Design, Synthesis, and Efficacy Testing of Nitroethylene- and 7-Nitrobenzoxadiazol-Based Flavodoxin Inhibitors against Helicobacter pylori Drug-Resistant Clinical Strains and in Helicobacter pylori -Infected Mice

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KEYWORDS

Novel antimicrobials; antimicrobial resistance; *Helicobacter pylori*; drug discovery; flavodoxin

ABSTRACT

Helicobacter pylori (*Hp*) infection is the main cause of peptic ulcer and gastric cancer. *Hp* eradication rates have fallen due to increasing bacterial resistance to currently used broad-spectrum antimicrobials. We have designed, synthesized and tested redox variants of nitroethylene- and 7-nitrobenzoxadiazole-based inhibitors of the essential *Hp* protein

flavodoxin. Derivatives of the 7-nitrobenzoxadiazole lead, carrying reduced forms of the nitro group and/or oxidized forms of a sulphur atom, display high therapeutic indexes against several reference *Hp* strains. These inhibitors are effective against metronidazole-, clarithromycin- and rifampicin-resistant *Hp* clinical isolates. Their toxicity for mice after oral administration is low and, when administered individually at single daily doses for 8 days in a mice model of *Hp* infection, they decrease significantly *Hp* gastric colonization rates and are able to eradicate the infection in up to 60% of the mice. These flavodoxin inhibitors constitute a novel family of *Hp*-specific antimicrobials that may help fight the constant increase of *Hp* antimicrobial-resistant strains.

INTRODUCTION

Helicobacter pylori (*Hp)* is a Gram-negative bacterium that establishes life-long infections in humans by colonising their gastric mucosa, usually during childhood.[1,2] *Hp* infection is the most common bacterial infection all over the world[2–4] with a prevalence that varies from 10 to 94 % in different countries.[5–8] *Hp* transmission follows person-to-person, oral-oral, faecal-oral, waterborne or iatrogenic routes.[5,9] In most cases, *Hp* infection is asymptomatic, but it can progressively damage the gastric mucosa[10] by inducing chronic gastritis[1] and then diseases from peptic ulcer to gastric mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma.[2,3,10] *Hp* is the main cause of gastric cancer all over the world.[10,11] Up to now, it is the only bacterium classified as a group I carcinogen.[4,5,9,11] Eradication of *Hp* infection is the gold standard in peptic ulcer disease treatment and might prevent gastric cancer,[11–13] the third most common cause of cancer death.[14] Effective and affordable treatments are needed to reduce the impact of *Hp* infection in those diseases. Moreover, *Hp* infection has been suggested to be involved in other extra-gastrointestinal disorders[11,15–17] and may modify the bioavailability and absorption of essential nutrients and the plasma levels of metabolic hormones.[15]

Conventional therapy against *Hp* consists of empirical triple or, more often now, quadruple treatments based on a proton pump inhibitor plus a combination of two or three broad spectrum antimicrobials such as clarithromycin (Cla), amoxicillin, metronidazole (Mnz), tetracycline and bismuth salts.[11,17–19] Increasing *Hp* resistance to these common antimicrobial drugs, especially to Cla and Mnz, is growing worldwide, reducing the effectiveness of available therapies.[11,18–21] Therefore, new specific treatments are required,[22] and eradication strategies need to be adapted geographically.[11,18,23] Ongoing research on vaccines against *Hp* has not been successful until now, probably due to *Hp*

genetic variability and to the complex host immune response against the bacterium.[9,24–26] Current alternatives proposed include the application of therapeutic regimes based on local patterns of antimicrobial susceptibility and the use of personalized treatments consisting of the pre-identification of *Hp* susceptibility/resistance to conventional antimicrobials by molecular or by cultured-guided methods.[12,18,20,27] The use of antimicrobial peptides and the development of novel compounds acting on specific *Hp* targets have also been proposed. Following the latter approach, specific *Hp* targets, essential for the bacterium and absent in humans, have been identified.[28–30] One such target is *Hp* flavodoxin (*Hp*-Fld), a small acidic redox protein that contains one molecule of tightly bound flavin mononucleotide cofactor.[30–33]

Flavodoxins[34,35] are bacterial proteins that take part in a variety of electron transfer reactions. In particular, *Hp*-Fld accepts electrons from the pyruvate ferredoxin oxidoreductase complex (PFOR), which catalyses the oxidative decarboxylation of pyruvate, and transfers them to flavodoxin quinone reductase.[30,36,37] Both *Hp*-Fld and *Hp*-PFOR are essential for the bacterium survival[30,38] and compounds interfering with this pathway might be suited for *Hp* eradication therapies (Figure 1).[30,39]

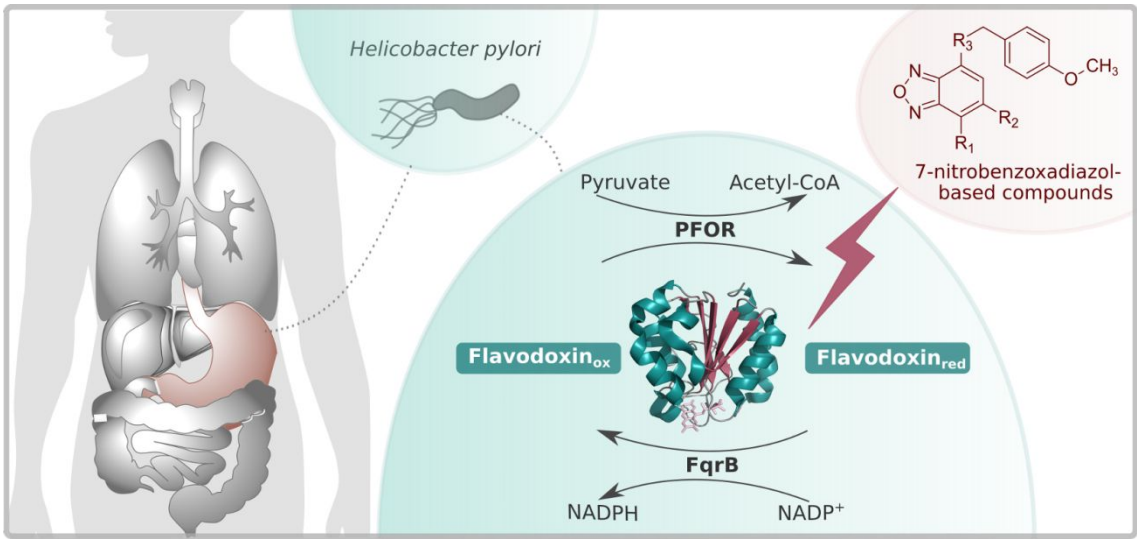


Figure 1. Graphical summary of the rational followed for the development of new flavodoxin inhibitors against *Helicobacter pylori*. *Hp* colonizes the gastric mucosa of over half of the world's population, establishing a life-long infection if untreated. Growing concern about the increasing *Hp* resistance to the conventional antibiotics used in therapy, has led to the proposal of new targets for fighting this global health problem. One of them is *Hp* flavodoxin, an electron transfer protein involved in a metabolic process essential for bacterial viability. We have designed and synthesized redox variants of a 7-nitrobenzoxadiazol *Hp* flavodoxin inhibitor and have tested them in *Hp* clinical isolates and in *Hp*-infected mice.

Unlike most other flavodoxins, *Hp*-Fld contains a pocket near the active site where small molecules could bind[30,31] interfering with binding or electron transfer to *Hp*-Fld partner proteins.[39] Because flavodoxins as such are not present in humans, and the Fld-like domain found in human cytochrome P450 reductase lacks an equivalent pocket near its active site, no side effects are anticipated for a therapy targeting *Hp*-Fld.[39]

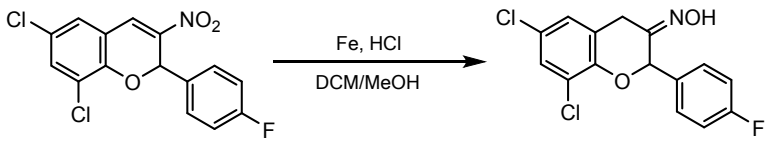
In earlier work we have characterized the biophysical properties of *Hp*-Fld[31–33,39,40] and have identified through experimental screening several small interacting molecules that bind to it. Three of them (named compounds **I**, **II** and **IV**) inhibit *Hp*-Fld-mediated pyruvate decarboxylation and act as specific bactericidal agents for *Hp*.[41] Based on those three hits, 123 new derivative molecules were developed, some of which showed better bactericidal properties than the three original ones.[42] Because the hits and many of the improved derivatives contain a nitro group and *Hp* is known to activate nitro groups in certain prodrugs through redox dependent reactions,[20,43–46] we have synthesized new anti-*Hp* compounds that contain partially or fully reduced forms of the nitro groups present in the parent molecules, and additional redox variants modifying sulphur and vinyl groups of the inhibitors. The efficacy of these compounds has been tested in reference *Hp* strains, *Hp* clinical isolates, and in *Hp*-infected mice.

RESULTS AND DISCUSSION

Synthetic reactions. Compounds **I-a**, **II-b**, **II-c**, **II-d**, **IV-c**, **IV-d**, **IV-e**, **IV-f** and **Mnz-a** could be readily synthesized using the routes described in Schemes 1-8.

The synthesis of compound **I-a** was performed by treatment of nitro compound **I** with iron in acidic conditions (HCl)[47] under ultrasonic activation, leading to the desired product in short reaction times (10-40 min) (Scheme 1).

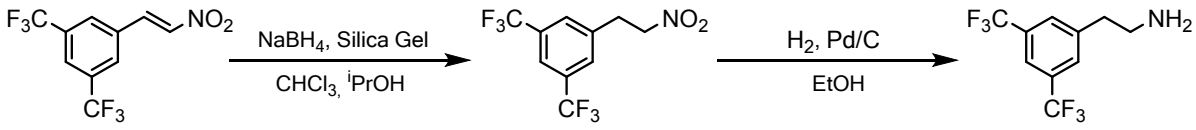
Scheme 1. Reduction of nitro-compound **I** to oxime **I-a**



I I-a

Reduction of nitrostyrene **II** with sodium borohydride in the presence of silica gel[48] and in a mixture of chloroform and 2-propanol led to the corresponding nitroalkane **II-b** (Scheme 2). Further reduction by catalytic hydrogenation using palladium-on-carbon as the catalyst led to aminoalkane**II-c**.

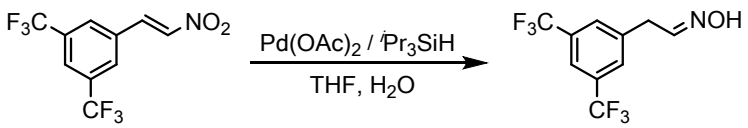
Scheme 2. Reduction of nitrostyrene **II** to nitroalkane **II-b** and reduction of this last one to aminoalkane **II-c**



II II-b II-c

Oxime **II-d** could be obtained by a palladium-catalysed reduction[49] with triisopropylsilane of nitro compound **II** in a mixture of THF and water (Scheme 3).

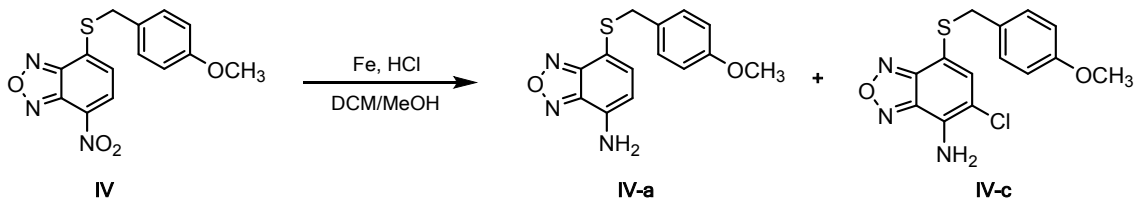
Scheme 3. Reduction of nitrostyrene **II** to oxime **II-d**



II II-d

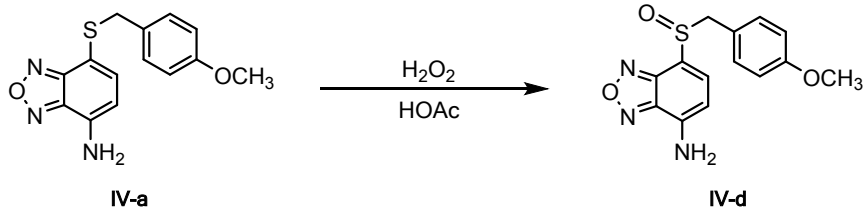
Reduction of nitro-compound **IV** with iron and hydrochloric acid, by using dichloromethane and methanol as solvents,[47] afforded the desired product **IV-c** and also some of the previously synthesized molecule **IV-a** (Scheme 4) from which it was separated by column chromatography.

Scheme 4. Reduction of nitro-compound **IV** to a mixture of amino-compound **IV-a** and amino-chlorinated compound **IV-c**

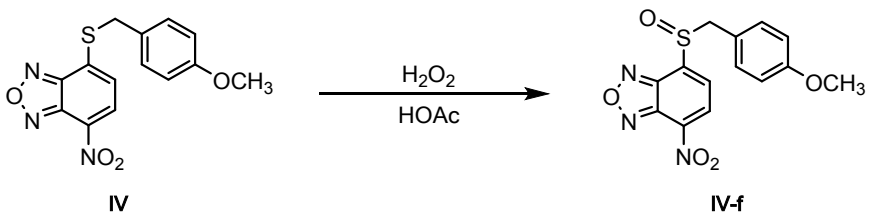


Amino-sulfoxide **IV-d** and amino-sulfone **IV-f** could be obtained by thio-ether oxidation of molecules **IV-a** and **IV**, respectively, with hydrogen peroxide in the presence of glacial acetic acid (Schemes 5 and 6). [50]

Scheme 5. Oxidation of sulphur compound **IV-a** to sulfoxide **IV-d**



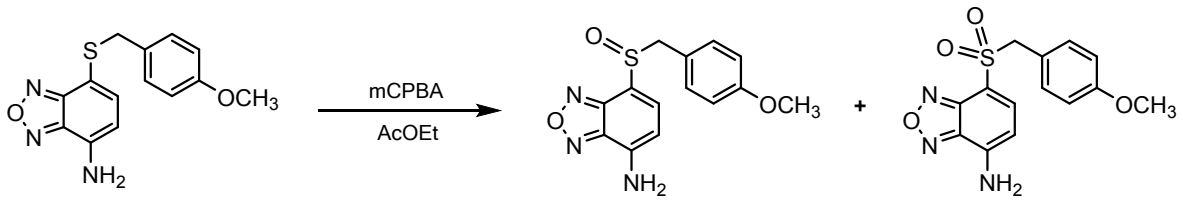
Scheme 6. Oxidation of sulphur compound **IV** to sulfoxide **IV-f**



IV IV-f

The amino-sulfone **IV-e** was synthesized by oxidation of compound **IV-a** using *meta*-chloroperbenzoic acid in ethyl acetate as the oxidant.[51] Compound **IV-e** was obtained as a mixture with compound**IV-d**, from which it was separated by flash chromatography (Scheme 7).

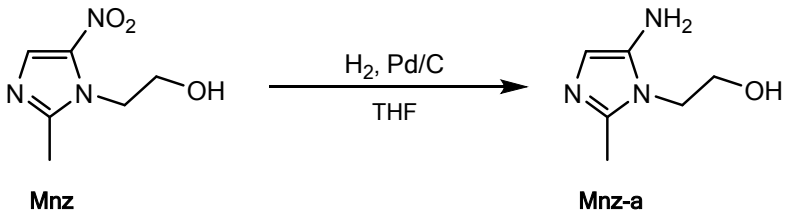
Scheme 7. Oxidation of sulphur **IV-a** to a mixture of sulfoxide **IV-d** and sulfone **IV-e**



IV-a IV-d IV-e

Commercially available metronidazole was transformed to **Mnz-a** by catalytic reduction using palladium-on-carbon as the catalyst (Scheme 8).[52] The resulting amino-imidazole proved to be sensitive to oxygen, heat and acid and therefore of limited utility; nevertheless, its toxicity and activity were determined.

Scheme 8. Reduction of metronidazole to amino-imidazole **Mnz-a**



Mnz Mnz-a

Improving therapeutic indexes by modifying the oxidation state of chemical groups present in the flavodoxin inhibitor molecules. Several new derivatives of compounds **I**, **II** and **IV** (Figure 2) have been synthesized and tested.

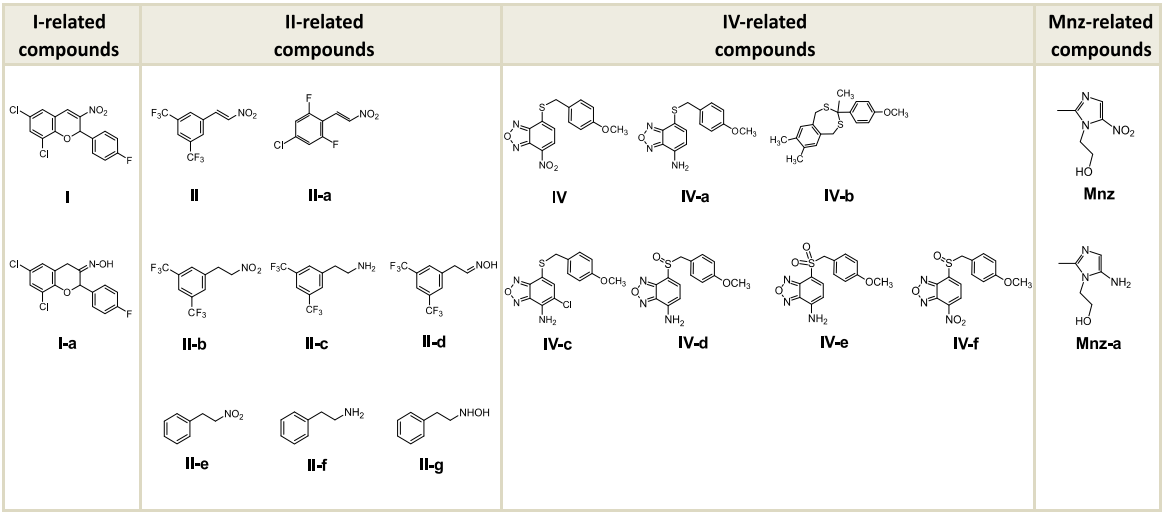
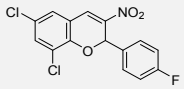
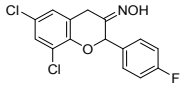
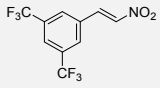
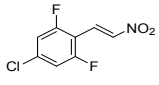
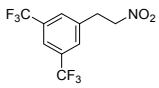
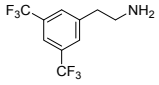
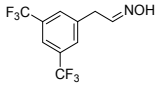
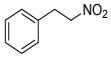
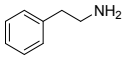
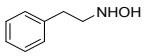
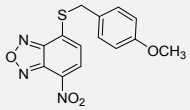
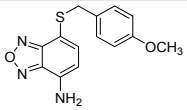
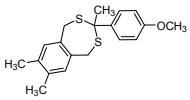
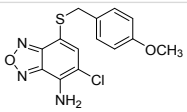
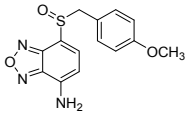


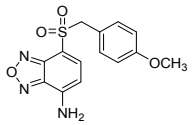
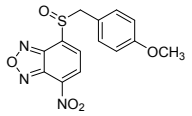
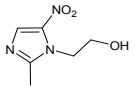
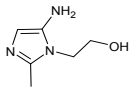
Figure 2. Chemical structure of 19 flavodoxin inhibitors grouped by similarity to lead compound (I, II, IV or Mnz). In each group the upper line provides the formula of the lead and derivatives preliminary tested *in vitro* in previous work.[41,42] The middle line gives the formula of newly synthesized derivatives (see Schemes 1-8 and Synthesis details below). The lower line for **II**-related compounds provides the formula of commercial compounds not previously tested as antimicrobials against *Hp*.

All of them are less cytotoxic toward human HeLa cells than their corresponding parent compounds (Table 1). In fact, most derivatives are not cytotoxic at concentrations as high as 1 mM (Figure S2). The *in vitro* anti-*Hp* activities of these derivatives have been evaluated using three *Hp* reference strains (Table 1). The specific impact on TIs brought about by modifying the oxidation state of the nitrogen and sulphur atoms and the vinyl group of the inhibitors has been evaluated from pair wise comparisons of the 19 compounds analysed.

Table 1. *In vitro* activity of flavodoxin inhibitors I, II and IV and related compounds against *H. pylori* reference strains[a]

Compound	Structure	MCC_{50}[b] (μM)	MIC[c] (μM)			TI[d] (MCC_{50}/MIC)		
		HeLa	SS1	NCTC 11637	26695	SS1	NCTC 11637	26695
I		5	0.37	0.37	0.37	14	14	14
I-a		117	12	6.1	6.1	10	19	19
II		15	1.8	3.5	1.8	8.3	4.3	8.3
II-a		36	2.3	2.3	2.3	16	16	16

II-b		>1000	223	56	56	>4.5	>18	>18
II-c		>500	>249	31	31	~2.0	>16	>16
II-d		>500	236	59	118	>2.1	>8.5	>4.2
II-e		>1000	212	212	106	>4.7	>4.7	>9.4
II-f		>1000	132	66	66	>7.6	>15	>15
II-g		>1000	>233	>233	>233	~4.3	~4.3	~4.3
IV		7	0.78	1.6	6.3	9.0	4.4	1.1
IV-a		>1000	13.9	27.8	27.8	>72	>36	>36
IV-b		>1000	6.1	3.0	3.0	>164	>333	>333
IV-c		>1000	5.9	11.8	5.9	>170	>85	>170
IV-d		>1000	105	53	26	>9.5	>19	>39

IV-e		>1000	100	100	100	>10	>10	>10
IV-f		179	12	6	6	15	30	30
Mnz		>1000	2.9	187	12	>345	>5.3	>83
Mnz-a		>1000	>453	227	453	~2.2	>4.4	>2.2

[a]Parent compounds **I**, **II** and **IV** are highlighted in grey

[b]The Minimal Cytotoxic Concentration (MCC_{50}) tested in HeLa cells

[c]Minimal Inhibitory Concentration (MIC) tested in three different *H. pylori* reference strains (NCTC 11637 (Mnz resistant), and 26695 and Sydney Strain 1 (Mnz susceptible))

[d]Therapeutic Indexes (TI) have been calculated as MCC_{50}/MIC

Compounds **I** and **II** share the presence of a nitroethylene moiety and their derivatives will be jointly discussed. In this family of compounds, reduction of the nitro groups to oxime lowers similarly cytotoxicity and antimicrobial activity, barely changing the TIs for the three *Hp* strains tested (Table 1). On the other hand, reduction of the ethylene group leads to a drastic decrease of toxicity with a notable reduction of antimicrobial activity, which results in a small increase of TI. Since reduction of the ethylene group lowers so much toxicity, differences in TIs among the ethylene-reduced variants cannot be properly evaluated. In Mnz, the reduction of the nitro group to amine clearly lowers the antimicrobial activity, while possible changes in cytotoxicity cannot be evaluated as neither

Mnz nor the amine variant are toxic at the higher concentration tested. Globally considered, reduction of the nitro or the ethylene group in the leads carrying a nitroethylene moiety (**I**, **II** and Mnz), decreases to a similar extent toxicity and antimicrobial activity. Only compounds **II-a** (previously tested by Galano et al., 2013[42] and here re-evaluated), **II-b**, **II-c** and **II-f** slightly improve (about 2-fold) the TI of their parent compound.

Compound **IV** also contains a nitro group, which, in this case, is attached to a benzoxadiazole ring. In this lead, reduction of the nitro group to amine lowers toxicity so much (>100-fold) that the concomitant moderate decrease in activity observed (around 10-fold) combines to a 10-fold higher TI in compound **IV-a**. The toxicity of compound **IV** is also greatly reduced (25-fold) by oxidation of the sulphur atom to sulfoxide, which causes a less pronounced decrease in activity and therefore also raises the TI. Oxidation of the sulphur atom to sulfoxide in **IV-a**, compound **IV-d**, similarly causes a smaller decrease in activity, while a possible further decrease in toxicity cannot be determined because **IV-a** is already non-toxic at the higher concentration tested (1 mM). Further oxidation of the sulphur atom in **IV-a** to sulfone, compound **IV-e**, lowers the activity. However, chlorination of **IV-a** increases the antimicrobial activity 2-4 fold and does not introduce toxicity, leading to the high TIs of compound **IV-c** (from >85 to >170, depending on the *Hp* reference strain tested). We have also tested an additional **IV**-related compound: **IV-b**, that was considered promising in previous assays[42]. Unlike all other **IV** variants, **IV-b** lacks the benzoxadiazol ring and, therefore, the nitro group. Its toxicity is very low (>1000 μM) and its anti-*Hp* activity is similar to that of compound **IV**. Therefore, the TI of **IV-b** is quite high (from 164 to 333 depending on the *Hp* strain).

Efficacy of compounds against antimicrobial-resistant *Hp*-strains. Most compounds in Table 1 display higher TI than Mnz for the Mnz-resistant strain NCTC 11637. In addition, several nitro-reduced derivatives of compound **IV** (i.e **IV-a**, **IV-c**, **IV-d**) and compound **IV-b** display, for the two Mnz-sensitive strains tested (strains SS1 and 26695), TIs close to that of Mnz, or even higher. To explore the potential usefulness of these flavodoxin inhibitors towards other *Hp* drug-resistant strains, we have determined the activity of 6 representative commonly used antimicrobials on six *Hp* strains obtained from patients who have relapsed or are refractory to, at least, two conventional therapies[53]. Three of these strains (isolates 1-3) are Mnz-resistant, two are both Mnz- and Cla-resistant (isolates 4 and 6) and one is rifampicin-resistant (isolate 5) (Table 2).

Table 2. Antimicrobial resistance profiles of *H. pylori* drug-resistant clinical isolates following the EUCAST criteria. (S = sensitive; R = resistant)

Compound	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
Amoxicillin	S	S	S	S	S	S
Clarithromycin	S	S	S	**R**	S	**R**
Tetracycline	S	S	S	S	S	S
Levofloxacin	S	S	S	S	S	S
Metronidazole	**R**	**R**	**R**	**R**	S	**R**
Rifampicin	S	S	S	S	**R**	S

Our analysis of the effect of the flavodoxin inhibitors on these clinical isolates (Table 3 and Table S2) confirms the activity trends previously observed when evaluated against reference strains (Table 1). While compounds **II-b**, **II-d**, **II-e**, **IV-d**, **IV-e** display low TIs,

with the exception of **II-e** on the isolate 4 (a Cla- and Mnz- resistant strain), compounds **IV-a** and **IV-c** show high TIs for most clinical strains. Indeed, according to EUCAST criteria, both **IV-c** and **IV-a** are effective against two Mnz-resistant strains (isolates 1 and 2) and against the two Cla- and Mnz-resistant strains (isolates 4 and 6). In addition, **IV-c** is also effective against the rifampicin-resistant strain (isolate 5). Only one Mnz-resistant strain (isolate 3) displayed low TIs for both **IV-c** and **IV-a** (Table 3).

Table 3. TI values (MCC_{50}/MIC) of some developed compounds against *H. pylori* drug-resistant clinical isolates [a]

Compound	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
II-b	9.0	9.0	9.0	9.0	9.0	9.0
II-d	4.2	4.2	4.2	34	4.2	4.2
II-e	4.7	4.7	4.7	605	4.7	4.7
IV-a	**144**	**72**	9.0	**144**	9.0	**72**
IV-c	**161**	**322**	10.0	**80**	**40**	**322**
IV-d	9.5	9.5	9.5	9.5	9.5	9.5
IV-e	10.0	10.0	20.0	20.0	10.0	10.0

[a]HeLa cells have been used to determine the Minimal Cytotoxic Concentration (MCC_{50}), as reported in Table 1. In bold, TI values indicative of effectivity, according to EUCAST criteria.

Efficacy of compounds against *Hp* gastric colonization in mice. The anti-*Hp* activity of these novel compounds has also been investigated in the mouse model of *Hp* infection.[54,55] First, the toxicity of three representative inhibitors (**II-a**, **IV** and **IV-a**) at doses of 10, 20

and 40 mg/100 g bw has been evaluated. Daily administration of compound **IV-a** for 8 days had no deleterious effects in stomach, liver, heart, lung and kidney at any dose, as determined by histopathological examination (Figure S3) and analysis of their biochemical parameters (data not shown). Compounds **IV** and **II-a** did not induce significant alterations at any dose in liver, heart, lung or kidney either, but they did provoke histological alterations in stomach when administered at the highest dose of 40 mg/100 g (compound **IV**) or at 20 or 40 mg/100 g (compound **II-a**) (Figure S3). The histological alterations found in stomach included hyperplasia, inflammation and necrosis of the epithelium and the gastric mucosa. None of these compounds were associated with increased levels of tested biochemical parameters when compared to animals which did not receive the compounds. The highest dose of compound **II-a** was, however, clearly toxic since it induced hair loss and weight loss (> 20%) in all mice treated. The lack of biochemical or histological alterations associated to the administration of high doses of **IV-a** indicates that reduction of the nitro group in compound **IV** to amine removes the toxicity exerted in the stomach by compound **IV** when orally administered to mice at high doses. The possibility that the histological alterations observed in stomach after administration of the highest dose of compound IV were associated to chemical instability of the compound at gastric pH has been tested (see "*In vitro* acid stability evaluation of compounds" section in Supplemental file). Compound IV (and the three derivatives tested: IV-a, IV-b and IV-d) are highly stable at pH 1.0. In the subsequent *in vivo* analysis on anti-*Hp* activity, the doses of compounds **IV** and **II-a** have been kept below those that produce histological alterations in mice stomachs.

Four experiments have been conducted in mice infected with *Hp* SS1 to test the anti-*Hp* activity of the leads (compounds **I**, **II** and **IV**) and of several of the best new derivatives:

I-a, related to **I**; **II-a**, **II-b**, **II-c** and **II-f**, related to **II**; and **IV-a**, **IV-b**, **IV-c** and **IV-d**, related to **IV**. The doses ranged from 0.1 to 20 mg/100 g bw. As expected, in the four experiments, all control mice have provided a clean background for interpretation: all mice in the infected non-treated groups have shown efficient stomach *Hp* colonization (10^5 to 10^6 CFU/g gastric tissue) and no *Hp* colonies have been found in any group corresponding to non-infected mice. The efficacy of the different compounds on *Hp* gastric colonization has been determined by comparing the CFU/g gastric tissue of each infected, treated-group with the corresponding infected, non-treated control group. Olive oil (90-95%)/DMSO (10-5%), used as vehicle to solubilise compounds, has not influenced the *Hp* gastric colonization (not shown). All the data gathered in the four experiments for the testing of compounds of the **I-II** series are presented in Figure 3, and data for the **IV** series in Figure 4. Data of gastric colonization for all compounds and doses tested are presented along with those of the corresponding control groups in Figures 3A and 4A, while the percentages of *Hp*–eradicated mice after treatment are shown in Figures 3B and 4B for the successful cases.

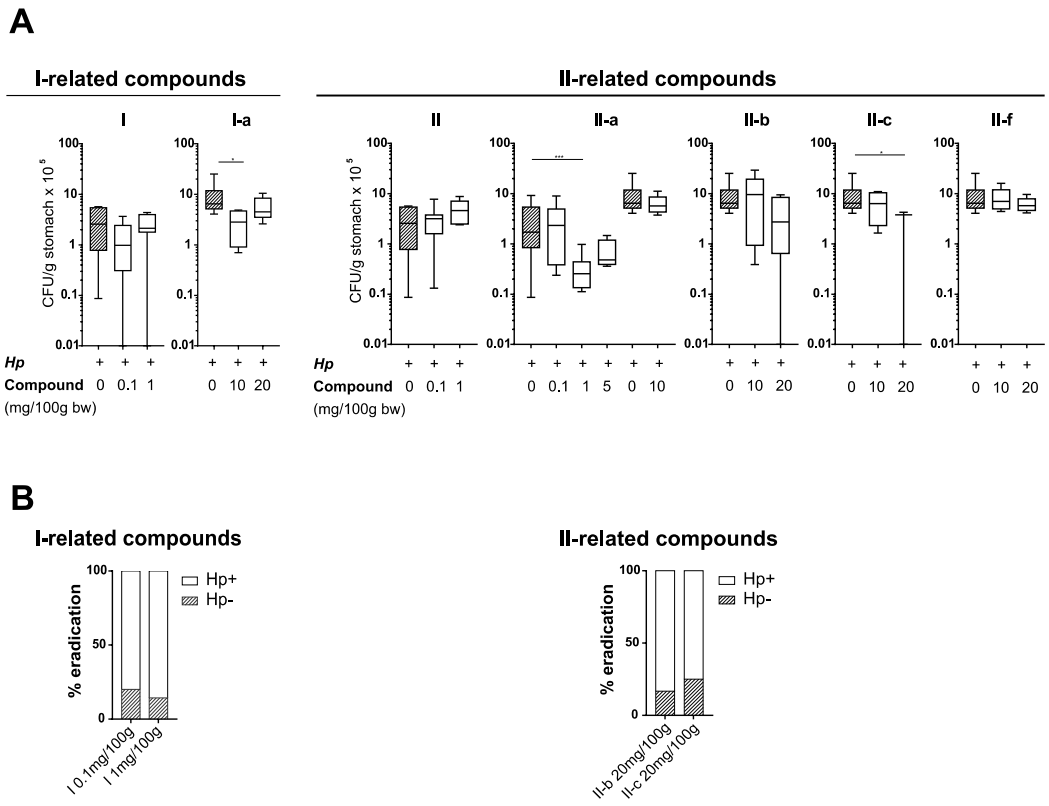


Figure 3. Inhibition of *Hp* SS1 gastric colonization of mice by treatment with I and II-related compounds (I, II, I-a, II-a, II-b, II-c and II-f). Four experiments have been carried out in order to test seven compounds at four doses (0.1, 1, 5, 10 and 20 mg/100 g body weight (bw)) as described in the methods section. All doses for each compound, except for compound **II-a**, have been tested in the same experiment, so they share the same control data. Two different experiments have been carried out in order to evaluate the effect of compound **II-a**. Its dose 10mg/100g bw was tested in the same experiment as that of **I-a**, **II-b**, **II-c** and **II-f** and this is why they share the same control. Gastric colonization was measured at three weeks post infection. Olive oil and DMSO, which were used as vehicles of the compounds, had no effect on the gastric colonization. **A)** Evaluation of *Hp* gastric colonization by colony counting. The number of viable colonies (CFU/g gastric tissue) are

represented for each compound dose tested. In all cases the CFU/g was related to that of the corresponding control group. Mann-Whitney U and Unpaired t tests were used for statistical analysis. Statistically significant inhibition of the gastric mucosa colonization by *Hp* has only been observed for compounds **I-a**, **II-a** and **II-c**. The inhibitory effect does not follow a clear dose dependency possibly related to the poor solubility of the compounds. *p≤0.05; **p≤0.01; ***p≤0.001. **B)** Eradication rates in *Hp*-infected mice treated with the indicated compounds able to eradicate the infection in some mice: **I**, **II-b** and **II-c**. Percentage of colonized (*Hp*+) and non-colonized (*Hp*-) mice are represented by white and grey bars, respectively.

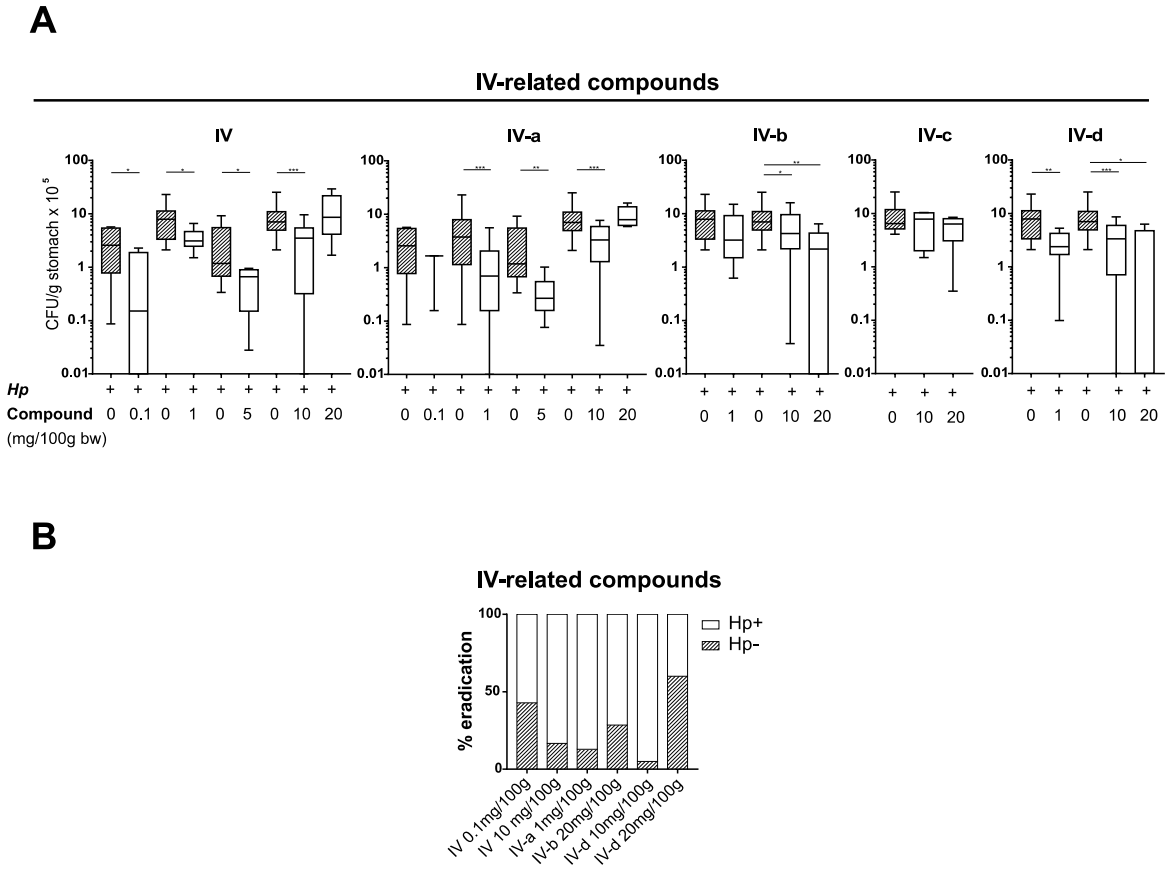


Figure 4. Inhibition of *Hp* SS1 gastric colonization in mice treated with IV-related compounds (IV, IV-a, IV-b, IV-c and IV-d). Four experiments have been carried out to test five compounds at five doses (0.1, 1, 5, 10 and 20 mg /100 g bw) as described in the methods section. Doses 10 and 20 mg/100 g of all compounds have been tested in the same experiment, so they share the same control data. All compounds were orogastrically administered during eight days at one week post *Hp* infection. Gastric colonization was measured at three weeks post infection. Olive oil and DMSO, which were used as vehicles of the compounds, had no effect on the gastric colonization. **A)** Evaluation of *Hp* gastric colonization inhibition by colony counting. The number of viable colonies (CFU/g gastric tissue) is represented for each compound dose tested. In all cases the CFU/g was related to that of the corresponding control group. Mann-Whitney U and Unpaired t tests were used for statistical analysis. A statistically significant inhibition of the gastric mucosa colonization has been observed for all compounds, except for **IV-c**, and especially for compounds **IV**, **IV-a** and **IV-d**, which were able to reduce the colonization rate at almost all doses. The inhibitory effect does not follow a clear dose dependency, possible related to the poor solubility of the compounds. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. **B)** Eradication rates in *Hp*-infected mice treated with the compounds able to eradicate the infection in some mice: **IV**, **IV-a**, **IV-b** and **IV-d**. Percentages of colonized (*Hp*+) and non-colonized (*Hp*-) mice are represented by white and grey bars, respectively.

As reported in Figure 3A, parent compound **I** was able to eradicate the *Hp* infection in some mice (1/5 and 1/7) at 0.1 mg and 1 mg/100 g bw daily doses, respectively, but the overall reduction of the gastric colonization rate observed was not statistically significant. The oxime variant **I-a** decreased significantly the colonization rate at 10 mg/100 g bw

(p=0,016), but did not eradicate *Hp* infection from any mouse. The performance of the **II**-related derivatives was also limited. Parent compound **II** had no inhibitory effect on the gastric colonization; however, the ethylene-reduced **II-b** eradicated *Hp* infection from some mice (1/6) at a 20 mg/100 g bw dose; the ethylene-reduced and nitro reduced **II-c** amine derivative also eradicated *Hp* infection from some mice (1/4) and significantly reduced the colonization rate (p=0.024) at 20 mg/100 g dose. In addition, compound **II-a** reduced the colonization (p=0.0009) at a 1 mg/100 g bw dose.

The best performance comes from **IV**-related compounds, in agreement with *in vitro* testing. Both the parent compound **IV**, the amine-derivatives **IV-a** and **IV-d**, and the non-benzoxadiazole containing compound **IV-b** significantly lowered the colonization rate at most of the doses tested (Figure 4A) and eradicated the infection in some mice (Figure 4B). Compound **IV** lowered colonization at 0.1, 1, 5 and 10 mg/100 g bw doses (p=0.044; 0.041; 0.048 and 0.0002, respectively) and eradicated infection in 43 % (3/7) and 17 % (3/18) of mice treated with 0.1 and 10 mg/100 g bw, respectively. Compound **IV-a** also inhibited the gastric colonization at 1, 5 and 10 mg/100 g bw doses (p=0.0001; 0.0018 and 0.0004, respectively) and eradicated the infection in 13 % (4/31) of treated mice at 1 mg/100 g bw. Compound **IV-b**, the derivative lacking the nitrobenzoxadiazole moiety, inhibited gastric colonization (p=0.0277 and 0.0042) at 10 and 20 mg/100 g bw, respectively, and eradicated the infection in 29 % (2/7) of mice at a 20 mg/100 g bw dose. Finally, compound **IV-d**, the sulfoxide version of compound **IV-a**, decreased colonization at 1, 10 and 20 mg/100 g bw doses (p=0.005, 0.0002 and 0.0122, respectively) and eradicate the infection in 60 % (3/5) of animals at the higher dose and in 5 % (1/20) of mice at 10 mg/100 g bw.

Perspectives for novel antimicrobials based in flavodoxin inhibitors. In previous work we discovered flavodoxin inhibitors **I**, **II** and **IV**[41,42] and explored the feasibility of improving their antibacterial activity by replacing some of their substituents.[42] To try to further improve their therapeutic indexes, we have synthesized new variants exhibiting different redox forms of the nitro, ethylene or sulphur groups present in the original inhibitors. Their potential for *Hp* eradication has been tested in the mice model of infection, and their activity against drug-resistant clinical *Hp* isolates have been determined. In all variants tested, partial or full reduction of the nitro group, reduction of the ethylene group, or partial or full oxidation of the sulphur atom largely reduces their cytotoxicity towards both HeLa cells and mice. While in the **I** and **II** series concomitant decreases in antibacterial activity are similarly large and the TIs are not improved, within the **IV** series, the activity decreases are only moderate and the resulting derivatives exhibit much larger TIs for *Hp* reference strains than the parent compound (Table 1). In fact, some of these novel flavodoxin inhibitors display TIs comparable to those of Mnz and are effective against a variety of *Hp* clinical isolates resistant to common antimicrobials such as Cla, Mnz or rifampicin. Importantly, these inhibitors, used as sole agents against *Hp* infection in the mice model, can significantly lower the gastric *Hp* load (Figures 3A and 4A) and, in some mice, they have eradicated the infection (Figures 3B and 4B). Thus, compound **IV** and its derivatives constitute a promising new family of antimicrobial chemicals with potential against the increase of *Hp* drug-resistant strains.

While their usefulness in novel combination therapies may be already worth investigating to provide alternatives for the existing drugs used against *Hp* infection, it is also clear that these inhibitors need to be improved before they can be used as stand-alone eradication drugs. Two research lines are being followed toward this end. On one hand, the mechanism

of action of these inhibitors needs to be clarified. As they were discovered using a simple binding assay to the target protein,[41,56] a straightforward inhibitory mechanism was proposed[41] consisting on the inhibitors sterically blocking the flavodoxin interaction with physiological partner proteins.[30,36,37] The fact that, besides the original inhibitors,[41] all the new derivatives herein tested bind to *Hp* flavodoxin (as determined by isothermal titration calorimetry and thermal shift assay, not shown) lends support to this mechanism and provides direct experimental evidence for *in vitro* target engagement. Work is in progress to obtain structural information on flavodoxin/inhibitor complexes as this could facilitate their rational redesign and improvement. In addition, the possibility that flavodoxin activates the compounds needs to be considered as the inhibitors contain nitro groups whose reduction could generate radical cytotoxic products ultimately responsible for the observed antibacterial activity. In this respect, the antibacterial activity of Mnz towards *Hp* and other microaerophilic pathogens is thought to be mediated by one such redox activation mechanism.[43,46,57–59] This alternative activating mechanism cannot be invoked, though, in the case of compound **IV-b**, the analogue of **IV** lacking a nitro group. Interestingly, the fact that inhibitors such as **IV-a** retain antibacterial activity when carrying an amine instead of the reducible nitro group of lead compound **IV** indicates that, if radical species played a role in the bactericidal activity, they would have to be generated in a reverse manner from cellular oxidation of the amine. On the other hand, a second line for improvement is suggested by the poor aqueous solubility of the inhibitors, reflected in their logS values (Table S1 and Figure S1), and possibly related to the toxicity observed in some of the mice stomachs at the highest dose of the parent compound **IV**, which we interpret as a chemical direct toxicity since no other alterations are observed. The solubility of the inhibitors needs to be increased by a judicious incorporation of polar groups in order to increase their

bioavailability and ensure a systemic effect. Work is in progress to synthesise and test more soluble variants of compound **IV**-derived inhibitors. Finally, unlike broad-spectrum antimicrobials targeting very fundamental cellular components such as DNA, ribosomes or the cell wall, flavodoxin inhibitors target a protein that is not essential for all bacteria and whose sequence conservation is not particularly high. Our preliminary analysis of the inhibitors' specificity revealed that compound **IV** was not effective against *H. pylori* distant bacteria such as *E. coli* or *S. aureus*.[41] We have now confirmed the same is true for the novel non-toxic inhibitors described here (not shown). These flavodoxin inhibitors thus appear to be selective and may be less damaging to the commensal microbiota than broad-spectrum antimicrobials.[60]

CONCLUSION

Several lead compounds previously shown to inhibit the flavodoxin from *Hp* have been redesigned to improve their antimicrobial activity. By synthesizing new variants exhibiting different redox forms of the nitro, ethylene or sulphur groups, a family of nitrobenzoxadiazol-based flavodoxin inhibitors with low toxicity for HeLa cells and for mice has been developed that exhibits high therapeutic indexes against reference *Hp* strains. Importantly, some of these new inhibitors are effective against drug-resistant clinical *Hp* isolates (including Cla-, Mnz- and rifampicin-resistant strains) and are able to significantly reduce *Hp* gastric colonization in the mouse model of infection and to eradicate the infection in some mice. After improvement of their pharmacokinetic properties, these compounds could constitute a new, *Hp*-specific family of antimicrobials that could be less damaging for the gut microbiota than currently used broad-spectrum

antibiotics and might be used on their own or in new combinations with existing antibiotics to fight *Hp* resistant strains.

EXPERIMENTAL SECTION

General synthetic and analytic procedures. All reagents were of analytical grade and were used as obtained from commercial sources. Compounds **II-e**, **II-f** and **II-g** are commercially available and were acquired from FCH group, Acros Organics and UkrOrgSyntez Ltd. (UORSY), respectively. Reactions were carried out using anhydrous solvents. ^{1}H NMR, ^{13}C NMR and ^{19}F NMR spectra were acquired at room temperature at 400, 100 and 376 MHz, respectively, using a 5 mm probe. Chemical shifts (δ) are reported in parts per million from tetramethylsilane with the solvent resonance as the internal standard. Coupling constants (J) are quoted in hertz. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), bs (broad singlet). Attached proton test spectra were acquired to determine the types of carbon signals, and Heteronuclear Multiple Bond Correlation spectra were acquired to confirm the position of heteroatoms. Special precautions such as degassing of the sample were not taken. All compounds used for biological assays are ≥ 95% purity based on NMR and HPLC chromatography. These last experiments have been performed using a Waters HPLC system equipped with a 600-E pump, a 2996 Photodiode Array detector, and a 2707 autosampler. The LC system was fitted with a C18 reversed phase column (VYDAC 238TP C18 5 μm, 4.6 mm × 250 mm) and operated, unless otherwise stated, using a linear gradient of buffer B (100% in 40 min) from 100% buffer A (buffer A consisting of 0.1% TFA in H2O, buffer B consisting of 0.085% TFA in CH3CN/H2O, 95:5 v/v) at a flow rate of 1 mL/min. High-resolution mass measurements were made using a microTOF (time-of-flight)

analyzer, and spectra were recorded from methanolic solutions using the positive electrospray ionization mode (ESI+). Reactions were magnetically stirred and whenever possible monitored by TLC. TLC was performed on precoated silica gel polyester plates and products were visualized using ultraviolet light (254 nm), ninhydrin, potassium permanganate and phosphomolybdic acid solution followed by heating. Column chromatography was performed using silica gel (Kiesegel 60, 230−400 mesh).

6,8-dichloro-2-(4-fluorophenyl)chroman-3-one oxime (I-a). Compound **I** (615 mg, 1.81 mmol) was dissolved in a mixture of dichloromethane (65 mL), conc. hydrochloric acid (4 mL) and methanol (29 mL). After the addition of iron powder (688 mg, 12.32 mmol), the mixture was sonicated (JP Selecta ultrasonic bath, 40 kHz) at room temperature for 10-40 minutes. TLC monitoring was done till completion of the reaction. The reaction mixture was filtered to remove excess iron, and the solvent was evaporated under reduced pressure to dryness. The residue was dissolved in water (35 mL) and extracted with ethyl acetate (3×50 mL). The organic layers were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. The crude product was then purified by flash chromatography (eluent: Hex/EtOAc: 8/2) to give 313 mg (53 % yield) of the desired product. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.54 (s, 1H), 7.45 (d, J = 2.5 Hz, 1H), 7.37-7.31 (m, 2H), 7.30 (d, J = 2.5 Hz, 1H), 7.23-7.18 (m, 2H), 5.93 (s, 1H), 3.94 (d, J = 22.2 Hz, 1H), 3.68 (d, J = 22.2 Hz, 1H) ppm. ^{19}F NMR (376 MHz, DMSO-d_6): δ -113.87 ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.9 (d, J = 244.6 Hz), 150.9, 148.0, 133.2, 128.6 (d, J = 8.5 Hz), 128.2, 127.3, 125.5, 124.6, 122.4, 115.4 (d, J = 21.7 Hz), 77.2, 24.4 ppm. HRMS (ESI$^+$) *m/z* [M+H]$^+$calc for $C_{15}H_{11}Cl_2FNO_2$ 326.0146, found 326.0125.

1-(2-nitroethyl)-3,5-bis(trifluoromethyl)benzene (II-b). To a vigorously stirred mixture of compound **II** (522 mg, 1.83 mmol), silica gel (3.7 g, column chromatography grade,

Sigma-Aldrich), 2-propanol (5.5 mL) and chloroform (30 mL), $NaBH_4$ (284 mg, 7.5 mmol) was added in about 40 mg portions over a period of 15 min at 25 ºC. The mixture was stirred for additional 2 hours or until completion of the reaction. Excess $NaBH_4$ was decomposed with diluted hydrochloric acid and the mixture was filtered. The filter was extracted with dichloromethane (3×50 mL) and the combined filtrates were washed with brine (50 mL), dried over anhydrous magnesium sulphate, filtered and evaporated *in vacuo* to dryness to give 504 mg (96 % yield) of the desired product which was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (s, 1H), 7.70 (s, 2H), 4.69 (t, J = 6.9 Hz, 2H), 3.46 (t, J = 6.9 Hz, 2H) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ −63.00 ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.4, 132.5 (q, J = 33.5 Hz), 129.0 (q, J = 3.6 Hz), 123.2 (q, J = 272.7 Hz), 121.8 (m, J = 3.8 Hz), 75.2, 32.8 ppm. HRMS (ESI$^+$) *m/z* $[M+H]^+$calc for $C_{10}H_8F_6NO_2$ 288.0454, found 288.0460.

2-(3,5-bis(trifluoromethyl)phenyl)ethan-1-amine (II-c). A solution of **II-b** (57.4 mg, 0.2 mmol) in ethanol (10 mL) was hydrogenated under hydrogen atmosphere (1 atm) at room temperature in the presence of 10 % palladium/carbon (10 mg). The reaction was stirred for 5 h or until complete as judged by TLC. Filtration of the catalyst and evaporation of the solvent afforded 51.4 mg (≈ 100 %) of pure desired product. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (s, 1H), 7.66 (s, 2H), 3.05 (t, J = 6.8 Hz, 2H), 2.90 (t, J = 6.8 Hz, 2H), 1.96 (bs, 1H) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ −62.83 ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 142.4, 131.8 (q, J = 33.2 Hz), 129.1 (q, J = 2.6 Hz), 123.5 (q, J = 272.2 Hz), 120.5 (m, J = 3.9 Hz), 43.0, 39.5 ppm. HRMS (ESI$^+$) *m/z* $[M+H]^+$calc for $C_{10}H_9F_6N$ 258.0712, found 258.0711.

2-(3,5-bis(trifluoromethyl)phenyl)acetaldehyde oxime (II-d). A round-bottom flask was charged with $Pd(OAc)_2$ (10 mg, 0.04mmol), the nitroarene **II** (214 mg, 0.75 mmol) and freshly distilled THF (4.5 mL). The flask was sealed and purged with Ar. Degassed H_2O

(1.75 mL) and $^{i}Pr_3SiH$ (1.1 g, 7.0 mmol) were sequentially, slowly added dropwise with a syringe. The reaction was stirred for 2 h or until completion as judged by TLC. At that time, the reaction flask was opened to the atmosphere and the reaction mixture was diluted with diethyl ether (25–50 mL). The layers were separated and the aqueous layer was back-extracted with diethyl ether (3×20 mL). The combined organic layers were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. Purification of the residue by flash chromatography (eluent: Hex/EtOAc: 8/2) gave 61 mg (30 % yield) of the desired product as a 1:1mixture of Z/E isomers. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (s, 1H) and 7.78 (s, 1H), 7.70 (s, 2H) and 7,68 (s, 2H), 7.58 (t, J = 6 Hz, 1H) and 6.92 (t, J = 5.6 Hz, 1H), 3.86 (d, J = 5.6 Hz, 2H) and 3.67 (d, J = 6 Hz, 2H) ppm. ^{19}F NMR (376 MHz, $CDCl_3$): δ −62.87 and −62.88 ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.8 and 148.5, 139.0 (q, J = 33.4 Hz), 132.2 (q, J = 33.4 Hz) and 132.2 (q, J = 33.3 Hz), 129.2 (m), 123.3 (q, J = 272.2 Hz) and 123.3 (q, J = 272.6 Hz), 121.2 (m, 3.8 Hz) and 121.0 (m, 3.8 Hz), 35.6 and 29.9 ppm. HRMS (ESI^+) *m/z* $[M]^+$calc for $C_{10}H_7F_6NO$ 271.0427, found 271.0433.

5-chloro-7-((4-methoxybenzyl)thio)benzo[c][1,2,5]oxadiazol-4-amine (IV-c). A mixture of dichloromethane (2.83 mL), conc. hydrochloric acid (170 µL) and methanol (1.29 mL) was set in a flask containing compound **IV** (25 mg, 0.08 mmol). After adding iron powder (30.42 mg, 0.54 mmol), the reaction was stirred at room temperature until disappearance of the nitro-compound, as judged by TLC. The mixture was poured into water (3 mL) and extracted with dichloromethane (4×3 mL). The organic layers were combined, dried over anhydrous magnesium sulphate, filtered and then evaporated *in vacuo*. TLC monitoring revealed that a mixture of two products had been obtained, one of them being a compound previously synthesised, namely **IV-a**, and the other one being **IV-c**. The crude was purified by column chromatography over silica gel (eluent: Hex/EtOAc: 6/4). Removal of solvents

under reduced pressure yielded 16 mg (61 % yield) of pure **IV-c**. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.23 (s, 1H), 7.14-7.12 (m, 2H), 6.94 (s, 2H), 6.83-6.81 (m, 2H), 4.19 (s, 2H), 3.70 (s, 3H) ppm. ^{13}C-NMR (100 MHz, DMSO-d_6): δ 158.3, 149.6, 144.6, 137.8, 132.2, 130.0, 129.1, 113.8, 108.1, 106.4, 55.0, 37.1 ppm. HRMS (ESI$^+$) *m/z* [M+Na]$^+$calc for $C_{14}H_{12}ClN_3NaO_2S$ 344.0231, found 344.0232.

7-((4-methoxybenzyl)sulfinyl)benzo[c][1,2,5]oxadiazol-4-amine (IV-d). Compound **IV-a** (495 mg, 1.72 mmol) was dissolved in a mixture of glacial acetic acid (20.70 mL) and 33 % aqueous hydrogen peroxide (1.38 mL), and kept at 25 ºC for 2.5 h. The yellowish-brown mixture obtained was quenched with saturated $NaHCO_3$ solution (50 mL) and extracted with dichloromethane (5×30 mL). The organic layers were dried over anhydrous magnesium sulphate, filtered and, once the solvent was evaporated under reduced pressure, the crude was purified by column chromatography over silica gel (eluent: Hex/AcOEt: 2/8) to give 378 mg (73 % yield) of the desired product. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.38 (d, J=8 Hz, 1H), 7.31 (s, 2H), 6.94-6.92 (m, 2H), 6.80-6.78 (m, 2H), 6.33 (d, J=8 Hz, 1H), 4.41 (d, J=12.8 Hz, 1H), 4.21 (d, J=12.8 Hz, 1H), 3.70 (s, 3H) ppm. ^{13}C-NMR (100 MHz, DMSO-d_6): δ 158.9, 145.7, 144.5, 140.3, 135.7, 131.3, 121.9, 113.6, 111.5, 103.1, 58.2, 55.0 ppm. HRMS (ESI$^+$) *m/z* [M+Na]$^+$calc for $C_{14}H_{13}N_3NaO_3S$ 326.0570, found 326.0575.

7-((4-methoxybenzyl)sulfonyl)benzo[c][1,2,5]oxadiazol-4-amine (IV-e). To a stirred solution of compound **IV-a** (29 mg, 0.1 mmol) in ethyl acetate (1.43 mL) at room temperature, 3-chloroperbenzoic acid (34.5 mg, 0.2 mmol)) was added. TLC monitoring was done till the completion of the reaction (1.5 h). Traces of 3-chloroperbenzoic acid and 3-chlorobenzoic acid were neutralized with saturated aqueous $NaHCO_3$ solution (2×2 mL). After extracting with ethyl acetate (3 x 3 mL), the combined organic layers were dried over anhydrous magnesium sulphate, filtered and the solvent was evaporated under reduced

pressure. TLC monitoring revealed that a mixture of **IV-d** and **IV-e** had been obtained. The crude was purified by flash chromatography (eluent: Hex/EtOAc: 3/7) to provide 27 mg (85 % yield) of pure **IV-e**.^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.95 (s, 2H), 7.57 (d, J=8 Hz, 1H), 7.01-6.98(m, 2H), 6.82-6.80 (m, 2H), 6.29 (d, J=8 Hz, 1H), 4.55 (s, 2H), 3.70 (s, 3H) ppm. ^{13}C-NMR (100 MHz, DMSO-d_6): δ 159.2, 146.0, 144.0, 143.5, 140.9, 131.9, 120.6, 113.7, 106.6, 102.0, 59.7, 55.0 ppm. HRMS (ESI$^+$) *m/z* [M+Na]$^+$calc for $C_{14}H_{13}N_3NaO_4S$ 342.0519, found 342.0523.

4-((4-methoxybenzyl)sulfinyl)-7-nitrobenzo[c][1,2,5]oxadiazole (IV-f). Compound **IV** (96 mg, 0.30 mmol) was dissolved in a mixture of glacial acetic acid (5.66 mL) and 33 % hydrogen peroxide (242 μL) and the reaction was stirred at 25 ºC for 24 h. The obtained orange-brown oily mixture was extracted with dichloromethane (3×10 mL). The combined organic layers were dried over anhydrous magnesium sulphate, filtered and the solvent was evaporated under reduced pressure. Flash chromatography of the mixture was done in order to purify the desired product. In this way 84.3 mg (88 % yield) of pure **IV-f**, which was stored in a sealed flask under an argon atmosphere at 4 °C until testing to avoid spontaneous decomposition because of its sensitivity to oxygen, were obtained. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.47 (d, J=7.2 Hz, 1H), 7.75 (d, J=7.2 Hz, 1H), 6.89-6.87 (m, 2H), 6.77-6.74 (m, 2H), 4.61 (d, J=13.6 Hz, 1H), 4.32 (d, J=13.6 Hz, 1H), 3.77 (s, 3H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 160.2, 146.1, 142.6, 142.3, 131.3, 129.1, 128.7, 119.6, 114.1, 59.5, 55.3 ppm. HRMS (ESI$^+$) *m/z* [M+Na]$^+$ calc for $C_{14}H_{11}N_3NaO_5S$ 356.0312, found 356.0308.

2-(5-amino-2-methyl-1H-imidazol-1-yl)ethan-1-ol (Mnz-a). Commercially available metronidazole (34.2 mg, 0.2 mmol) and 25 % Pd/C (9 mg) were placed in a 50 mL two-necked round-bottomed flask under argon atmosphere. Anhydrous, degassed THF (10 mL)

was added. The reaction was stirred for 24 h at room temperature and, after checking for complexion, the reaction mixture was filtered through Celite® and washed a few times with anhydrous THF (3×3 mL). The solvent was removed under reduced pressure to afford the 26,5 mg (94 %) of crude product as an oil. The thus formed amino-imidazole is sensitive to O_2 and heat, and therefore it was stored without further purification in a sealed flask under an argon atmosphere at –20 °C until its MCC_{50} and MIC determinations. Just before MIC determinations, the crude was purified by flash chromatography (First eluent: dichloromethane /methanol: 1/1, second eluent: methanol, third eluent: methanol containing 0.1% triethylamine) and used immediately. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.91 (s, 1H), 4.16 (bs, 2H), 3.75 (t, J = 5.6 Hz, 2H), 3.54 (t, J = 5.6 Hz, 2H), 2.16 (s, 3H) ppm.

Minimal Inhibitory Concentrations (MIC) on reference *Hp* strains (NCTC 11637, 26695 and SS1). For microdilution MIC testing, *Hp* reference strains NCTC 11637 (ATCC 43504, Mnz-resistant strain), 26695 (ATCC 700392)[61] and SS1 (Sydney Strain 1)[54] (Biosafety Level 2 pathogen) were grown in Brain-Heart Infusion (BHI) broth supplemented with 4 % fetal bovine serum at 37 °C under microaerophilic conditions and then diluted to an optical density at 600 nm of 0.01. Samples of 100 µL of the diluted bacterial suspension were dispensed in a 96-well plate, except for the first well of each row in which 200 µL of the bacterial suspension along with 2 µL of the compound (from a stock solution in dimethyl sulfoxide (DMSO) at 6.4 µg/µL) were added. Two-fold serial dilutions were made as described,[41,42] which allowed to test the compounds in a concentration range from 64 to 0.031 µg/mL. Positive and negative controls consisted of brain-heart infusion broth supplemented with 4 % fetal bovine serum and inoculated or not with the diluted *Hp* bacterial suspension, respectively. We ensured that DMSO concentration was kept at 1 % v/v or below, so that no toxic effect was found for *Hp* cells.

Plates were incubated at 37 ºC for 48 hours under a microaerophilic atmosphere. MICs correspond to the lowest concentrations of compounds leading to a complete inhibition of *Hp* growth. The MIC values were determined by recording the colour change observed after addition of 30 µL of resazurin (0.1 mg/ml; Sigma-Aldrich) to each well in the 96-well dish. The MICs were additionally confirmed by spotting 10 µL of two consecutive wells (one with the highest concentration of compound that allowed bacterial growth, and the adjacent well with the lowest concentration of compound that prevented bacterial growth) onto Columbia Agar with Sheep Blood plates (Oxoid) followed by incubation at 37 °C for 48 h under microaerophilic conditions. Growth or absence of *Hp* growth allowed to determine the Minimal Bactericidal Concentration of the compounds. Each experiment was performed twice in triplicate. Both assays confirmed the MIC values obtained by the microdilution method.

MIC on drug-resistant *Hp* strains. Six *Hp* drug-resistant strains obtained at the University Hospital Lozano Blesa (Spain) from patients with gastric pathologies (dyspepsia and/or peptic ulcer disease) who had failed to at least two conventional therapies recommended in national guidelines[53] were selected. These strains were isolated from gastric biopsies obtained during routine upper gastrointestinal endoscopic procedures and cultured. According to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints,[62] three of these *Hp* strains were Mnz-resistant, two were Mnz/Cla resistant, and one was rifampicin-resistant. Compounds **II-b**, **II-d**, **II-e**, **IV-a**, **IV-c**, **IV-d** and **IV-e** were tested on all of these strains. The determination of their MIC was performed by microdilution and followed by the colorimetric method using resazurin, as explained above. Written informed consent was obtained from patients and the procedures were approved by the Regional Ethical Committee of Aragón.

Minimal Cytotoxic Concentrations (MCC_{50}). HeLa cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere. The toxicity of the compounds towards HeLa cells was determined by the XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulphenyl)-*2H*-tetrazolium-5-carboxanilide sodium salt) method using the *Cell Proliferation Kit II* (Roche) as described.[41,42] Briefly, 0.1-μL volumes of compound dissolved in DMSO were added to give final compound concentrations of 25, 50, 75, 100, 250, 500, 750, and 1000 μM, in 96-well plates (with 30,000 cells in 100 μL in each well). The untreated control consisted in 30,000 cells per well in 100 μL of complete medium (with 0.1 % DMSO). Cell viability was calculated from the wells' absorbance at 450 and 650 nm, as described.[41,42] Cytotoxicity curves (i.e percentage of cell viability versus compound concentration) were used to calculate the compounds concentration producing 50 % cell lysis (MCC_{50}), as described.[41,42] All experiments were performed twice in triplicate. Therapeutic Indexes (TI) were calculated as MCC_{50}/MIC.

Toxicity assays in mice. The *in vivo* toxicity of compounds was tested in 48 twenty-week old specific-pathogen-free female C57BL/6J mice obtained from Charles River Laboratories España (Barcelona, Spain), fed with a standard commercial rodent diet and water *ad libitum*. Females were selected because of their less aggressiveness. All procedures followed for toxicity analyses in mice were approved by the Ethics Committee for Animal Experiments of the University of Zaragoza, and all the experiments were performed according to the approved ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines for the care and manipulation of laboratory animals. Toxicity of compounds **IV**, **II-a**, and **IV-a** was tested at three different doses: 10, 20 and 40 mg/100 g bw. Assuming the administered compounds are uniformly distributed throughout the

animal body, these doses range from 12 to 1600 times the MICs of the compounds. Ten groups of 4-5 animals were used. To nine of them, and for each compound, the corresponding daily dose was administered in volumes of 100 µl by oral gavage for 7 days. To the control group, vehicle alone (olive oil, 5 % DMSO) was administered. After treatment, mice were sacrificed by CO_2 asphyxiation and blood was collected by cardiac puncture. An automatic analyser (VetScan, Abaxis) was used to determine biochemical alterations. Histopathology evaluation was done on heart, stomach, liver, lung and kidney samples by haematoxylin-eosin staining of tissue sections.

Antibacterial assays in mice. *In vivo* analysis of the anti-*Hp* activity of the compounds was carried out in 6-week old (SPF) NMRI (Naval Medical Research Institute) mice (Charles River laboratories, France). The level of *Hp* gastric colonization in the mice model is similar in male and female, so assays were carried out in females because they are less aggressive than males under the hosting conditions. Experiments were performed according to the European Directives (2010/63/UE), approved by the Committee of Central Animal Facility board of the Institute Pasteur and following the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. The project was authorized by the Comité d'Éthique en Expérimentation Animale, Institut Pasteur (Ref 00317.02). Four different experiments were performed to test compounds **I**, **II**, **IV**, **I-a**, **II-a**, **II-b**, **II-c**, **II-f**, **IV-a**, **IV-b**, **IV-c** and **IV-d** at some of the following doses of 0.1, 1, 5, 10 and 20 mg/100 g bw. In all experiments, 7 mice were analysed for each studied condition. Mice were orogastrically infected at days 1 and 3 during the first week of the experiment with 100 µL of a suspension of *Hp* strain SS1 ($5 \cdot 10^8$ CFU/ml) known to colonize efficiently the mouse gastric mucosa.[54] According to a previously established model of antimicrobial treatment of *Hp* infection,[55] administration of the compounds started at day 8. One-hundred µL of each

compound solubilised in 5-10 % DMSO (Euromedex)/95-90 % olive oil (Sigma-Aldrich) as vehicle at the defined doses were daily administered by oral gavage for 8 consecutive days. Non-infected control groups received orally 100 µL of peptone trypsin broth alone. In addition, infected and non-treated control groups were administered 100 µL of vehicle. No toxicity signs associated to the vehicle or the compounds treatment were noticed: i.e. no weight variations (Figure S1) and no appetite alterations. At 8 days post-treatment (day 24), mice were sacrificed by CO_2 inhalation, stomachs were isolated and *Hp* gastric colonization determined as described.[63] Plates were incubated for 5 days at 37 °C under a microaerophilic atmosphere created by the Anoxomat AN2CTS system (MART Microbiology B.V.). The antibacterial activity of the compounds was evaluated by counting the number of viable bacterial colonies expressed as CFU per gram of gastric tissue.[63]

Statistical analysis. Statistical analyses of efficacy assays in mice were performed with GraphPad Prism 7 Software (GraphPad Software, Inc. CA, USA). Mann-Whitney U and Unpaired t tests were used to assess differences in mouse gastric colonization between *Hp*-infected vehicle-treated and compound-treated groups. Differences were considered statistically significant for $p \leq 0.05$.

ASSOCIATED CONTENT

Supporting information

The supporting information is available free of charge on the ACS Publication website.

Compound characterization data (by NMR, mass spectrometry, HPLC and theoretical determination of logS values), additional information about *in vitro* and *in vivo* efficacy experiments (a table where compound doses for mice experiment are detailed, the MIC values for the developed compounds against clinical *Hp* isolates and the mice weight

variation during efficacy experiments), *in vitro* and *in vivo* toxicity data (viability of HeLa cells in the presence of the compounds, histological study of *in vivo* toxicity of some compounds, *in vitro* acid stability evaluation of compounds), and SMILES format representations of compounds (Molecular Formula Strings).

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Notes

The authors declare that a patent has been filled concerning derivatives of compound IV.

ETHICS APPROVAL

Ethics Committee for Animal Experiments of the University of Zaragoza (Spain) and Committee of Central Animal Facility board of the Institute Pasteur (Paris, France).

ACKNOWLEDGMENTS

JS was supported by grant BFU2016-78232-P (MINECO, Spain) and E45_17R (Gobierno de Aragón, Spain). AL was supported by grant PI11/02578 (MICINN, Spain). S.S. and A.M. are recipients of predoctoral fellowships from the Aragonese and Spanish Governments, respectively. The authors thank Dr Francis Mégraud (Bordeaux. France) for advice on culturing human *Hp* isolates and infecting animals with human strains.

ABBREVIATIONS USED

ATCC, American Type Culture Collection; bw, body weigh; CFU, colony forming units; Cla, clarithromycin; DMSO, dimethyl sulfoxide; ESI, electrospray; EUCAST, European Committee on Antimicrobial Susceptibility Testing; Fld, flavodoxin; g, grams; *Hp, Helicobacter pylori;* HPLC, High-performance liquid chromatography; HRMS, high-resolution mass spectrometry*;* MCC, minimal cytotoxic concentration*;* MIC, minimal inhibitory concentration*;* Mnz, metronidazole; NCTC, National Collection of Type Cultures; NMR, nuclear magnetic resonance; SS1, Sydney Strain 1*;*THF, tetrahydrofuran; TI, therapeutic index; TLC, thin layer chromatography.

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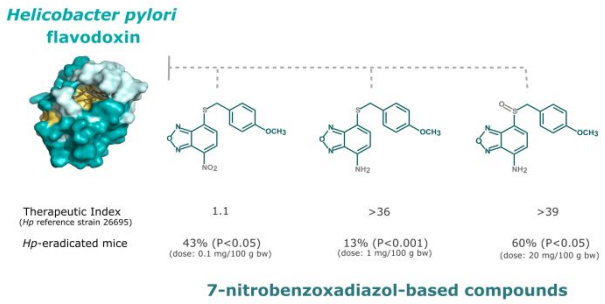
Table of Contents Graphic



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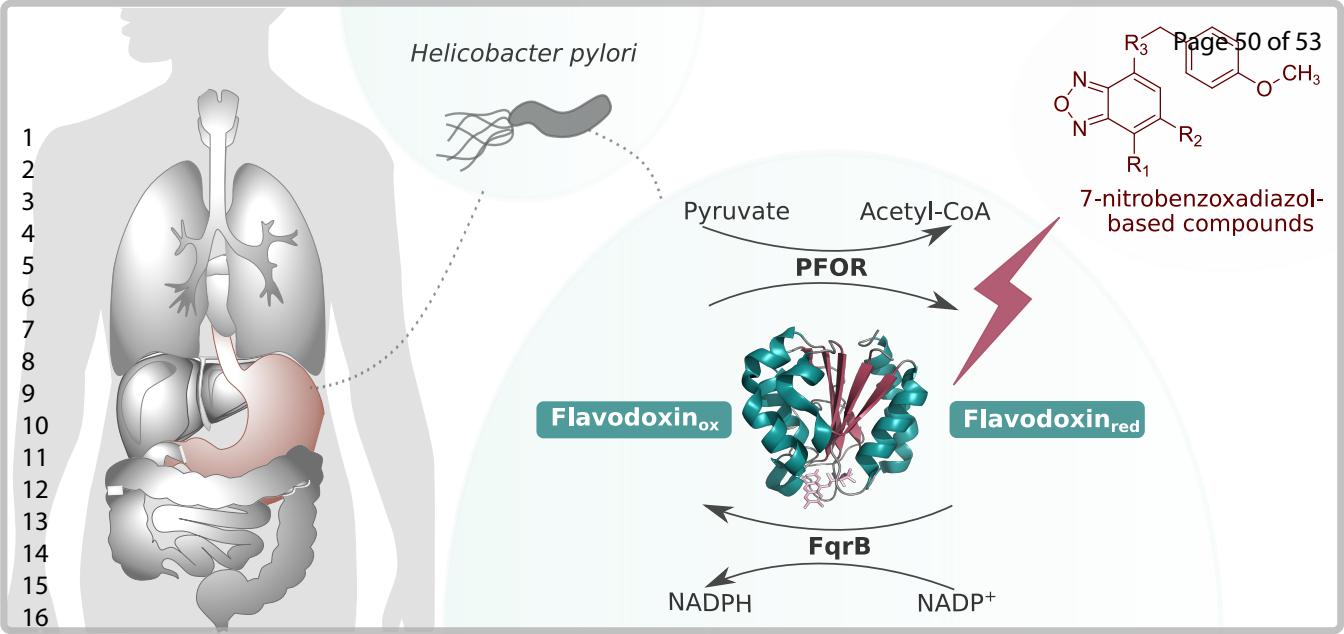
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FqrB
NADPH
NADP$^+$
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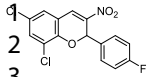
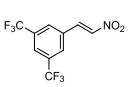
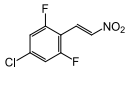
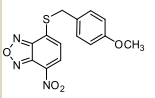
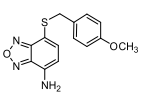
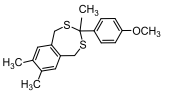
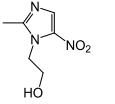
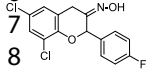
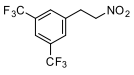
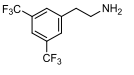
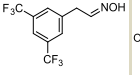
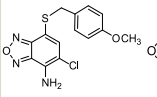
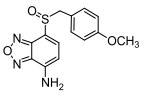
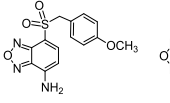
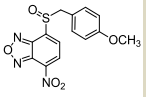
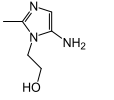
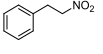
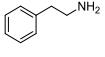
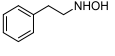
R_1
CH_3
7-nitrobenzoxadiazol-based compounds

I-related compounds	II-related compounds		IV-related compounds				Mnz-related compounds
 I	 II	 II-a	 IV	 IV-a	 IV-b		 Mnz
 I-a	 II-b	 II-c	 II-d	 IV-c	 IV-d	 IV-e	 IV-f	 Mnz-a
	 II-e	 II-f	 II-g					

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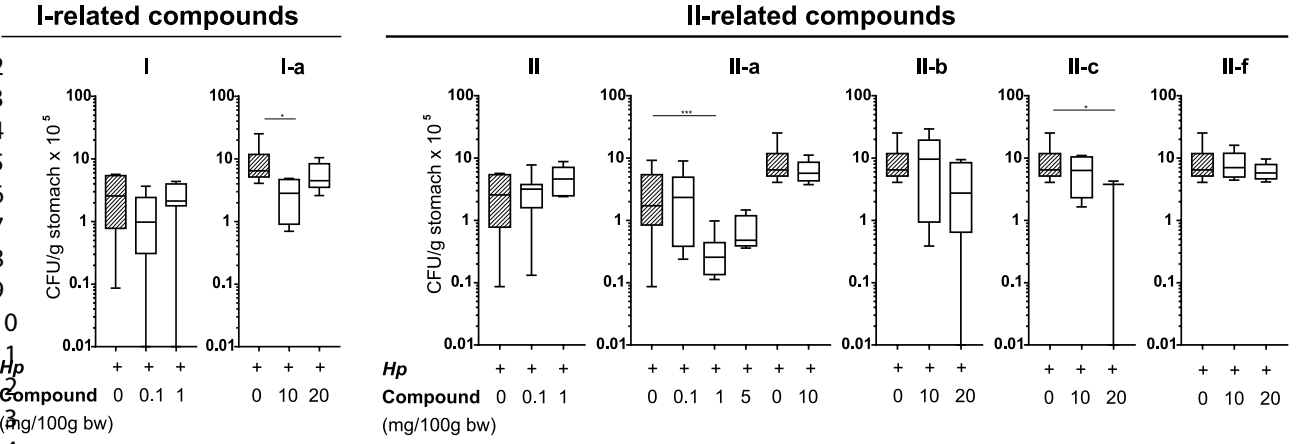
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II
II-a
II-b
II-c
II-f
CFU/g stomach x 10^5
Hp
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(mg/100g bw)
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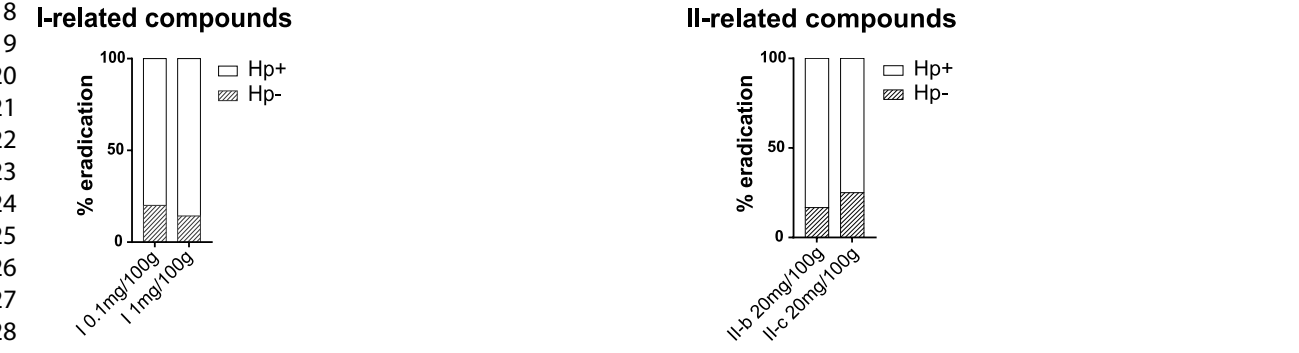
0 0.1 1 5 0 10
0 10 20
0 10 20
0 10 20

B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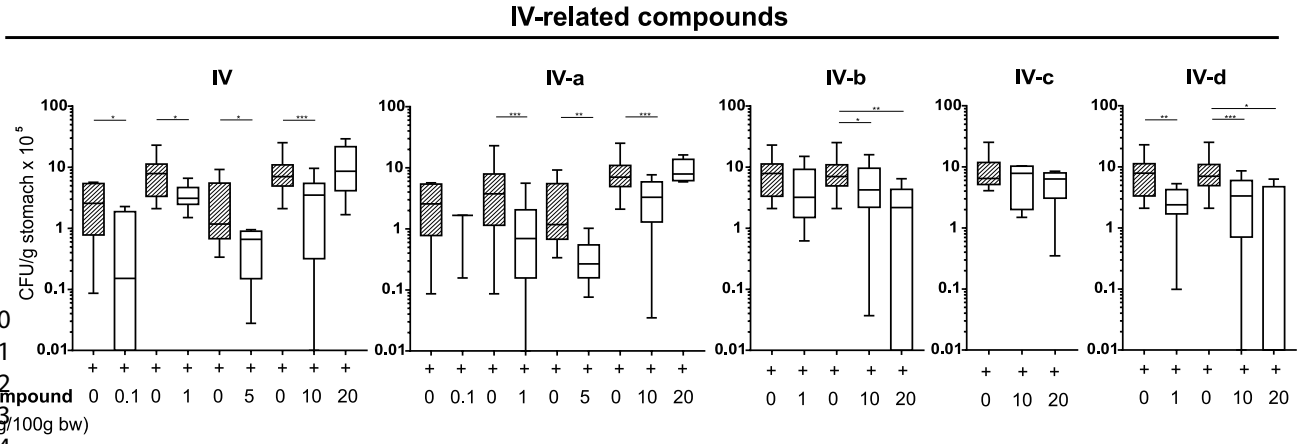
II-c 20mg/100g

A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Hp
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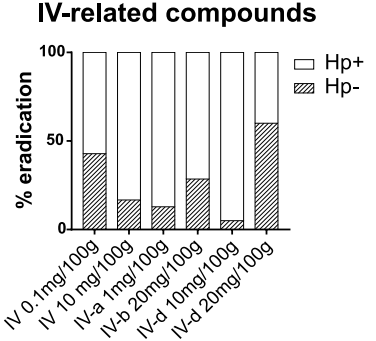
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