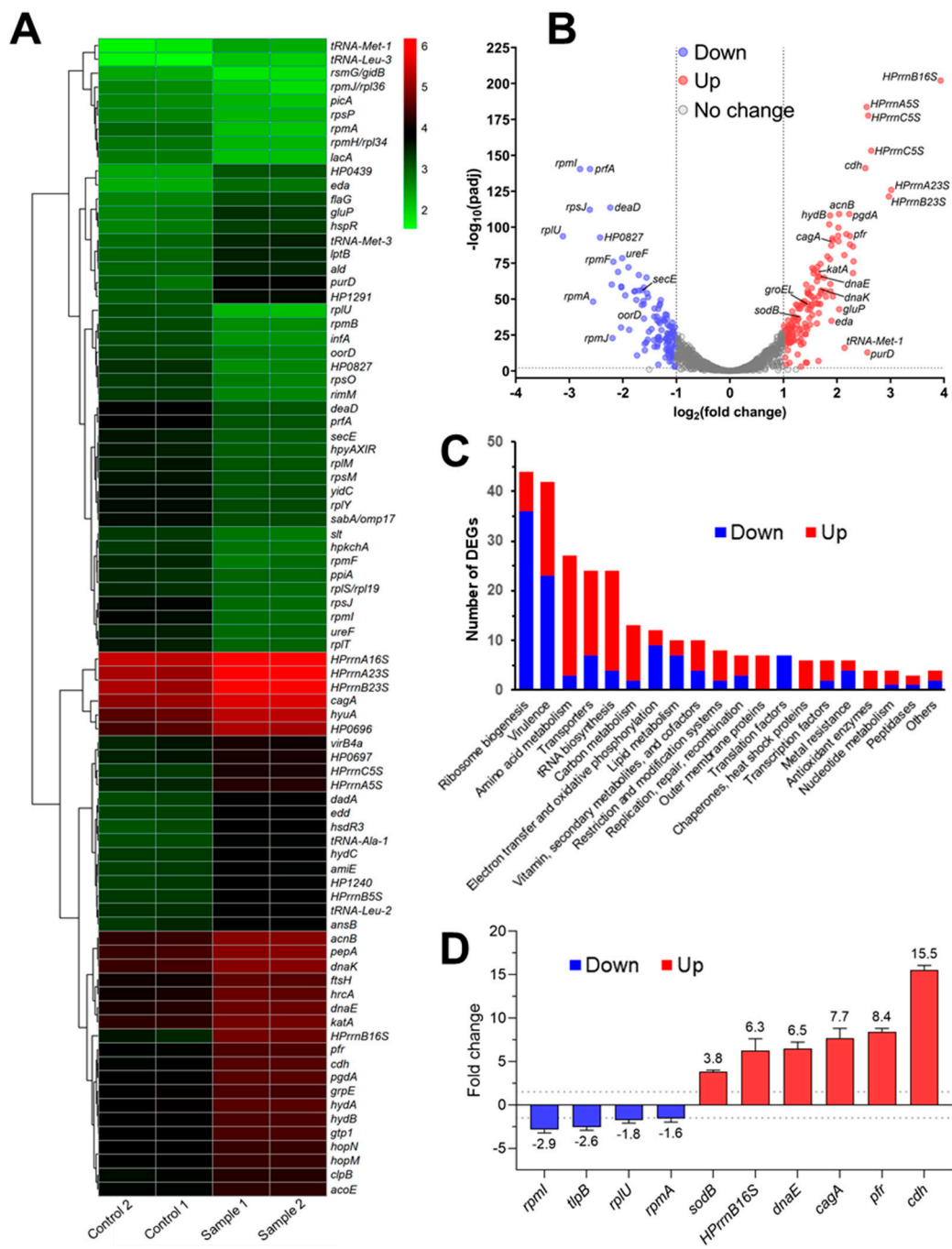


Figure 7. Transcriptomic analysis of *H. pylori* cells exposed to lethal concentrations of HsrA bactericidal inhibitor V. Fresh cultures of *H. pylori* 26695 adjusted to 10^7 CFU/mL were exposed in triplicate to $4 \times \text{MIC}$ of HsrA inhibitor V and incubated under controlled conditions (microaerobiosis, 37°C) until \log_{10} CFU/mL resulted diminished in one unit. **(A)** Heat map showing hierarchical cluster analysis of selected differentially expressed genes (DEG, absolute \log_2 fold change > 1 and p -value < 0.05). Each row represents a gene, and each column represents a sample. Red and green indicate up-regulated and down-regulated genes, respectively. The gradient colour barcode at the top right indicates the \log_{10} normalized hit counts. **(B)** Volcano plot indicating distribution of DEGs. **(C)** Distribution of up-regulated (column in red) and down-regulated (column in blue) known-function genes by functional categories. **(D)** qPCR analysis of selected genes. Dashed grey lines indicate fold change cut-off values of ± 1.5 .

Unexpectedly, transcriptomic analysis revealed that at least 42 genes related to *H. pylori* pathogenicity significantly changed their expression upon *in vivo* inhibition of HsrA, including *cag*

pathogenicity island genes, flagellar proteins, adhesins, toxins and enzymes, among others (Figure 7C, Supplementary Table S3). In addition, a plethora of genes involved in other functional categories such as carbon and lipid metabolisms, biosynthesis of vitamins and cofactors, nucleotide metabolism, transcription and translation factors, DNA replication and repair, outer membrane proteins and membrane transporters, as well as metal-resistance, exhibited significant changes in their transcript abundances.

To validate the transcriptome analysis, we evaluated the transcription levels of 10 selected genes, including both upregulated and downregulated DEGs according to RNA-seq. As shown in Figure 7D and Supplementary Table S3, all the genes evaluated by qPCR showed the same variation trend observed in RNA-seq upon *in vivo* inhibition of HsrA.

3. Discussion

Infection with *H. pylori* constitutes a silent pandemic of global concern. About 50% of the world's population is estimated to be infected with this carcinogenic bacterial pathogen [5,62], although this prevalence reaches 80% or more in several countries of Americas, Eastern Mediterranean, and Eastern Europe [63]. *H. pylori* is considered the most important risk factor for development of gastric cancer [7], the fifth most common malignancy and the fourth leading cause of all cancer-related deaths worldwide [64]. Despite eradication of *H. pylori* infection significantly reduces both incidence and mortality of gastric cancer [65–67], the increasing resistome accumulated by this pathogen in the last years has led to a considerable drop in the efficacy of current eradication therapies [10,68,69]. To face this concerning trend, efforts have been made to discover novel treatment strategies.

We previously validated the essential OmpR-like orphan response regulator HsrA as an effective therapeutic target against *H. pylori* [40–42]. HsrA affinity-based high-throughput screening (HTS) of 1,200 clinically approved drugs from Prestwick Chemical Library® revealed that some highly prescribed 1,4-dihydropyridine (DHP) calcium channel blockers, as well as several natural flavonoids, acted as HsrA low-molecular weight ligands leading to inhibition of the *in vitro* DNA-binding activity of the regulator. Some of these potentially repurposable drugs exhibited noticeable bactericidal effects against different antibiotic-resistant strains of *H. pylori* with MIC ranging 4 - 16 mg/L [40,41], and significantly decreased gastric colonization of *H. pylori* in mice [41]. Notably, flavonoids such as chrysin and hesperetin demonstrated strong synergistic bactericidal action against *H. pylori* in combination with clarithromycin or metronidazole, supporting the potential inclusion of these natural phytochemicals as valuable adjuvants in novel eradication therapies [40,70].

In the present work, we have identified novel low-molecular weight inhibitors of HsrA through the screening of 12,000 drug-like compounds from the Maybridge HitFinder™ chemical library. The collection is highly compliant with the Lipinski's rule-of-five, a rule of thumb to evaluate drug-likeness of novel potential medicines [52,53]. According to the Lipinski's rule-of-five, those compounds with molecular weight < 500 Da, calculated logarithm of the octanol-water partition coefficient (clog *P*) < 5, number of hydrogen bond acceptors ≤ 10, and number of hydrogen bond donors ≤ 5, will have better oral bioavailability and pharmacokinetics. Thus, molecules that violate more than one of these physicochemical parameters may show problems in terms of bioavailability after oral administration.

At least six of the novel drug-like HsrA ligands identified by affinity-based HTS of the HitFinder™ collection, denoted as I, IV, V, VIII, XI and XII, noticeably inhibited the DNA binding activity of the response regulator *in vitro* and demonstrated strong bactericidal activities against antibiotic-resistant strains of *H. pylori*. With MIC values ranging from 0.031 to 4 mg/L, some of these novel antimicrobial candidates constituted the most potent bactericidal HsrA inhibitors discovered to date. In addition, at least four of these highly bactericidal HsrA ligands exhibited narrow-spectrum antimicrobial activities against *H. pylori*, with MBC values ≥ 64 mg/L against several representative Gram-positive and Gram-negative members of normal human microbiota. Since protein HsrA is unique among members of phylum *Campylobacterota* (former class *Epsilonproteobacteria*) [45], the results suggest that certain low-molecular weight inhibitors of HsrA could specifically bind this response regulator without affecting biological activities of essential proteins from other bacterial

species. These evidences push up the value of HsrA as an effective and selective therapeutic target for development of novel precision antibiotics against *H. pylori*, moving away the risk of dysbiosis observed with current eradication therapies [28,31].

Notably, one of the novel drug-like HsrA inhibitor described here, denoted as ligand V and named N'-[[[(4-chloroanilino)carbonyl]]oxy-5-nitrothiophene-3-carboximidamide at the ChemSpider database (<https://www.chemspider.com>), showed significantly higher antimicrobial activities than the rest of compounds against all *H. pylori* strains used in the study. With MIC values ranging from 0.031 to 0.125 mg/L, the antimicrobial potency of ligand V was at least 4-fold higher than the second most active bactericidal HsrA inhibitor, the ligand XII, and more than 10-fold stronger than metronidazole against the same strains. In addition, ligand V demonstrated the highest therapeutic index among all highly bactericidal HsrA inhibitors described here, and showed additive antimicrobial action in combination with clarithromycin and metronidazole.

Several factors could be contributing to the higher antimicrobial potency exhibited by ligand V on *H. pylori* cells. For instance, a favourable balance among the physicochemical properties of this compound could make a difference in the microbial membrane translocation and intracellular concentration compared with other HsrA bactericidal ligands. In this context, the lipophilicity/hydrophilicity ratio, the molecular weight, the structural flexibility (rotatable bonds), the number of H-bond donors and acceptors, but also the polar surface area of small molecules may greatly influence their permeability through bacterial membranes as well as their possible efflux outside the pathogen [71–74]. Despite the contribution of each physicochemical property to membrane permeation and efflux varies among different bacteria species [74], it is well recognized that the polar nature of the outer membrane of Gram-negative pathogens hinders passive translocation of highly hydrophobic molecules [71,75]. Thus, decreasing the lipophilicity/hydrophilicity ratio of a low-molecular weight inhibitor of *E.coli* DNA gyrase by reducing logD from 2.59 to 1.75 led to 4-fold increase in its antibacterial potency [72]. Likewise, fine-tuning the physicochemical properties of a topoisomerase inhibitor in *Pseudomonas aeruginosa* by lowering logD from 2.0 to 0.9 resulted in 8-fold increase of its antibacterial activity [75]. On the other hand, Gram-negative pathogens express a plethora of broad-specific efflux pumps that actively export a variety of foreign molecules out of the cell, thereby decreasing the effective antibiotic concentration in the cytoplasm [76]. Some evidences suggested that highly polar and small compounds or very large and zwitterionic molecules are less susceptible to efflux [71].

Herein, time-kill kinetic studies demonstrated that ligand V and VIII produced a significant faster decline in bacterial counts compared to the rest of HsrA bactericidal inhibitors, suggesting similar permeability through *H. pylori* membrane. However, the polar surface area (TPSA) of ligand V almost duplicates this property in ligand VIII, which could be linked with a higher propensity of the last compound to be expelled by *H. pylori* efflux pumps. In fact, almost 30 genes related to different efflux pumps have been identified in the *H. pylori* genome [77].

The antimicrobial potency of HsrA ligands could also be influenced by the binding affinity of each molecule to its target protein. According to our ITC studies, the interaction of ligand V with HsrA occurs with > 3-fold higher affinity than the interaction of ligand XII, the second most potent bactericidal compound. Despite ligand IV bound HsrA with the highest affinity, its larger molecular weight and lipophilicity could negatively affect membrane permeation.

Besides its potent antimicrobial activity against *H. pylori*, ligand V stood up for its strong bactericidal effect against *C. jejuni*, another clinically relevant pathogen of phylum *Campylobacterota*. *C. jejuni* is considered the leading cause of food-borne bacterial gastroenteritis worldwide [78]. Infection in humans usually occurs by consumption of contaminated foods (mainly poultry) or direct contact with animal hosts (including pets) and environmental reservoirs [79,80]. Despite most cases of human campylobacteriosis produces mild and self-limiting diarrhoea and may not require antimicrobial therapy [78,81], the use of antibiotics could be necessary to shorten the duration of illness and to prevent severe complications in more susceptible populations, including very young children, elderly people and immunocompromised patients [78,82]. In addition, antibiotics must be

used in cases of invasive or extra-gastrointestinal manifestations such as meningitis, bacteraemia and endocarditis, among others [81,83,84].

When antibiotics are needed, the recommended first-line treatment of campylobacteriosis consists of macrolides such as azithromycin and erythromycin [85]. Fluoroquinolones like ciprofloxacin can be used as alternative therapy, though intravenous aminoglycosides or carbapenems are recommended in severe systemic cases. Tetracyclines and chloramphenicol are also alternative therapies, but they are usually avoided in young children due to their adverse effects [82,85]. As in the case of *H. pylori*, the increasing rates of antibiotic resistance in *Campylobacter* strains isolated from humans and animals worldwide, especially to fluoroquinolones but also to macrolides [86–88], prompted the WHO to include *C. jejuni* as a high priority pathogen in the search of novel antimicrobials [24].

Among all the novel drug-like HsrA bactericidal inhibitors described in this work, only the ligand V exhibited a strong antimicrobial effect on *C. jejuni*, with MIC = 0.25 mg/L. This antimicrobial potency is similar to that observed with ciprofloxacin, and stronger than those observed with doxycycline, erythromycin and gentamicin against the same *C. jejuni* strain used herein [89]. Molecular interaction of ligand V with CosR was demonstrated by ITC analysis, while this compound noticeably inhibited the *in vitro* DNA binding activity of CosR according to EMSA experiments. Hence, the strong antimicrobial activity of ligand V on *C. jejuni* appears to rely on the inhibition of the essential biological activity of CosR [90–93], thereby validating this protein as an effective therapeutic target against *C. jejuni* [44]. The dual inhibition triggered by the same low-molecular weight ligand on the biological activities of HsrA and CosR could be expected given the high sequence identity of both orthologue proteins, especially in their C-terminal DNA binding domains (60% overall identity, 85% identity in the effector domain) [45].

Transcriptomic analysis was carried out in order to discern the global effects of lethal concentrations of ligand V on the *H. pylori* physiology. The results observed here appeared in correspondence with an effective *in vivo* inhibition of HsrA, and confirmed previous experimental evidences of Pelliciani and colleagues [49] about the key role of this essential response regulator in the control of protein biosynthesis and energy metabolism. Exposure of *H. pylori* to the highly bactericidal HsrA inhibitor V particularly disturbed transcription of genes involved in ribosome biogenesis [94]. The strong detriment observed in the expression of a plethora of ribosomal proteins could certainly arrest translation and protein biosynthesis leading to cell death [95]. In addition, the *in vivo* inhibition of HsrA led to a significant decrease in the transcription of several components of the respiratory chain and ATP synthase. Proteome imbalance in the electron transport chain could impair redox reactions and proton translocation across cytoplasmic membrane, shrinking the transmembrane electrochemical proton gradient that drives the synthesis of ATP [96,97]. Furthermore, disruption of ATP biosynthesis could be additionally exacerbated by an altered expression of ATP synthase subunits. On the other hand, imbalance in the respiratory chain may increase the production of reactive oxygen species leading to oxidative stress [98]. This deleterious condition could induce the expression of antioxidant enzymes, heat shock proteins, and other molecular chaperones [99,100], a transcriptional response observed in this work upon exposure of *H. pylori* to the HsrA inhibitor V. Taking together, the bactericidal effect triggered by the *in vivo* inhibition of the essential regulatory role of HsrA seems to be a consequence of synergistic deleterious disturbances in the *H. pylori* physiology, including the arrest of protein biosynthesis, malfunction of respiratory chain, detriment in ATP generation, and oxidative stress.

Due to the unsuccessful attempts to manipulate the expression of the essential *hsrA* gene *in vivo* [45,47], the elucidation of the HsrA regulon has represented a challenge. Previous studies have identified several dozens of putative target genes and a handful of well-characterized targets [46–49]. Our transcriptomic data suggest that HsrA acts as a global transcriptional regulator, modulating directly or indirectly the expression of up to 23% of the ORFs encoded by the *H. pylori* 26696 genome. Further analyses must be conducted in order to completely unravel the global regulatory role of HsrA and its crucial contribution to viability and pathogenicity of *H. pylori*.

In conclusion, the results presented here strongly support the use of HsrA and its orthologue proteins as effective and selective therapeutic targets for development of novel antimicrobial strategies against clinically relevant pathogenic members of phylum *Campylobacterota*. The novel drug-like highly bactericidal inhibitors of HsrA described here constitute valuable candidates to a new family of narrow-spectrum antibiotics that allow overcoming the current resistome, protecting from dysbiosis, and enhancing the battery of therapeutic options for novel personalized treatments against *H. pylori*.

4. Materials and Methods

4.1. Bacterial Strains and Culture Conditions

H. pylori reference strains ATCC 700392 (aka 26695), ATCC 43504 (metronidazole-resistant), and ATCC 700684 (clarithromycin-resistant), as well as *Campylobacter jejuni* ATCC 33560 were purchased from the American Type Culture Collection (Rockville, MA, USA). The *H. pylori* strain Donostia 2 (levofloxacin-resistant) was isolated from gastroduodenal biopsies and kindly donated by Dr. Milagrosa Montes from Donostia University Hospital (San Sebastian, Spain). *Helicobacter* sp. and *Campylobacter* sp. strains were routinely grown in Blood Agar Base No. 2 (OXOID, Basingstoke, UK) supplemented with 8% defibrinated horse blood (OXOID) at 37 °C for 48-72 h, under microaerobic conditions (85% N₂, 10% CO₂, 5% O₂). In some determinations, bacteria were cultured in brain heart infusion broth (OXOID) supplemented with 4% foetal bovine serum (Gibco, Carlsbad, CA, USA) at 37 °C for 48-72 h, under microaerobiosis.

Reference strains *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 700603, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, and *Streptococcus agalactiae* ATCC 12386 were kindly donated by the Microbiology Service of University Clinic Hospital Lozano Blesa (Zaragoza, Spain). These strains were routinely grown in Mueller-Hilton agar/broth (PanReac AppliChem, Barcelona, Spain), overnight at 37°C.

4.2. Chemicals

The Maybridge HitFinder™ chemical library was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All compounds of the chemical library were provided as 10 mM solutions dissolved in 100% dimethyl sulfoxide (DMSO). Selected compounds of the library were purchased in additional amounts from Thermo Fisher Scientific, dissolved in 100% DMSO, and stored at -20°C until use. Conventional antibiotics including clarithromycin (CLR), metronidazole (MTZ), levofloxacin (LVX), and ampicillin (AMP) were purchased from Merck (Rahway, NJ, USA). Stock solutions of these drugs at 10.24 g/L in 100% DMSO were stored at -20 °C for up to 30 days.

4.3. Recombinant Expression and Purification of Response Regulators

The *H. pylori* 26695 HsrA protein was overexpressed in *E. coli* BL21(DE3) (EMD Biosciences) and purified by immobilized metal-affinity chromatography (IMAC) as previously described [40]. The complete coding sequence of the *cosR* gene from *C. jejuni* ATCC 33560 was amplified by PCR and cloned into the expression vector pET-28a (Novagen). Sequences of primers used for cloning *hsrA* and *cosR* are described in Supplementary Table S4. Recombinant His-tagged CosR was overexpressed in *E. coli* BL21(DE3) and purified by IMAC-Ni²⁺, using an imidazole gradient for elution. Purified protein was dialyzed in 50 mM Tris-HCl (pH 8), 300 mM NaCl, and 10% glycerol. Concentration of purified recombinant proteins were estimated using the BCA™ Protein Assay kit (Thermo Fisher Scientific, Bothell, WA, United States). DNA binding activities of purified recombinant response regulators HsrA and CosR were assessed by EMSA, as described below.

4.4. High-Throughput Screening

High-throughput screening (HTS) of the Maybridge HitFinder™ chemical library for *H. pylori* HsrA ligands was assessed by a fluorescence-based thermal shift assay, according to previous

described procedures [40,54]. Briefly, a reaction mixture containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 10% glycerol, 5 mM DTT, SYPRO® Orange ready-to-use fluorescent stain (Thermo Fisher Scientific) at a final concentration of $10 \times$, and 10 μ M HsrA was freshly prepared. Next, 90 μ L was dispensed in all wells of V-shape 96-well plates ABgene™ Thermo-Fast™ 96 (Thermo Fisher Scientific). Wells of columns 1 and 12 received 10 μ L of DMSO (vehicle) and were used as reference controls. The rest of wells (columns 2 to 11) received 2.5 μ L of four different compounds from the HitFinder library (250 μ M as final concentration each). The unfolding curve corresponding to each well was registered from 25°C to 75°C in 1°C steps using an Mx3005P™ qPCR System (Agilent Technologies, Inc., CA, USA), and analysed by Origin 7.0 (OriginLab, Northampton, MA, USA) using a homemade script that determines the midpoint temperature of unfolding (T_m). Those compounds that clearly increased the thermal stability of HsrA observed in reference controls ($T_m \geq 5^\circ\text{C}$) were considered as HsrA ligands.

4.5. Electrophoretic Mobility Shift Assays

DNA binding activities of recombinant HsrA and CosR were assessed *in vitro* by electrophoretic mobility shift assays (EMSAs). A 300-bp promoter region of the *H. pylori* 26695 operon *porGDAB* was used as target sequence of HsrA in all EMSA experiments [48]. Likewise, a 420-bp sequence of the *C. jejuni* *sodB* promoter (ATCC 33560) was used as target DNA of CosR [90]. The oligonucleotides used for the synthesis of these promoter regions are described in Supplementary Table S4. Response regulators HsrA (6 μ M) or CosR (700 nM) were mixed with 120 ng of their target promoters in reaction buffer 10 mM bis-Tris (pH 7.5), 40 mM KCl, 100 mg/L BSA, 1 mM DTT, and 5% glycerol. For the *in vitro* inhibition analyses, mixtures of target DNA and response regulator were exposed to 2, 1, 0.5 and 0.1 mM of each compound of interest, incubated at room temperature for 20 min, and then separated on a 6% non-denaturing electrophoresis. DMSO instead of inhibitors was included as vehicle controls, while an internal sequence of the *Anabaena* sp. *pkn22* gene was used as non-specific competitor in all assays. For DNA-protein binding visualization, gels were stained with SYBR Safe DNA gel stain (Invitrogen) and analysed with a Gel Doc 2000 Image Analyser (Bio-Rad).

4.6. Minimal Inhibitory and Bactericidal Concentrations

The antimicrobial activity of HsrA inhibitors was evaluated by determination of minimal inhibitory concentration (MIC) against four different strains of *H. pylori*: ATCC 700392 (aka 26695), ATCC 43504 (MTZ-resistant reference strain), ATCC 700684 (CLR-resistant reference strain), and Donostia 2 (LVX-resistant clinical isolate). MIC were determined by the broth microdilution method, as previously described [42]. Briefly, *H. pylori* strains were grown in blood agar at 37 °C for 72 h under microaerobic conditions. Fresh inoculums adjusted at $\text{OD}_{600} = 0.01$ [$\sim 10^6$ colony forming units (CFU) per mL] in BHI broth supplemented with 4% foetal bovine serum (BHI + FBS) were prepared and immediately exposed to final concentrations between 64 to 0.031 mg/L of each compound of interest. Compound solvent (DMSO) as well as conventional antibiotics (MTZ, CLR, LVX) were included as controls. MIC values were determined colourimetrically after 48 h at 37°C by using resazurin. For minimal bactericidal concentration (MBC) determinations, aliquots from MIC plates were aseptically seeded on blood agar and incubated for 72 h at 37 °C under microaerobic conditions. Each experiment was performed twice in triplicate. MIC and MBC values of selected compounds against *C. jejuni* ATCC 33560 were determined by using the same method and culture conditions described for *H. pylori* strains.

Antimicrobial activities of selected compounds against several Gram-negative and Gram-positive representative species of normal human microbiota were determined according to the EUCAST Guidelines [101]. Briefly, standardized inoculums (0.5 McFarland) were freshly prepared from overnight Mueller–Hinton agar plates of reference strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, and *S. agalactiae* ATCC 12386. Next, bacterial suspensions adjusted to 5×10^5 CFU/mL in Mueller–Hinton broth were exposed to a range of concentrations from 64 to 0.031 mg/L of selected compounds. DMSO and conventional antibiotics LVX and AMP were included as controls. MBC were determined by

seeding aliquots from dilutions around the MIC values on Mueller-Hinton agar plates, which were incubated for 24 - 48 h at 37 °C. Experiments were performed twice in triplicate.

4.7. Time-Kill Kinetic Assays

Time-kill kinetics of selected HsrA inhibitors were carried out as previously described [42], with slight modifications. Briefly, fresh grown cells of *H. pylori* strain ATCC 700392 were resuspended in BHI + FBS at 1.0×10^4 CFU/mL and exposed to $4 \times$ MIC of each compound. Bacteria exposed to DMSO (vehicle) instead HsrA inhibitors was included as control. All cultures were incubated at 37 °C under microaerobic conditions, and aliquots were taken after 0, 2, 4, 8, and 24 h of exposure for CFU determination. Experiments were performed twice in triplicate. Results were presented as \log_{10} CFU/mL versus hours of exposure. Differences in *H. pylori* survival were subjected to statistical analysis by using the Mann-Whitney *U* test.

4.8. Checkerboard Assays

Potential synergisms between selected HsrA inhibitors and first-line antibiotics were evaluated by using checkerboard assays, as previous described [40,42]. Briefly, the antimicrobials to be tested were two-fold serially diluted in BHI + FBS using independent sterile microtiter plates. Then, concentration gradients from two antimicrobials were aseptically mixed at equal volumes in a new microtiter plate resulting in a matrix of 77 unique combinations of both compounds. Next, freshly prepared inoculum of *H. pylori* ATCC 700392 adjusted at 2×10^6 CFU/mL in BHI+ FBS were added to all wells, and plates were incubated for 72 h at 37 °C under microaerophilic conditions. Microbial growth was revealed by adding filter-sterilized resazurin up to 0.01 mg/mL and further incubation for 6 hours. Fractional inhibitory concentration index (FICI) for each antimicrobial combination was calculated as previous described. Values of $FICI \leq 0.5$, $0.5 < FICI \leq 1$, $1 < FICI \leq 4$, and $FICI > 4$ were considered as indicatives of synergistic, additive, neutral or antagonist interactions, respectively [40,42].

4.9. In Vivo Inhibition of HsrA

Bacterial growth from three blood agar plates of *H. pylori* ATCC 700392 (aka 26695) incubated for 48 h at 37 °C under microaerobic conditions was aseptically resuspended in BHI + FBS and adjusted to 10^7 CFU/mL. BHI broth cultures were incubated microaerobically at 37 °C for 24 h. Next, aliquots of 12 mL were exposed in triplicate to $4 \times$ MIC of HsrA inhibitor V and further incubated in the same above conditions until \log_{10} CFU/mL resulted diminished in one unit. At this time, bacterial growth was stopped by adding 1.5 mL ice-cold RNA stop solution (95% absolute ethanol, 5% acid-buffered phenol), and subsequently incubated on ice. Control samples were exposed to the same amount of DMSO instead inhibitor. *Helicobacter* cells from all samples were harvested by centrifugation (4,000 rpm at 4 °C for 10 min), pellets were quickly frozen by immersion in liquid nitrogen and stored at -80 °C.

4.10. RNA Sequencing

RNA-seq analysis was performed by GENEWIZ (Azenta Life Sciences, Leipzig, Germany). Frozen cell pellets from two biological replicates of each treatment condition were properly submitted to GENEWIZ in dry ice. RNA-seq libraries were prepared according to standard protocols via rRNA depletion, RNA fragmentation and random priming, reverse transcription, 5'-phosphorylation and dA-tailing of cDNAs, adaptor ligation and PCR enrichment. Once the quality of each library was confirmed, they were sequenced on Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) with 150-bp paired-ends. Raw sequence reads were filtered to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36 [102]. The trimmed reads were subsequently mapped to the *H. pylori* strain 26695 reference genome NC_000915.1 available on NCBI, by using the Bowtie2 aligner v.2.2.6 [103]. Unique gene hit counts were calculated by using featureCounts [104] from the Subread package v.1.5.2. Only unique reads that fell within gene regions

were counted. Using DESeq2 [105], a comparison of gene expression between control and treated groups of samples was performed. The Wald test was used to generate *p*-values and log2 fold changes. Genes with an adjusted *p*-value < 0.05 and absolute log2 fold change > 1 were considered as differentially expressed genes (DEG) for each comparison. The RNA-seq data generated in this study have been deposited in ArrayExpress with the accession number E-MTAB-14255.

4.11. Quantitative Real-Time PCR

For RNA extraction to qPCR analyses, frozen pellets were quickly resuspended in 850 µL lysis buffer containing 20 mM sodium acetate (pH 5.2), 0.5% SDS, 1 mM Na₂-EDTA. Then, 850 µL of acid phenol preheated at 65°C were immediately added to cell suspensions. Samples were vigorously mixed by vortex and incubated at 65°C for 10 min. Cell extracts were centrifuged at 13,000 rpm during 5 min at room temperature and aqueous phase was transferred to clean RNase-free tubes. Next, samples were treated with 1 mL of TRIzol™ Reagent (Thermo Fisher Scientific) for 5 min, after which 200 µL of chloroform were added and vigorously mixed. Colourless upper aqueous phase was sequentially washed three times with 1V of chloroform, and total RNA was precipitated overnight at -80°C with 2.5 V of ice-cold absolute ethanol. RNA samples were resuspended in 50 µL RNase-free water and genomic DNA was subsequently removed by using the TURBO DNA-free™ Kit (Thermo Fisher Scientific). Purity and integrity of RNA samples were checked by both NanoDrop spectrophotometer (Thermo Fisher Scientific) and electrophoresis. Absence of residual DNA was determined by qPCR.

Reverse transcription was carried out with SuperScript retrotranscriptase (Invitrogen) in reaction buffer containing 150 ng of random primers (Invitrogen), 1 mM dNTP mix (GE Healthcare), and 10 mM DTT. Quantitative PCRs were performed by using a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) in 30 µL of reaction mixtures containing 10 ng of cDNA, 12.5 µL of SYBR Green PCR Master Mix (Thermo Fisher Scientific), and 0.4 µL of primers at 25 µM in water. The sequences of primers used for each gene are described in Supplementary Table S4. Relative quantification was performed according to the $\Delta\Delta C_t$ method [106]. Expression levels were normalized using the *H. pylori* 26695 *glnA* gene as housekeeping [107].

4.12. Cytotoxicity and Therapeutic Index

The *in vitro* toxicity of selected HsrA inhibitors was determined on HeLa cells by the PrestoBlue™ assay, as previously described [42]. Briefly, cells were cultured at 37 °C with 5% CO₂ in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, 1% L-GlutaMAX™ solution (Thermo Fisher Scientific) and 1% penicillin/streptomycin. When 80% confluence was achieved, cells were detached, counted, seeded in 96-well microplates at a density of 10,000 cells per well, and allowed to adhere for 24 h. Next, the mammalian cells were exposed to selected compounds at a range of 0.125 to 128 mg/L. After 24 h of exposure, cell viability was measured with PrestoBlue™ cell viability reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. Experiments were performed twice in triplicate. The 50 % cytotoxic concentration (CC₅₀) of each compound was calculated by regression analysis using Microsoft Excel. Therapeutic index (TI) values were calculated as the ratio of the CC₅₀ to the higher MIC observed with each compound [108].

4.13. Isothermal Titration Calorimetry

Molecular interactions of selected ligands with proteins HsrA or CosR were studied by isothermal titration calorimetry (ITC), as previously described [42]. Titrations were assessed in a high-sensitivity MicroCal Auto-iTC200 calorimeter (Malvern Panalytical, Malvern, UK). Experiments were carried out at 25 °C in freshly prepared buffer containing 50 mM Tris-HCl [pH 8], 150 mM NaCl, 10% glycerol, and 1% DMSO. A 20 µM solution of protein was located in the calorimetric cell. Then, a sequence of 19 injections of 2 µL volume each with 200 µM solution of the corresponding ligand was programmed with a time spacing of 150 s, a stirring speed of 750 rpm, and a reference power of 10 µcal/s in the sample cell. Thermodynamic properties such as binding stoichiometry, dissociation

constants and binding enthalpies were calculated by nonlinear least-squares regression data analysis using the Origin 7.0 software.

4.14. Molecular Docking

Predicted models of interaction between the response regulator HsrA and selected compounds were determined by molecular docking analyses using AutoDock Vina [109]. The 3D structures of compounds were built in Corina Classic (www.mn-am.com) and energy was minimized using the web server AMMOS2 [110]. 3D structure of HsrA (2HQR, model 1, chain A) was obtained from Protein Data Bank. The protein structure was considered rigid while ligands were treated as flexible molecules with free rotatable bonds. Interaction energy was estimated by AutoGrid4 [111] and top-ranked pose for each ligand was considered as the predicted model of interaction. Protein-ligand complexes were visualized by PyMOL (www.pymol.org).

5. Patents

The authors declare that a patent has been filed concerning the use of compounds for the treatment and/or prevention of an infection or disease caused by *Helicobacter* or *Campylobacter*.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Isothermal titration calorimetry (ITC) analyses of the interaction between the *H. pylori* response regulator HsrA and its low-molecular weight ligands I, IV, V, VIII, XI and XII.; Figure S2: ITC analysis of the interaction between the *C. jejuni* response regulator CosR and compound V.; Table S1: Compounds from the Maybridge HitFinder™ chemical collection identified as HsrA ligands according the fluorescent thermal shift-based HTS.; Table S2: Differential expression analysis of *H. pylori* 26695 exposed to lethal concentrations of the highly bactericidal HsrA ligand V; Table S3: Functional classification of DEGs.; Table S4: List of oligonucleotides used in this study.

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Data Availability Statement: RNA-seq data have been deposited in ArrayExpress under accession number E-MTAB-14255, and are available for reviewers through this link: <https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-14255?key=5f27c51b-0a47-402f-a663-8fee6c188cc7>. The rest of data supporting the findings of this study are available within the paper and its Supplementary Information files.

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