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## 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

Sandra Salillas, Miriam Alías, Valérie Michel, Alejandro Mahía, Ainhoa Lucía, Liliana Rodrigues, Jessica Bueno, Juan José Galano-Frutos, Hilde de Reuse, Adrián Velázquez-Campoy, et al.

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8 nitroethylene- and 7-nitrobenzoxadiazol-based  
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11 flavodoxin inhibitors against *Helicobacter pylori*  
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16 drug-resistant clinical strains and in *Helicobacter*  
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21 *pylori*-infected mice  
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### 36 37 38 **KEYWORDS**

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40 Novel antimicrobials; antimicrobial resistance; *Helicobacter pylori*; drug discovery;  
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42 flavodoxin  
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### 46 47 **ABSTRACT**

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49 *Helicobacter pylori* (*Hp*) infection is the main cause of peptic ulcer and gastric cancer. *Hp*  
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51 eradication rates have fallen due to increasing bacterial resistance to currently used broad-  
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53 spectrum antimicrobials. We have designed, synthesized and tested redox variants of  
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55 nitroethylene- and 7-nitrobenzoxadiazole-based inhibitors of the essential *Hp* protein  
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3 flavodoxin. Derivatives of the 7-nitrobenzoxadiazole lead, carrying reduced forms of the  
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5 nitro group and/or oxidized forms of a sulphur atom, display high therapeutic indexes  
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7 against several reference *Hp* strains. These inhibitors are effective against metronidazole-,  
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9 clarithromycin- and rifampicin-resistant *Hp* clinical isolates. Their toxicity for mice after  
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11 oral administration is low and, when administered individually at single daily doses for 8  
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13 days in a mice model of *Hp* infection, they decrease significantly *Hp* gastric colonization  
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15 rates and are able to eradicate the infection in up to 60% of the mice. These flavodoxin  
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17 inhibitors constitute a novel family of *Hp*-specific antimicrobials that may help fight the  
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19 constant increase of *Hp* antimicrobial-resistant strains.  
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## INTRODUCTION

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

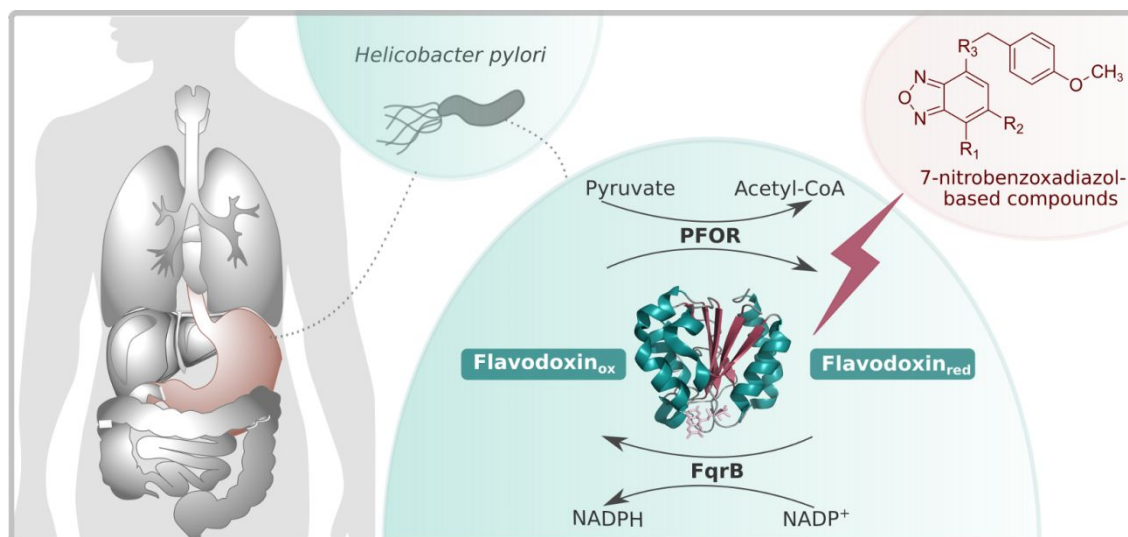
Conventional therapy against *Hp* consists of empirical triple or, more often now, quadruple treatments based on a proton pump inhibitor plus a combination of two or three broad spectrum antimicrobials such as clarithromycin (Cla), amoxicillin, metronidazole (Mnz), tetracycline and bismuth salts.<sup>11,17-19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

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3 genetic variability and to the complex host immune response against the bacterium.<sup>9,24–26</sup>

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

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

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**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<sup>30,31</sup> interfering with binding or electron transfer to *Hp*-Fld partner proteins.<sup>39</sup> 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.<sup>39</sup>

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

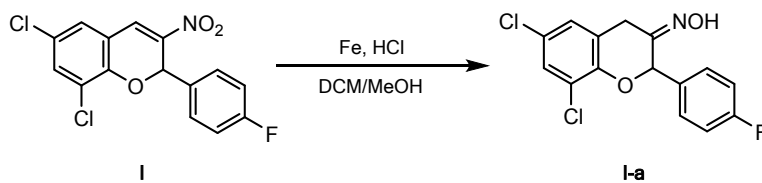
## 31 32 33 **RESULTS AND DISCUSSION**

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

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

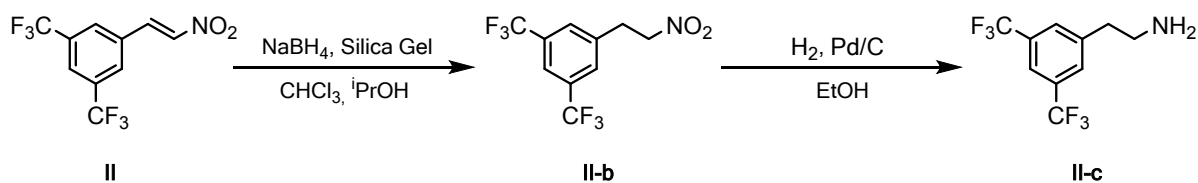
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49 **Scheme 1.** Reduction of nitro-compound **I** to oxime **I-a**  
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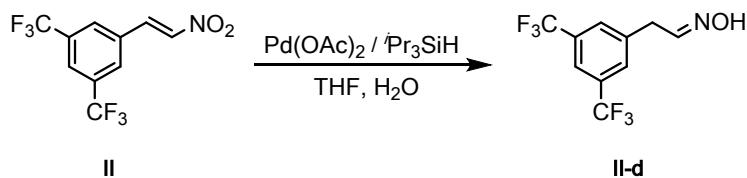
10 Reduction of nitrostyrene **II** with sodium borohydride in the presence of silica gel<sup>48</sup> and in  
11 a mixture of chloroform and 2-propanol led to the corresponding nitroalkane **II-b** (Scheme  
12 2). Further reduction by catalytic hydrogenation using palladium-on-carbon as the catalyst  
13 led to aminoalkane **II-c**.  
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21 **Scheme 2.** Reduction of nitrostyrene **II** to nitroalkane **II-b** and reduction of this last one to  
22 aminoalkane **II-c**  
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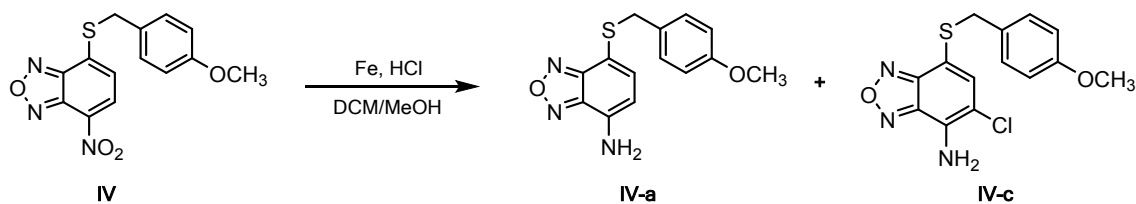
36 Oxime **II-d** could be obtained by a palladium-catalysed reduction<sup>49</sup> with triisopropylsilane  
37 of nitro compound **II** in a mixture of THF and water (Scheme 3).  
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43 **Scheme 3.** Reduction of nitrostyrene **II** to oxime **II-d**  
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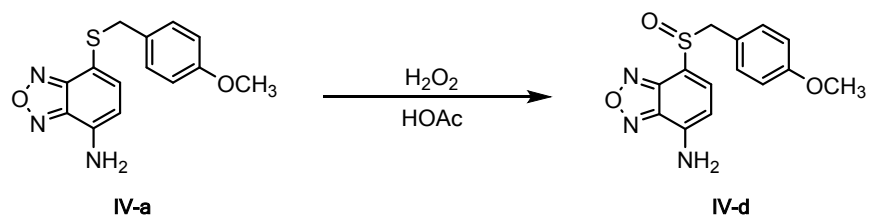
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3 Reduction of nitro-compound **IV** with iron and hydrochloric acid, by using  
4 dichloromethane and methanol as solvents,<sup>47</sup> afforded the desired product **IV-c** and also  
5 some of the previously synthesized molecule **IV-a** (Scheme 4) from which it was separated  
6 by column chromatography.  
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14 **Scheme 4.** Reduction of nitro-compound **IV** to a mixture of amino-compound **IV-a** and  
15 amino-chlorinated compound **IV-c**  
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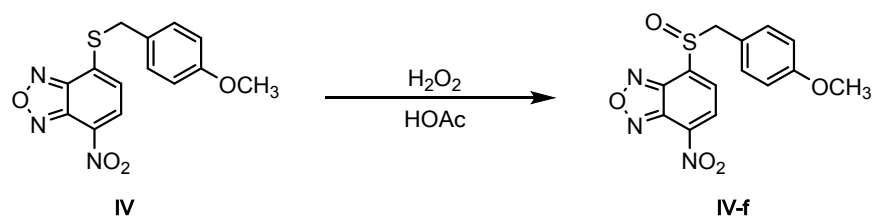


38 Amino-sulfoxide **IV-d** and amino-sulfone **IV-f** could be obtained by thio-ether oxidation of  
39 molecules **IV-a** and **IV**, respectively, with hydrogen peroxide in the presence of glacial  
40 acetic acid (Schemes 5 and 6).<sup>50</sup>  
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51 **Scheme 5.** Oxidation of sulphur compound **IV-a** to sulfoxide **IV-d**  
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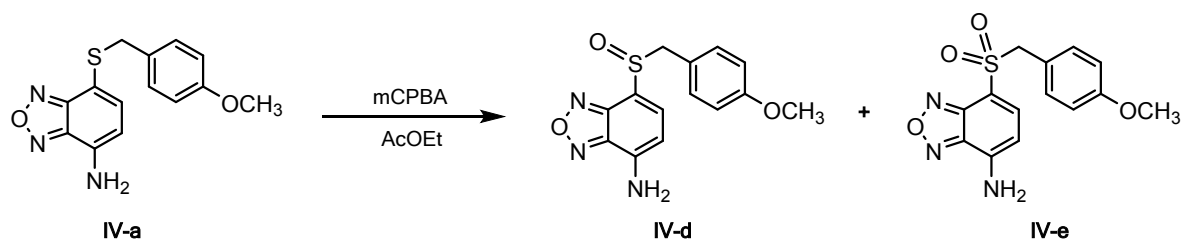


51 **Scheme 6.** Oxidation of sulphur compound **IV** to sulfoxide **IV-f**  
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11 The amino-sulfone **IV-e** was synthesized by oxidation of compound **IV-a** using *meta*-  
12 chloroperbenzoic acid in ethyl acetate as the oxidant.<sup>51</sup> Compound **IV-e** was obtained as a  
13 mixture with compound **IV-d**, from which it was separated by flash chromatography  
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18 (Scheme 7).

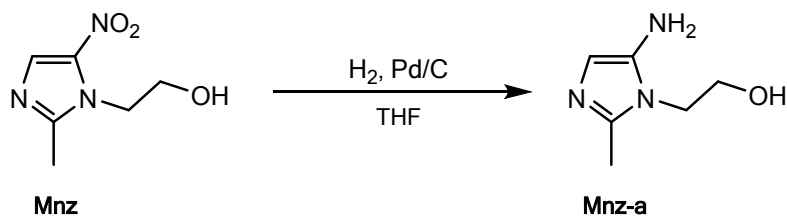
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23 **Scheme 7.** Oxidation of sulphur **IV-a** to a mixture of sulfoxide **IV-d** and sulfone **IV-e**



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

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



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

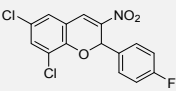
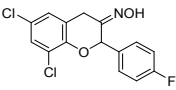
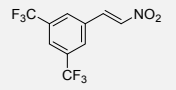
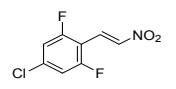
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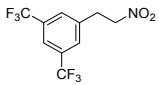
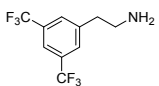
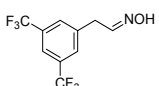
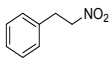
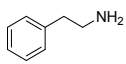
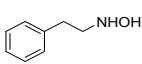
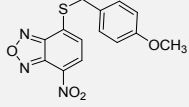
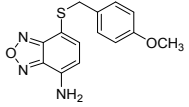
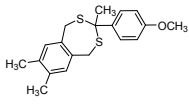
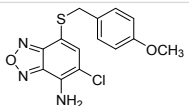
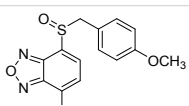
I-related compounds	II-related compounds			IV-related compounds				Mnz-related compounds
<b>I</b>	<b>II</b>	<b>II-a</b>	<b>II-c</b>	<b>IV</b>	<b>IV-a</b>	<b>IV-b</b>	<b>Mnz</b>	
<b>I-a</b>	<b>II-b</b>	<b>II-c</b>	<b>II-d</b>	<b>IV-c</b>	<b>IV-d</b>	<b>IV-e</b>	<b>IV-f</b>	<b>Mnz-a</b>
	<b>II-e</b>	<b>II-f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

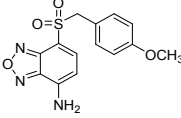
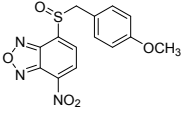
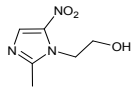
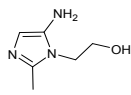
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41 **Figure 2. Chemical structure of 19 flavodoxin inhibitors grouped by similarity to lead**  
42 **compound (I, II, IV or Mnz).** In each group the upper line provides the formula of the  
43 lead and derivatives preliminary tested *in vitro* in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

Compound	Structure	MCC <sub>50</sub> <sup>b</sup>	MIC <sup>c</sup>			TI <sup>d</sup>		
		( $\mu$ M)	(μM)			(MCC <sub>50</sub> /MIC)		
		HeLa	SS1	NCTC 11637	26695	SS1	NCTC 11637	26695
<b>I</b>		5	0.37	0.37	0.37	14	14	14
<b>I-a</b>		117	12	6.1	6.1	10	19	19
<b>II</b>		15	1.8	3.5	1.8	8.3	4.3	8.3
<b>II-a</b>		36	2.3	2.3	2.3	16	16	16

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4	<b>II-b</b>		>1000	223	56	56	>4.5	>18	>18
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8	<b>II-c</b>		>500	>249	31	31	~2.0	>16	>16
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10									
11									
12									
13	<b>II-d</b>		>500	236	59	118	>2.1	>8.5	>4.2
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15									
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17									
18	<b>II-e</b>		>1000	212	212	106	>4.7	>4.7	>9.4
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20									
21									
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23	<b>II-f</b>		>1000	132	66	66	>7.6	>15	>15
24									
25									
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27									
28	<b>II-g</b>		>1000	>233	>233	>233	~4.3	~4.3	~4.3
29									
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31									
32	<b>IV</b>		7	0.78	1.6	6.3	9.0	4.4	1.1
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37	<b>IV-a</b>		>1000	13.9	27.8	27.8	>72	>36	>36
38									
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42	<b>IV-b</b>		>1000	6.1	3.0	3.0	>164	>333	>333
43									
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46	<b>IV-c</b>		>1000	5.9	11.8	5.9	>170	>85	>170
47									
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51	<b>IV-d</b>		>1000	105	53	26	>9.5	>19	>39
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<b>IV-e</b>		>1000	100	100	100	>10	>10	>10
<b>IV-f</b>		179	12	6	6	15	30	30
<b>Mnz</b>		>1000	2.9	187	12	>345	>5.3	>83
<b>Mnz-a</b>		>1000	>453	227	453	~2.2	>4.4	>2.2

<sup>a</sup>Parent compounds **I**, **II** and **IV** are highlighted in grey

<sup>b</sup>The Minimal Cytotoxic Concentration (MCC<sub>50</sub>) tested in HeLa cells

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

<sup>d</sup>Therapeutic Indexes (TI) have been calculated as MCC<sub>50</sub>/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

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3 Mnz nor the amine variant are toxic at the higher concentration tested. Globally considered,  
4 reduction of the nitro or the ethylene group in the leads carrying a nitroethylene moiety (**I**,  
5 **II** and Mnz), decreases to a similar extent toxicity and antimicrobial activity. Only  
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10 compounds **II-a** (previously tested by Galano et al., 2013<sup>42</sup> and here re-evaluated), **II-b**, **II-**  
11  
12 **c** and **II-f** slightly improve (about 2-fold) the TI of their parent compound.

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14 Compound **IV** also contains a nitro group, which, in this case, is attached to a  
15 benzoxadiazole ring. In this lead, reduction of the nitro group to amine lowers toxicity so  
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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<sup>42</sup>. Unlike all other **IV** variants, **IV-b** lacks  
the benzoxadiazol ring and, therefore, the nitro group. Its toxicity is very low (>1000  $\mu$ M)  
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<sup>53</sup>. 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
<b>Amoxicillin</b>	S	S	S	S	S	S
<b>Clarithromycin</b>	S	S	S	<b>R</b>	S	<b>R</b>
<b>Tetracycline</b>	S	S	S	S	S	S
<b>Levofloxacin</b>	S	S	S	S	S	S
<b>Metronidazole</b>	<b>R</b>	<b>R</b>	<b>R</b>	<b>R</b>	S	<b>R</b>
<b>Rifampicin</b>	S	S	S	S	<b>R</b>	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<sub>50</sub>/MIC) of some developed compounds against *H. pylori* drug-resistant clinical isolates <sup>a</sup>**

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

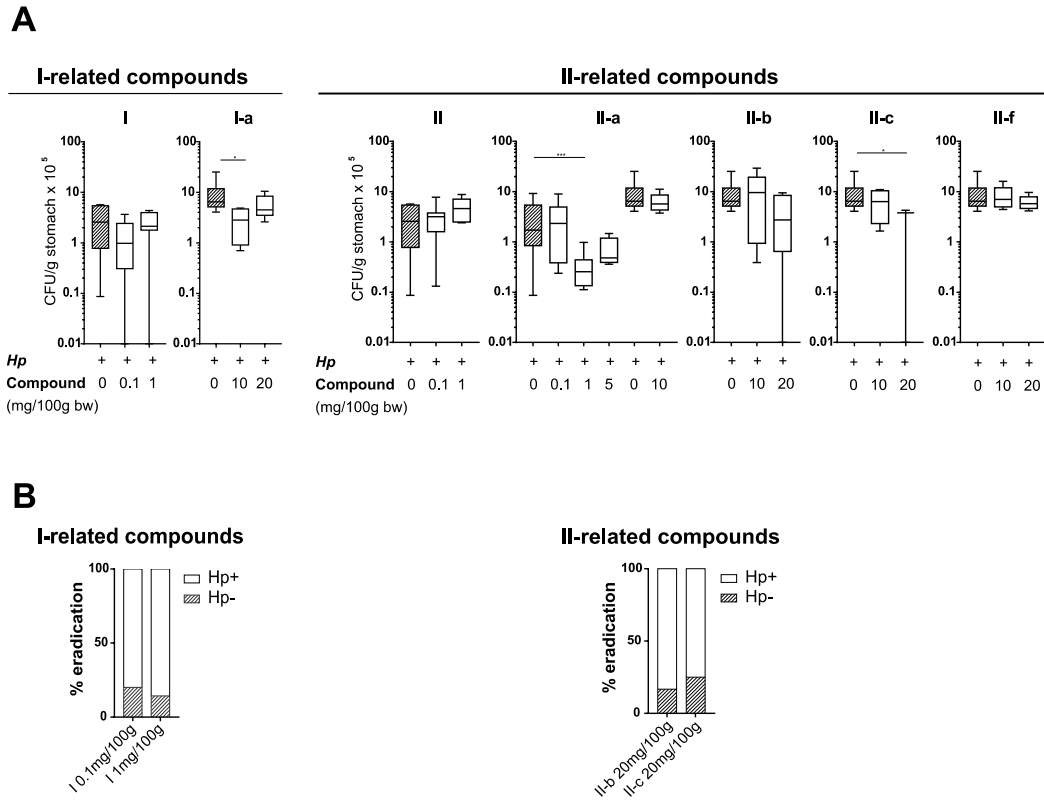
<sup>a</sup>HeLa cells have been used to determine the Minimal Cytotoxic Concentration (MCC<sub>50</sub>), as reported in Table 1. In bold, TI values indicative of effectivity, according to EUCAST criteria.

**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.<sup>54,55</sup> First, the toxicity of three representative inhibitors (**II-a**, **IV** and **IV-a**) at doses of 10, 20

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3 and 40 mg/100 g bw has been evaluated. Daily administration of compound **IV-a** for 8 days  
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5 had no deleterious effects in stomach, liver, heart, lung and kidney at any dose, as  
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7 determined by histopathological examination (Figure S3) and analysis of their biochemical  
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9 parameters (data not shown). Compounds **IV** and **II-a** did not induce significant alterations  
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11 at any dose in liver, heart, lung or kidney either, but they did provoke histological  
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13 alterations in stomach when administered at the highest dose of 40 mg/100 g (compound  
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15 **IV**) or at 20 or 40 mg/100 g (compound **II-a**) (Figure S3). The histological alterations  
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17 found in stomach included hyperplasia, inflammation and necrosis of the epithelium and  
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19 the gastric mucosa. None of these compounds were associated with increased levels of  
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21 tested biochemical parameters when compared to animals which did not receive the  
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23 compounds. The highest dose of compound **II-a** was, however, clearly toxic since it  
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25 induced hair loss and weight loss (> 20%) in all mice treated. The lack of biochemical or  
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27 histological alterations associated to the administration of high doses of **IV-a** indicates that  
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29 reduction of the nitro group in compound **IV** to amine removes the toxicity exerted in the  
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31 stomach by compound **IV** when orally administered to mice at high doses. The possibility  
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33 that the histological alterations observed in stomach after administration of the highest dose  
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35 of compound **IV** were associated to chemical instability of the compound at gastric pH has  
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37 been tested (see “*In vitro* acid stability evaluation of compounds” section in Supplemental  
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39 file). Compound **IV** (and the three derivatives tested: **IV-a**, **IV-b** and **IV-d**) are highly stable  
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41 at pH 1.0. In the subsequent *in vivo* analysis on anti-*Hp* activity, the doses of compounds  
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43 **IV** and **II-a** have been kept below those that produce histological alterations in mice  
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45 stomachs.  
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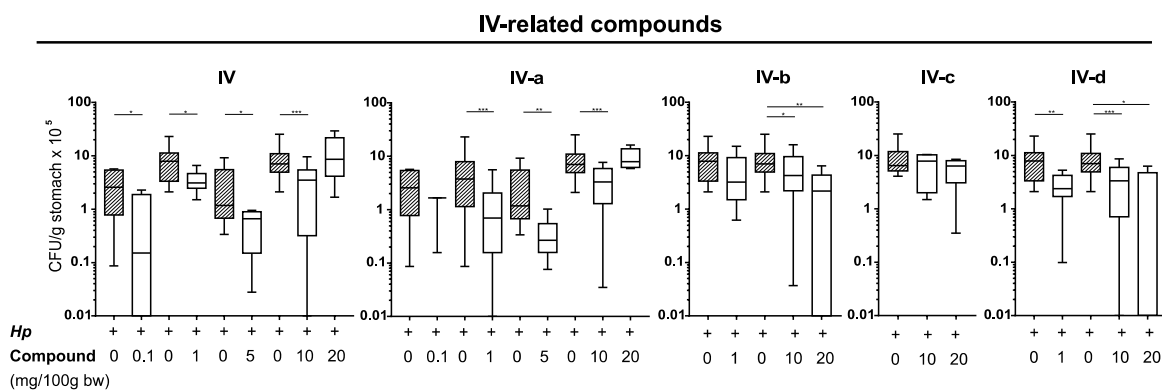
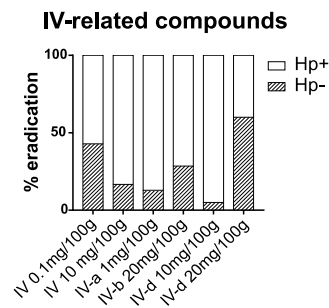
53 Four experiments have been conducted in mice infected with *Hp* SS1 to test the anti-*Hp*  
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55 activity of the leads (compounds **I**, **II** and **IV**) and of several of the best new derivatives:  
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3 **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**,  
4 related to **IV**. The doses ranged from 0.1 to 20 mg/100 g bw. As expected, in the four  
5 experiments, all control mice have provided a clean background for interpretation: all mice  
6 in the infected non-treated groups have shown efficient stomach *Hp* colonization ( $10^5$  to  
7  $10^6$  CFU/g gastric tissue) and no *Hp* colonies have been found in any group corresponding  
8 to non-infected mice. The efficacy of the different compounds on *Hp* gastric colonization  
9 has been determined by comparing the CFU/g gastric tissue of each infected, treated-group  
10 with the corresponding infected, non-treated control group. Olive oil (90-95%)/DMSO (10-  
11 5%), used as vehicle to solubilise compounds, has not influenced the *Hp* gastric  
12 colonization (not shown). All the data gathered in the four experiments for the testing of  
13 compounds of the **I-II** series are presented in Figure 3, and data for the **IV** series in Figure  
14 4. Data of gastric colonization for all compounds and doses tested are presented along with  
15 those of the corresponding control groups in Figures 3A and 4A, while the percentages of  
16 *Hp*-eradicated mice after treatment are shown in Figures 3B and 4B for the successful  
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**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

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

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49 As reported in Figure 3A, parent compound **I** was able to eradicate the *Hp* infection in  
50 some mice (1/5 and 1/7) at 0.1 mg and 1 mg/100 g bw daily doses, respectively, but the  
51 overall reduction of the gastric colonization rate observed was not statistically significant.  
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56 The oxime variant **I-a** decreased significantly the colonization rate at 10 mg/100 g bw  
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3 (p=0,016), but did not eradicate *Hp* infection from any mouse. The performance of the **II**-  
4 related derivatives was also limited. Parent compound **II** had no inhibitory effect on the  
5 gastric colonization; however, the ethylene-reduced **II-b** eradicated *Hp* infection from some  
6 mice (1/6) at a 20 mg/100 g bw dose; the ethylene-reduced and nitro reduced **II-c** amine  
7 derivative also eradicated *Hp* infection from some mice (1/4) and significantly reduced the  
8 colonization rate (p=0.024) at 20 mg/100 g dose. In addition, compound **II-a** reduced the  
9 colonization (p=0.0009) at a 1 mg/100 g bw dose.

19 The best performance comes from **IV**-related compounds, in agreement with *in vitro*  
20 testing. Both the parent compound **IV**, the amine-derivatives **IV-a** and **IV-d**, and the non-  
21 benzoxadiazole containing compound **IV-b** significantly lowered the colonization rate at  
22 most of the doses tested (Figure 4A) and eradicated the infection in some mice (Figure 4B).  
23 Compound **IV** lowered colonization at 0.1, 1, 5 and 10 mg/100 g bw doses (p=0.044;  
24 0.041; 0.048 and 0.0002, respectively) and eradicated infection in 43 % (3/7) and 17 %  
25 (3/18) of mice treated with 0.1 and 10 mg/100 g bw, respectively. Compound **IV-a** also  
26 inhibited the gastric colonization at 1, 5 and 10 mg/100 g bw doses (p=0.0001; 0.0018 and  
27 0.0004, respectively) and eradicated the infection in 13 % (4/31) of treated mice at 1  
28 mg/100 g bw. Compound **IV-b**, the derivative lacking the nitrobenzoxadiazole moiety,  
29 inhibited gastric colonization (p=0.0277 and 0.0042) at 10 and 20 mg/100 g bw,  
30 respectively, and eradicated the infection in 29 % (2/7) of mice at a 20 mg/100 g bw dose.  
31 Finally, compound **IV-d**, the sulfoxide version of compound **IV-a**, decreased colonization  
32 at 1, 10 and 20 mg/100 g bw doses (p=0.005, 0.0002 and 0.0122, respectively) and  
33 eradicate the infection in 60 % (3/5) of animals at the higher dose and in 5 % (1/20) of mice  
34 at 10 mg/100 g bw.  
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**Perspectives for novel antimicrobials based in flavodoxin inhibitors.** In previous work we discovered flavodoxin inhibitors **I**, **II** and **IV**<sup>41,42</sup> and explored the feasibility of improving their antibacterial activity by replacing some of their substituents.<sup>42</sup> 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

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3 of action of these inhibitors needs to be clarified. As they were discovered using a simple  
4 binding assay to the target protein,<sup>41,56</sup> a straightforward inhibitory mechanism was  
5 proposed<sup>41</sup> consisting on the inhibitors sterically blocking the flavodoxin interaction with  
6 physiological partner proteins.<sup>30,36,37</sup> The fact that, besides the original inhibitors,<sup>41</sup> all the  
7 new derivatives herein tested bind to *Hp* flavodoxin (as determined by isothermal titration  
8 calorimetry and thermal shift assay, not shown) lends support to this mechanism and  
9 provides direct experimental evidence for *in vitro* target engagement. Work is in progress to  
10 obtain structural information on flavodoxin/inhibitor complexes as this could facilitate their  
11 rational redesign and improvement. In addition, the possibility that flavodoxin activates the  
12 compounds needs to be considered as the inhibitors contain nitro groups whose reduction  
13 could generate radical cytotoxic products ultimately responsible for the observed  
14 antibacterial activity. In this respect, the antibacterial activity of Mnz towards *Hp* and other  
15 microaerophilic pathogens is thought to be mediated by one such redox activation  
16 mechanism.<sup>43,46,57-59</sup> This alternative activating mechanism cannot be invoked, though, in  
17 the case of compound **IV-b**, the analogue of **IV** lacking a nitro group. Interestingly, the fact  
18 that inhibitors such as **IV-a** retain antibacterial activity when carrying an amine instead of  
19 the reducible nitro group of lead compound **IV** indicates that, if radical species played a  
20 role in the bactericidal activity, they would have to be generated in a reverse manner from  
21 cellular oxidation of the amine. On the other hand, a second line for improvement is  
22 suggested by the poor aqueous solubility of the inhibitors, reflected in their logS values  
23 (Table S1 and Figure S1), and possibly related to the toxicity observed in some of the mice  
24 stomachs at the highest dose of the parent compound **IV**, which we interpret as a chemical  
25 direct toxicity since no other alterations are observed. The solubility of the inhibitors needs  
26 to be increased by a judicious incorporation of polar groups in order to increase their  
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3 bioavailability and ensure a systemic effect. Work is in progress to synthesise and test more  
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5 soluble variants of compound **IV**-derived inhibitors. Finally, unlike broad-spectrum  
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7 antimicrobials targeting very fundamental cellular components such as DNA, ribosomes or  
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9 the cell wall, flavodoxin inhibitors target a protein that is not essential for all bacteria and  
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11 whose sequence conservation is not particularly high. Our preliminary analysis of the  
12  
13 inhibitors' specificity revealed that compound **IV** was not effective against *H. pylori* distant  
14  
15 bacteria such as *E. coli* or *S. aureus*.<sup>41</sup> We have now confirmed the same is true for the  
16  
17 novel non-toxic inhibitors described here (not shown). These flavodoxin inhibitors thus  
18  
19 appear to be selective and may be less damaging to the commensal microbiota than broad-  
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21 spectrum antimicrobials.<sup>60</sup>  
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## 28 **CONCLUSION**

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30 Several lead compounds previously shown to inhibit the flavodoxin from *Hp* have been  
31  
32 redesigned to improve their antimicrobial activity. By synthesizing new variants exhibiting  
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34 different redox forms of the nitro, ethylene or sulphur groups, a family of  
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36 nitrobenzoxadiazol-based flavodoxin inhibitors with low toxicity for HeLa cells and for  
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38 mice has been developed that exhibits high therapeutic indexes against reference *Hp*  
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40 strains. Importantly, some of these new inhibitors are effective against drug-resistant  
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42 clinical *Hp* isolates (including Cla-, Mnz- and rifampicin-resistant strains) and are able to  
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44 significantly reduce *Hp* gastric colonization in the mouse model of infection and to  
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46 eradicate the infection in some mice. After improvement of their pharmacokinetic  
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48 properties, these compounds could constitute a new, *Hp*-specific family of antimicrobials  
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50 that could be less damaging for the gut microbiota than currently used broad-spectrum  
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3 antibiotics and might be used on their own or in new combinations with existing antibiotics  
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5 to fight *Hp* resistant strains.  
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## 9 10 **EXPERIMENTAL SECTION**

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12 **General synthetic and analytic procedures.** All reagents were of analytical grade and  
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14 were used as obtained from commercial sources. Compounds **II-e**, **II-f** and **II-g** are  
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16 commercially available and were acquired from FCH group, Acros Organics and  
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18 UkrOrgSyntez Ltd. (UORSY), respectively. Reactions were carried out using anhydrous  
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20 solvents. <sup>1</sup>H NMR, <sup>13</sup>C NMR and <sup>19</sup>F NMR spectra were acquired at room temperature at  
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22 400, 100 and 376 MHz, respectively, using a 5 mm probe. Chemical shifts ( $\delta$ ) are reported  
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24 in parts per million from tetramethylsilane with the solvent resonance as the internal  
25  
26 standard. Coupling constants (J) are quoted in hertz. The splitting patterns are reported as s  
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28 (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), bs  
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30 (broad singlet). Attached proton test spectra were acquired to determine the types of carbon  
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32 signals, and Heteronuclear Multiple Bond Correlation spectra were acquired to confirm the  
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34 position of heteroatoms. Special precautions such as degassing of the sample were not  
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36 taken. All compounds used for biological assays are  $\geq$  95% purity based on NMR and  
37  
38 HPLC chromatography. These last experiments have been performed using a Waters HPLC  
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40 system equipped with a 600-E pump, a 2996 Photodiode Array detector, and a 2707  
41  
42 autosampler. The LC system was fitted with a C18 reversed phase column (VYDAC 238TP  
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44 C18 5  $\mu$ m, 4.6 mm  $\times$  250 mm) and operated, unless otherwise stated, using a linear gradient  
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46 of buffer B (100% in 40 min) from 100% buffer A (buffer A consisting of 0.1% TFA in  
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48 H<sub>2</sub>O, buffer B consisting of 0.085% TFA in CH<sub>3</sub>CN/H<sub>2</sub>O, 95:5 v/v) at a flow rate of 1  
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50 mL/min. High-resolution mass measurements were made using a microTOF (time-of-flight)  
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3 analyzer, and spectra were recorded from methanolic solutions using the positive  
4 electrospray ionization mode (ESI+). Reactions were magnetically stirred and whenever  
5 possible monitored by TLC. TLC was performed on precoated silica gel polyester plates  
6 and products were visualized using ultraviolet light (254 nm), ninhydrin, potassium  
7 permanganate and phosphomolybdic acid solution followed by heating. Column  
8 chromatography was performed using silica gel (Kiesegel 60, 230–400 mesh).

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17 **6,8-dichloro-2-(4-fluorophenyl)chroman-3-one oxime (I-a)**. Compound **I** (615 mg, 1.81  
18 mmol) was dissolved in a mixture of dichloromethane (65 mL), conc. hydrochloric acid (4  
19 mL) and methanol (29 mL). After the addition of iron powder (688 mg, 12.32 mmol), the  
20 mixture was sonicated (JP Selecta ultrasonic bath, 40 kHz) at room temperature for 10-40  
21 minutes. TLC monitoring was done till completion of the reaction. The reaction mixture  
22 was filtered to remove excess iron, and the solvent was evaporated under reduced pressure  
23 to dryness. The residue was dissolved in water (35 mL) and extracted with ethyl acetate  
24 (3×50 mL). The organic layers were dried over anhydrous magnesium sulphate, filtered and  
25 concentrated *in vacuo*. The crude product was then purified by flash chromatography  
26 (eluent: Hex/EtOAc: 8/2) to give 313 mg (53 % yield) of the desired product. <sup>1</sup>H NMR  
27 (400 MHz, DMSO-*d*<sub>6</sub>): δ 11.54 (s, 1H), 7.45 (d, J = 2.5 Hz, 1H), 7.37-7.31 (m, 2H), 7.30  
28 (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 =  
29 22.2 Hz, 1H) ppm. <sup>19</sup>F NMR (376 MHz, DMSO-*d*<sub>6</sub>): δ -113.87 ppm. <sup>13</sup>C NMR (100 MHz,  
30 DMSO-*d*<sub>6</sub>): δ 161.9 (d, J = 244.6 Hz), 150.9, 148.0, 133.2, 128.6 (d, J = 8.5 Hz), 128.2,  
31 127.3, 125.5, 124.6, 122.4, 115.4 (d, J = 21.7 Hz), 77.2, 24.4 ppm. HRMS (ESI+) *m/z*  
32 [M+H]<sup>+</sup>calc for C<sub>15</sub>H<sub>11</sub>Cl<sub>2</sub>FNO<sub>2</sub> 326.0146, found 326.0125.

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54 **1-(2-nitroethyl)-3,5-bis(trifluoromethyl)benzene (II-b)**. To a vigorously stirred mixture  
55 of compound **II** (522 mg, 1.83 mmol), silica gel (3.7 g, column chromatography grade,  
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3 Sigma-Aldrich), 2-propanol (5.5 mL) and chloroform (30 mL), NaBH<sub>4</sub> (284 mg, 7.5 mmol)  
4  
5 was added in about 40 mg portions over a period of 15 min at 25 °C. The mixture was  
6  
7 stirred for additional 2 hours or until completion of the reaction. Excess NaBH<sub>4</sub> was  
8  
9 decomposed with diluted hydrochloric acid and the mixture was filtered. The filter was  
10  
11 extracted with dichloromethane (3×50 mL) and the combined filtrates were washed with  
12  
13 brine (50 mL), dried over anhydrous magnesium sulphate, filtered and evaporated *in vacuo*  
14  
15 to dryness to give 504 mg (96 % yield) of the desired product which was used in the next  
16  
17 step without further purification. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.81 (s, 1H), 7.70 (s, 2H),  
18  
19 4.69 (t, J = 6.9 Hz, 2H), 3.46 (t, J = 6.9 Hz, 2H) ppm. <sup>19</sup>F NMR (376 MHz, CDCl<sub>3</sub>): δ  
20  
21 -63.00 ppm. <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 138.4, 132.5 (q, J = 33.5 Hz), 129.0 (q, J =  
22  
23 -3.6 Hz), 123.2 (q, J = 272.7 Hz), 121.8 (m, J = 3.8 Hz), 75.2, 32.8 ppm. HRMS (ESI<sup>+</sup>) *m/z*  
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25 [M+H]<sup>+</sup>calc for C<sub>10</sub>H<sub>8</sub>F<sub>6</sub>NO<sub>2</sub> 288.0454, found 288.0460.

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28 **2-(3,5-bis(trifluoromethyl)phenyl)ethan-1-amine (II-c)**. A solution of **II-b** (57.4 mg, 0.2  
29  
30 mmol) in ethanol (10 mL) was hydrogenated under hydrogen atmosphere (1 atm) at room  
31  
32 temperature in the presence of 10 % palladium/carbon (10 mg). The reaction was stirred for  
33  
34 5 h or until complete as judged by TLC. Filtration of the catalyst and evaporation of the  
35  
36 solvent afforded 51.4 mg (≈ 100 %) of pure desired product. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  
37  
38 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)  
39  
40 ppm. <sup>19</sup>F NMR (376 MHz, CDCl<sub>3</sub>): δ -62.83 ppm. <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 142.4,  
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42 131.8 (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  
44 43.0, 39.5 ppm. HRMS (ESI<sup>+</sup>) *m/z* [M+H]<sup>+</sup>calc for C<sub>10</sub>H<sub>9</sub>F<sub>6</sub>N 258.0712, found 258.0711.

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46  
47 **2-(3,5-bis(trifluoromethyl)phenyl)acetaldehyde oxime (II-d)**. A round-bottom flask was  
48  
49 charged with Pd(OAc)<sub>2</sub> (10 mg, 0.04mmol), the nitroarene **II** (214 mg, 0.75 mmol) and  
50  
51 freshly distilled THF (4.5 mL). The flask was sealed and purged with Ar. Degassed H<sub>2</sub>O  
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(1.75 mL) and  $i\text{Pr}_3\text{SiH}$  (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 1:1 mixture of Z/E isomers.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  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.  $^{19}\text{F}$  NMR (376 MHz,  $\text{CDCl}_3$ ):  $\delta$  -62.87 and -62.88 ppm.  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  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<sup>+</sup>)  $m/z$  [M]<sup>+</sup> calc for  $\text{C}_{10}\text{H}_7\text{F}_6\text{NO}$  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  $\mu\text{L}$ ) 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

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3 under reduced pressure yielded 16 mg (61 % yield) of pure **IV-c**. <sup>1</sup>H-NMR (400 MHz,  
4 DMSO-*d*<sub>6</sub>): 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),  
5 3.70 (s, 3H) ppm. <sup>13</sup>C-NMR (100 MHz, DMSO-*d*<sub>6</sub>): δ 158.3, 149.6, 144.6, 137.8, 132.2,  
6 130.0, 129.1, 113.8, 108.1, 106.4, 55.0, 37.1 ppm. HRMS (ESI<sup>+</sup>) *m/z* [M+Na]<sup>+</sup>calc for  
7 C<sub>14</sub>H<sub>12</sub>ClN<sub>3</sub>NaO<sub>2</sub>S 344.0231, found 344.0232.  
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12 **7-((4-methoxybenzyl)sulfinyl)benzo[c][1,2,5]oxadiazol-4-amine (IV-d)**. Compound **IV-a**  
13 (495 mg, 1.72 mmol) was dissolved in a mixture of glacial acetic acid (20.70 mL) and 33 %  
14 aqueous hydrogen peroxide (1.38 mL), and kept at 25 °C for 2.5 h. The yellowish-brown  
15 mixture obtained was quenched with saturated NaHCO<sub>3</sub> solution (50 mL) and extracted  
16 with dichloromethane (5×30 mL). The organic layers were dried over anhydrous  
17 magnesium sulphate, filtered and, once the solvent was evaporated under reduced pressure,  
18 the crude was purified by column chromatography over silica gel (eluent: Hex/AcOEt: 2/8)  
19 to give 378 mg (73 % yield) of the desired product. <sup>1</sup>H-NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 7.38  
20 (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),  
21 4.41 (d, J=12.8 Hz, 1H), 4.21 (d, J=12.8 Hz, 1H), 3.70 (s, 3H) ppm. <sup>13</sup>C-NMR (100 MHz,  
22 DMSO-*d*<sub>6</sub>): δ 158.9, 145.7, 144.5, 140.3, 135.7, 131.3, 121.9, 113.6, 111.5, 103.1, 58.2,  
23 55.0 ppm. HRMS (ESI<sup>+</sup>) *m/z* [M+Na]<sup>+</sup>calc for C<sub>14</sub>H<sub>13</sub>N<sub>3</sub>NaO<sub>3</sub>S 326.0570, found 326.0575.  
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42 **7-((4-methoxybenzyl)sulfonyl)benzo[c][1,2,5]oxadiazol-4-amine (IV-e)**. To a stirred  
43 solution of compound **IV-a** (29 mg, 0.1 mmol) in ethyl acetate (1.43 mL) at room  
44 temperature, 3-chloroperbenzoic acid (34.5 mg, 0.2 mmol) was added. TLC monitoring  
45 was done till the completion of the reaction (1.5 h). Traces of 3-chloroperbenzoic acid and  
46 3-chlorobenzoic acid were neutralized with saturated aqueous NaHCO<sub>3</sub> solution (2×2 mL).  
47 After extracting with ethyl acetate (3 x 3 mL), the combined organic layers were dried over  
48 anhydrous magnesium sulphate, filtered and the solvent was evaporated under reduced  
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3 pressure. TLC monitoring revealed that a mixture of **IV-d** and **IV-e** had been obtained. The  
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5 crude was purified by flash chromatography (eluent: Hex/EtOAc: 3/7) to provide 27 mg  
6  
7 (85 % yield) of pure **IV-e**. <sup>1</sup>H-NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 7.95 (s, 2H), 7.57 (d, J=8 Hz,  
8  
9 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)  
10  
11 ppm. <sup>13</sup>C-NMR (100 MHz, DMSO-*d*<sub>6</sub>): δ 159.2, 146.0, 144.0, 143.5, 140.9, 131.9, 120.6,  
12  
13 113.7, 106.6, 102.0, 59.7, 55.0 ppm. HRMS (ESI<sup>+</sup>) *m/z* [M+Na]<sup>+</sup>calc for C<sub>14</sub>H<sub>13</sub>N<sub>3</sub>NaO<sub>4</sub>S  
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15 342.0519, found 342.0523.  
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19 **4-((4-methoxybenzyl)sulfinyl)-7-nitrobenzo[c][1,2,5]oxadiazole (IV-f)**. Compound **IV**  
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21 (96 mg, 0.30 mmol) was dissolved in a mixture of glacial acetic acid (5.66 mL) and 33 %  
22  
23 hydrogen peroxide (242 μL) and the reaction was stirred at 25 °C for 24 h. The obtained  
24  
25 orange-brown oily mixture was extracted with dichloromethane (3×10 mL). The combined  
26  
27 organic layers were dried over anhydrous magnesium sulphate, filtered and the solvent was  
28  
29 evaporated under reduced pressure. Flash chromatography of the mixture was done in order  
30  
31 to purify the desired product. In this way 84.3 mg (88 % yield) of pure **IV-f**, which was  
32  
33 stored in a sealed flask under an argon atmosphere at 4 °C until testing to avoid  
34  
35 spontaneous decomposition because of its sensitivity to oxygen, were obtained. <sup>1</sup>H-NMR  
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37 (400 MHz, CDCl<sub>3</sub>): δ 8.47 (d, J=7.2 Hz, 1H), 7.75 (d, J=7.2 Hz, 1H), 6.89-6.87 (m, 2H),  
38  
39 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. <sup>13</sup>C-  
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41 NMR (100 MHz, CDCl<sub>3</sub>): δ 160.2, 146.1, 142.6, 142.3, 131.3, 129.1, 128.7, 119.6, 114.1,  
42  
43 59.5, 55.3 ppm. HRMS (ESI<sup>+</sup>) *m/z* [M+Na]<sup>+</sup> calc for C<sub>14</sub>H<sub>11</sub>N<sub>3</sub>NaO<sub>5</sub>S 356.0312, found  
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45 356.0308.  
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51 **2-(5-amino-2-methyl-1H-imidazol-1-yl)ethan-1-ol (Mnz-a)**. Commercially available  
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53 metronidazole (34.2 mg, 0.2 mmol) and 25 % Pd/C (9 mg) were placed in a 50 mL two-  
54  
55 necked round-bottomed flask under argon atmosphere. Anhydrous, degassed THF (10 mL)  
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3 was added. The reaction was stirred for 24 h at room temperature and, after checking for  
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5 complexation, the reaction mixture was filtered through Celite® and washed a few times with  
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7 anhydrous THF (3×3 mL). The solvent was removed under reduced pressure to afford the  
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9 26,5 mg (94 %) of crude product as an oil. The thus formed amino-imidazole is sensitive to  
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11 O<sub>2</sub> and heat, and therefore it was stored without further purification in a sealed flask under  
12  
13 an argon atmosphere at -20 °C until its MCC<sub>50</sub> and MIC determinations. Just before MIC  
14  
15 determinations, the crude was purified by flash chromatography (First eluent:  
16  
17 dichloromethane /methanol: 1/1, second eluent: methanol, third eluent: methanol containing  
18  
19 0.1% triethylamine) and used immediately. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 5.91 (s, 1H),  
20  
21 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.  
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26 **Minimal Inhibitory Concentrations (MIC) on reference *Hp* strains (NCTC 11637,**  
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28 **26695 and SS1).** For microdilution MIC testing, *Hp* reference strains NCTC 11637 (ATCC  
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30 43504, Mnz-resistant strain), 26695 (ATCC 700392)<sup>61</sup> and SS1 (Sydney Strain 1)<sup>54</sup>  
31  
32 (Biosafety Level 2 pathogen) were grown in Brain-Heart Infusion (BHI) broth  
33  
34 supplemented with 4 % fetal bovine serum at 37 °C under microaerophilic conditions and  
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36 then diluted to an optical density at 600 nm of 0.01. Samples of 100 μL of the diluted  
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38 bacterial suspension were dispensed in a 96-well plate, except for the first well of each row  
39  
40 in which 200 μL of the bacterial suspension along with 2 μL of the compound (from a  
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42 stock solution in dimethyl sulfoxide (DMSO) at 6.4 μg/μL) were added. Two-fold serial  
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44 dilutions were made as described,<sup>41,42</sup> which allowed to test the compounds in a  
45  
46 concentration range from 64 to 0.031 μg/mL. Positive and negative controls consisted of  
47  
48 brain-heart infusion broth supplemented with 4 % fetal bovine serum and inoculated or not  
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50 with the diluted *Hp* bacterial suspension, respectively. We ensured that DMSO  
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52 concentration was kept at 1 % v/v or below, so that no toxic effect was found for *Hp* cells.  
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3 Plates were incubated at 37 °C for 48 hours under a microaerophilic atmosphere. MICs  
4 correspond to the lowest concentrations of compounds leading to a complete inhibition of  
5 *Hp* growth. The MIC values were determined by recording the colour change observed  
6 after addition of 30 µL of resazurin (0.1 mg/ml; Sigma-Aldrich) to each well in the 96-well  
7 dish. The MICs were additionally confirmed by spotting 10 µL of two consecutive wells  
8 (one with the highest concentration of compound that allowed bacterial growth, and the  
9 adjacent well with the lowest concentration of compound that prevented bacterial growth)  
10 onto Columbia Agar with Sheep Blood plates (Oxoid) followed by incubation at 37 °C for  
11 48 h under microaerophilic conditions. Growth or absence of *Hp* growth allowed to  
12 determine the Minimal Bactericidal Concentration of the compounds. Each experiment was  
13 performed twice in triplicate. Both assays confirmed the MIC values obtained by the  
14 microdilution method.

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

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3 **Minimal Cytotoxic Concentrations (MCC<sub>50</sub>).** HeLa cells were cultured in Dulbecco's  
4 Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin  
5 and 100 µg/mL streptomycin at 37 °C in a 5% CO<sub>2</sub> atmosphere. The toxicity of the  
6 compounds towards HeLa cells was determined by the XTT (2,3-Bis-(2-methoxy-4-nitro-5-  
7 sulphenyl)-2*H*-tetrazolium-5-carboxanilide sodium salt) method using the *Cell*  
8 *Proliferation Kit II* (Roche) as described.<sup>41,42</sup> Briefly, 0.1-µL volumes of compound  
9 dissolved in DMSO were added to give final compound concentrations of 25, 50, 75, 100,  
10 250, 500, 750, and 1000 µM, in 96-well plates (with 30,000 cells in 100 µL in each well).  
11 The untreated control consisted in 30,000 cells per well in 100 µL of complete medium  
12 (with 0.1 % DMSO). Cell viability was calculated from the wells' absorbance at 450 and  
13 650 nm, as described.<sup>41,42</sup> Cytotoxicity curves (i.e percentage of cell viability versus  
14 compound concentration) were used to calculate the compounds concentration producing  
15 50 % cell lysis (MCC<sub>50</sub>), as described.<sup>41,42</sup> All experiments were performed twice in  
16 triplicate. Therapeutic Indexes (TI) were calculated as MCC<sub>50</sub>/MIC.

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19 **Toxicity assays in mice.** The *in vivo* toxicity of compounds was tested in 48 twenty-week  
20 old specific-pathogen-free female C57BL/6J mice obtained from Charles River  
21 Laboratories España (Barcelona, Spain), fed with a standard commercial rodent diet and  
22 water *ad libitum*. Females were selected because of their less aggressiveness. All  
23 procedures followed for toxicity analyses in mice were approved by the Ethics Committee  
24 for Animal Experiments of the University of Zaragoza, and all the experiments were  
25 performed according to the approved ARRIVE (Animal Research: Reporting of *In Vivo*  
26 Experiments) guidelines for the care and manipulation of laboratory animals. Toxicity of  
27 compounds **IV**, **II-a**, and **IV-a** was tested at three different doses: 10, 20 and 40 mg/100 g  
28 bw. Assuming the administered compounds are uniformly distributed throughout the  
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3 animal body, these doses range from 12 to 1600 times the MICs of the compounds. Ten  
4 groups of 4-5 animals were used. To nine of them, and for each compound, the  
5 corresponding daily dose was administered in volumes of 100  $\mu$ l by oral gavage for 7 days.  
6  
7 To the control group, vehicle alone (olive oil, 5 % DMSO) was administered. After  
8 treatment, mice were sacrificed by CO<sub>2</sub> asphyxiation and blood was collected by cardiac  
9 puncture. An automatic analyser (VetScan, Abaxis) was used to determine biochemical  
10 alterations. Histopathology evaluation was done on heart, stomach, liver, lung and kidney  
11 samples by haematoxylin-eosin staining of tissue sections.  
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21 **Antibacterial assays in mice.** *In vivo* analysis of the anti-*Hp* activity of the compounds  
22 was carried out in 6-week old (SPF) NMRI (Naval Medical Research Institute) mice  
23 (Charles River laboratories, France). The level of *Hp* gastric colonization in the mice model  
24 is similar in male and female, so assays were carried out in females because they are less  
25 aggressive than males under the hosting conditions. Experiments were performed according  
26 to the European Directives (2010/63/UE), approved by the Committee of Central Animal  
27 Facility board of the Institut Pasteur and following the ARRIVE (Animal Research:  
28 Reporting of *In Vivo* Experiments) guidelines. The project was authorized by the Comité  
29 d'Éthique en Expérimentation Animale, Institut Pasteur (Ref 00317.02). Four different  
30 experiments were performed to test compounds **I, II, IV, I-a, II-a, II-b, II-c, II-f, IV-a,**  
31 **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  
32 all experiments, 7 mice were analysed for each studied condition. Mice were orogastrically  
33 infected at days 1 and 3 during the first week of the experiment with 100  $\mu$ L of a  
34 suspension of *Hp* strain SS1 ( $5 \cdot 10^8$  CFU/ml) known to colonize efficiently the mouse  
35 gastric mucosa.<sup>54</sup> According to a previously established model of antimicrobial treatment of  
36 *Hp* infection,<sup>55</sup> administration of the compounds started at day 8. One-hundred  $\mu$ L of each  
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3 compound solubilised in 5-10 % DMSO (Euromedex)/95-90 % olive oil (Sigma-Aldrich)  
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5 as vehicle at the defined doses were daily administered by oral gavage for 8 consecutive  
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7 days. Non-infected control groups received orally 100  $\mu$ L of peptone trypsin broth alone. In  
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9 addition, infected and non-treated control groups were administered 100  $\mu$ L of vehicle. No  
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11 toxicity signs associated to the vehicle or the compounds treatment were noticed: i.e. no  
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13 weight variations (Figure S1) and no appetite alterations. At 8 days post-treatment (day 24),  
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15 mice were sacrificed by CO<sub>2</sub> inhalation, stomachs were isolated and *Hp* gastric colonization  
16  
17 determined as described.<sup>63</sup> Plates were incubated for 5 days at 37 °C under a  
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19 microaerophilic atmosphere created by the Anoxomat AN2CTS system (MART  
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21 Microbiology B.V.). The antibacterial activity of the compounds was evaluated by counting  
22  
23 the number of viable bacterial colonies expressed as CFU per gram of gastric tissue.<sup>63</sup>  
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28 **Statistical analysis.** Statistical analyses of efficacy assays in mice were performed with  
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30 GraphPad Prism 7 Software (GraphPad Software, Inc. CA, USA). Mann-Whitney U and  
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32 Unpaired t tests were used to assess differences in mouse gastric colonization between *Hp*-  
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34 infected vehicle-treated and compound-treated groups. Differences were considered  
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36 statistically significant for  $p \leq 0.05$ .  
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## 42 ASSOCIATED CONTENT

### 43 Supporting information

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

45  
46  
47 Compound characterization data (by NMR, mass spectrometry, HPLC and theoretical  
48  
49 determination of logS values), additional information about *in vitro* and *in vivo* efficacy  
50  
51 experiments (a table where compound doses for mice experiment are detailed, the MIC  
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53 values for the developed compounds against clinical *Hp* isolates and the mice weight  
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3 variation during efficacy experiments), *in vitro* and *in vivo* toxicity data (viability of HeLa  
4 cells in the presence of the compounds, histological study of *in vivo* toxicity of some  
5 compounds, *in vitro* acid stability evaluation of compounds), and SMILES format  
6 representations of compounds (Molecular Formula Strings).  
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## 14 **AUTHOR INFORMATION**

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### 26 **Author Contributions**

27  
28 Study concept and design: JS. Acquisition of data: SS, MA, VM, AM, AL, LR, JB, CS.  
29  
30 Analysis and interpretation of data: SS, MA, VM, AM, AL, LR, JB, JJG, AV-C, JAC, CS,  
31  
32 JC, JAA, MDDV, AL, ET, JS. Drafting of the manuscript: SS, JAA, MDDV, AL, ET, JS.  
33  
34 Obtained funding: HR, AV\_C, JC, JAA, MDDV, AL, ET, JS. Study supervision: JAA,  
35  
36 MDDV, AL, ET, JS.  
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### 40 **Notes**

41  
42 The authors declare that a patent has been filled concerning derivatives of compound IV.  
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## 47 **ETHICS APPROVAL**

48  
49 Ethics Committee for Animal Experiments of the University of Zaragoza (Spain) and  
50  
51 Committee of Central Animal Facility board of the Institute Pasteur (Paris, France).  
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7  
8 Governments, respectively. The authors thank Dr Francis Mégraud (Bordeaux, France) for  
9  
10 advice on culturing human *Hp* isolates and infecting animals with human strains.  
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### 17 **ABBREVIATIONS USED**

18  
19 ATCC, American Type Culture Collection; bw, body weigh; CFU, colony forming units;  
20  
21 Cla, clarithromycin; DMSO, dimethyl sulfoxide; ESI, electrospray; EUCAST, European  
22  
23 Committee on Antimicrobial Susceptibility Testing; Fld, flavodoxin; g, grams; *Hp*,  
24  
25 *Helicobacter pylori*; HPLC, High-performance liquid chromatography; HRMS, high-  
26  
27 resolution mass spectrometry; MCC, minimal cytotoxic concentration; MIC, minimal  
28  
29 inhibitory concentration; Mnz, metronidazole; NCTC, National Collection of Type  
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31 Cultures; NMR, nuclear magnetic resonance; SS1, Sydney Strain 1; THF, tetrahydrofuran;  
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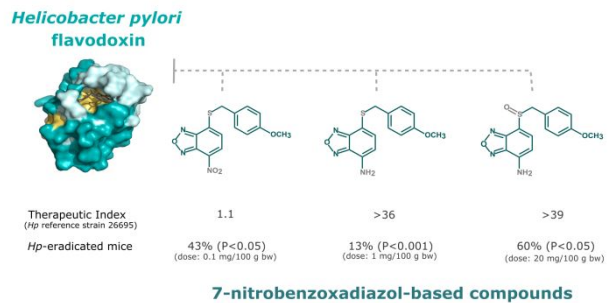


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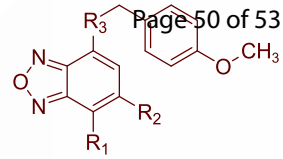
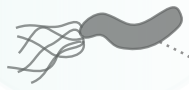
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Table of Contents Graphic



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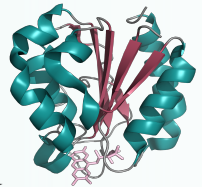
*Helicobacter pylori*



Pyruvate → Acetyl-CoA

**PFOR**

**Flavodoxin<sub>ox</sub>**

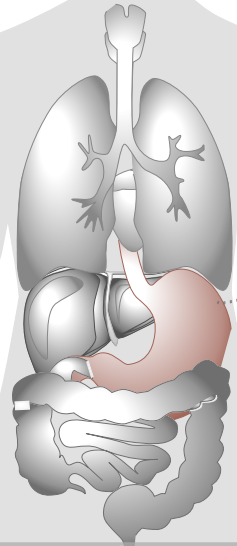


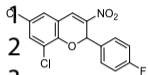
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**FqrB**

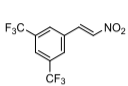
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NADP<sup>+</sup>

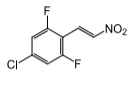




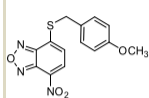
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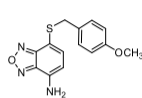
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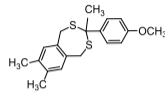
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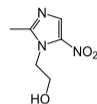
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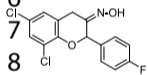
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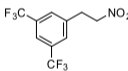
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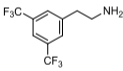
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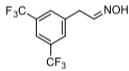
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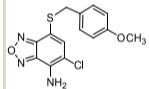
**II-b**



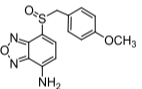
**II-c**



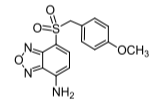
**II-d**



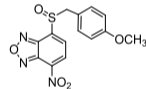
**IV-c**



**IV-d**



**IV-e**



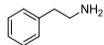
**IV-f**



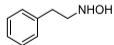
**Mnz-a**



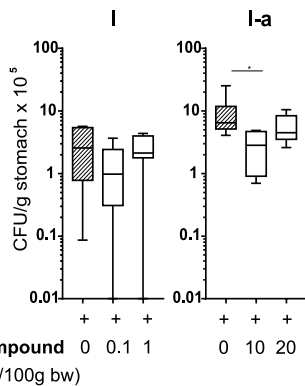
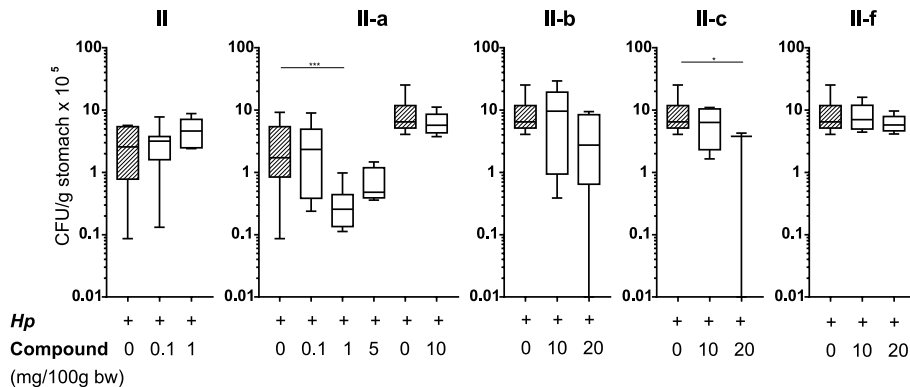
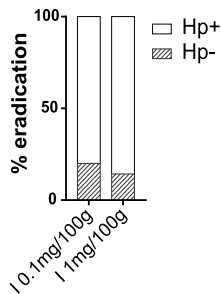
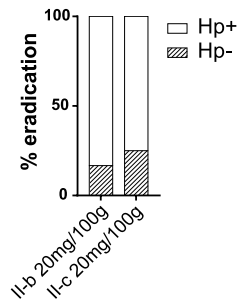
**II-e**



**II-f**



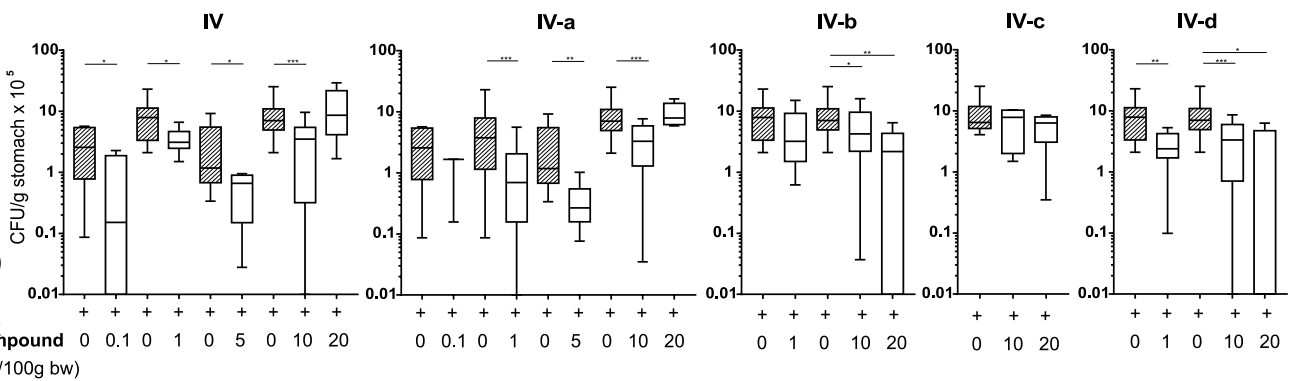
**II-g**

**A****I-related compounds****II-related compounds****B****I-related compounds****II-related compounds**

**A**

**IV-related compounds**

1  
2  
3  
4  
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**B**

**IV-related compounds**

