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Anti-*Helicobacter pylori* Agents Endowed with H_2-Antagonist Properties

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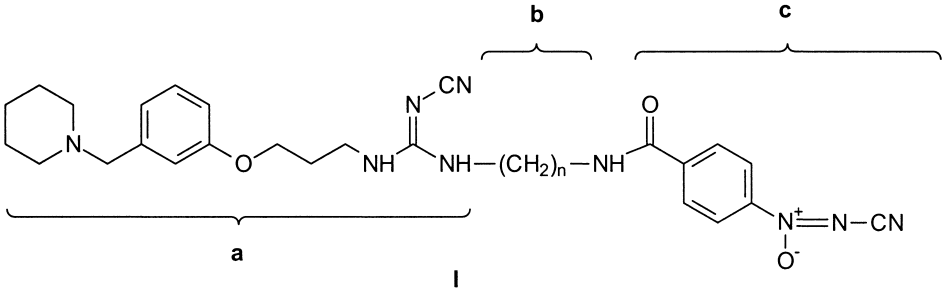
Abstract—New anti-*Helicobacter pylori* (*H. pylori*) agents endowed with H_2-antagonists properties were obtained by combining the lamtidine derived pharmacophoric group with the antibiotic calvatic acid. All the compounds were tested for their irreversible H_2-antagonist properties and for their ability to inhibit 20 *H. pylori* strains, two of them metronidazole resistant. The most active derivative (compound **4**) displayed antimicrobial activity similar to metronidazole.

Today it is widely accepted that *Helicobacter pylori* (*H. pylori*), a spiral microaerophilic S-shaped Gram negative bacterium which colonises the gastric mucosa, is a major causative factor of a number of gastric pathologies such as gastritis, peptic ulcers and certain gastric cancers.[1] Multiple therapeutic regimens are generally used for the treatment of infections by this microrganism;[2] all of which imply the frequent combination of antimicrobial agents with antisecretory drugs, such as H_2-receptor antagonists or proton pump inhibitors. A number of problems are associated with multiple therapy, among these drug resistance, side effects and non-compliance. Therefore great interest is devoted to novel agents suitable for a single therapy treatment. A possible strategy for obtaining these products is to combine an antisecretory pharmacophore with an anti-*H. pylori* agent in a single molecule. Recently we found that calvatic acid **1**,



2, n = 2; **3**, n = 4; **4**, n = 6

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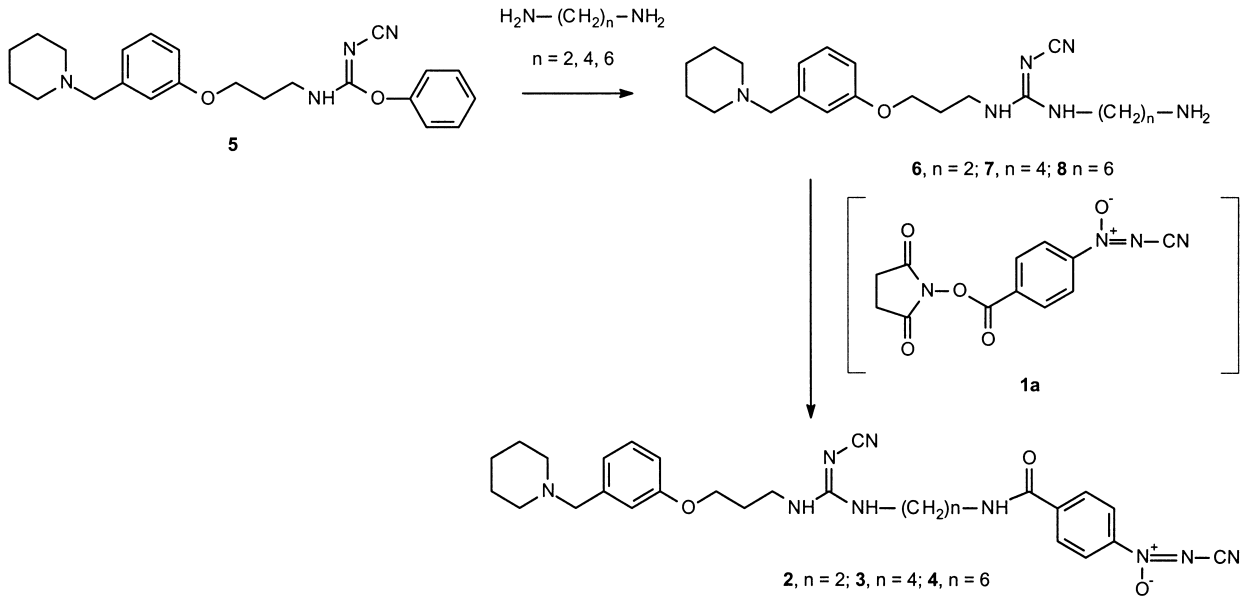
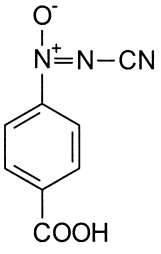


Scheme 1.



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an antibiotic isolated from culture broth of the gastromycete *Calvatia lilacina* (Berk.) Henn. P., and some of its analogues display potent action against many strains of *H. pylori*, including two that are metronidazole resistant.[3] On these bases we designed structures **2**, **3**, **4** of general formula **I**.

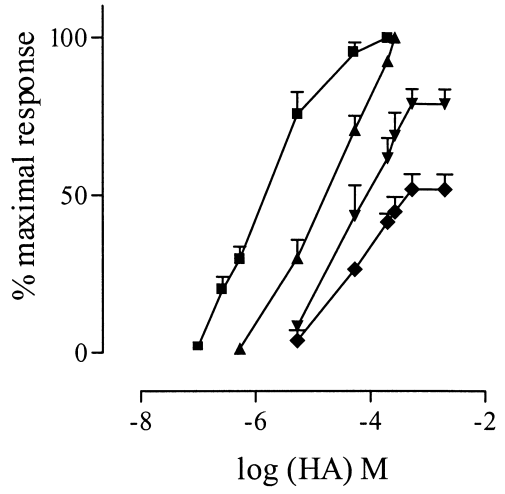

Figure 1. Concentration–response curves for histamine (HA) after incubation with **2** (30 μM) at different times: ■ (control); ▲ (t = 20 min); ▼ (t = 80 min); ◆ (t = 180 min).

Block **a** is similar to the moiety present in lamtidine, a well known H_2-antagonist, and identical to the moiety present in a new class of ligands with high affinity and selectivity for the histamine H_2-receptor.[4] Block **b** is a suitable polymethylene spacer and block **c** is represented by the acyl residue of calvatic acid. Here we report the synthesis, irreversible H_2-antagonist properties and the anti-*H. pylori* activity of these derivatives.

The synthetic pathway used to obtain the final compounds is reported in Scheme 1. Amines **6**–**8** were synthesized by action of appropriate alkanediamine on **5**,[4] according to a method reported in the literature and modified in some aspects. These intermediates were allowed to react with the activated ester **1a**, obtained from calvatic acid, *N*-hydroxysuccinimide and *N*,*N*′-dicyclohexyl-carbodiimide (DCC), to afford the expected final compounds **2**–**4**. The pharmacological profile of the products at histamine H_2-receptor was assessed on isolated guinea pig right atria. Irreversible inactivation of H_2-binding sites was studied by incubating the compounds for various times at fixed concentrations. The tissues were then washed free of drug, and histamine cumulative concentration–response curve was recorded. At 30 μM concentration, after 20 min of incubation with guinea pig right atrium, **2** was able to shift the curve of histamine irreversibly to the right without reducing the maximal response (Fig. 1). A progressive reduction of this response was observed after 80 min (20%) and 3 h (50%) of incubation, respectively. Working at a lower concentration (10 μM) an irreversible rightwards shift of histamine curve without reduction of the maximal response occurred (data not shown). The length increase of the spacer in **2** was accompanied by a drop of the irreversible action. In fact, after 3 h of incubation a less marked shift to the right of the histamine curve and only 10% and 30% reduction of the maximum was observed for derivatives **3** and **4**, respectively (Fig. 2). The irreversible block of

receptor by these compounds could be explained by the presence of cyano-NNO-azoxy function which is able to react in physiological conditions with -SH groups.[5] In fact, studies with a series of tetramine disulphides related to benextramine indicate that a thiol group able to bind irreversibly the disulphide moiety of the drugs is present on the surface of the histamine H_2-receptor.[6] This process may be expected to occur in two consecutive steps, as clearly shown for other irreversible antagonists at other receptors.[7] The first is the reversible interaction of the lamtidine part of the drugs, able to recognise the receptor, with the target active sites; the second the covalent bond formation between cyano-NNO-azoxy function and an adjacent thiol group. Thus, the drop of the irreversible activity observed on increasing the length of the spacer could be due to the fact that the shorter the chain the better the placement of the reactive N(O)NCN function at the -SH group. Obviously, during the walk to the receptor the compounds could also react with other -SH groups. However, this interaction should only influence their accessibility to the target but not the selectivity for it, this latter being due to the lamtidine moiety. In fact calvatic acid, when tested alone with the same protocol (30 μM, 3 h), did not display any effect on H_2-receptor.

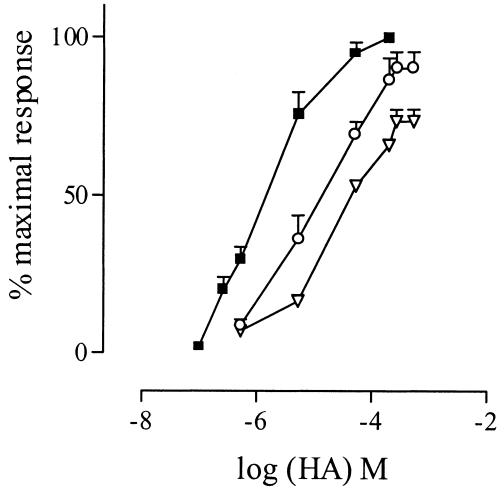

Figure 2. Concentration–response curves for histamine (HA) after 180 min of incubation with: ■ (control); ○ (**3**, 30 μM); ▽ (**4**, 30 μM).

Table 1. Minimal inhibitory concentration (MIC) of compounds **1**, **2**, **3**, **4** and metronidazole against 20 *H. pylori* strains, including two metronidazole resistant strains

Compound	MIC (μg/mL)[a]				
	All strains			Metronidazole resistant	
	Range[b]	MIC_{50}	MIC_{90}	NCTC11637	102R
1	<0.0039–0.062	0.016	0.031	0.0078	0.031
2	0.25–16	8	16	16	8
3	0.25–8	2	4	4	8
4	0.25–2	1	2	1	2
Metronidazole	0.031–16	0.25	8	16	8

[a]MICs are expressed as μg/mL of calvatic acid (see Experimental).
[b]Range: range of minimal inhibitory concentrations.

Activity against *H. pylori* was assessed on 20 clinical strains, two of these (NCTC 11637 and 102R) were metronidazole resistant. Metronidazole was taken as reference. The results expressed as minimal inhibitory concentrations MIC_{50} and MIC_{90}, namely the minimal concentration needed to inhibit 50% and 90% of the used strains, respectively, are reported in Table 1. The antibacterial activity of our compounds is much lower than calvatic acid activity, but comparable to the one shown by metronidazole. The activity of the synthesized hybrids ranks the order **4**>**3**>**2**. The most active compound (**4**) shows a MIC_{50} value four times higher than metronidazole, by contrast its MIC_{90} value is four times lower than the reference. Furthermore compound **4** displays good activity against the two metronidazole resistant strains. In conclusion the compounds described in the present work represent a new class of products endowed with both H_2-antagonist and anti-*H. pylori* activity. In vivo studies to evaluate the ability of **2**–**4** to exert both antisecretory and anti-*H. pylori* activity are being conducted.

Experimental[8]

Synthesis

***N*-(Aminoalkyl)-*N′*-cyano-*N″*-(3-(3-(1-piperidylmethyl)-phenoxy)propyl)guanidines 6–8.** A solution of **5** (1.96 g, 5.00 mmols) in a mixture of ethyl acetate (20 mL) and methanol (5 mL) was added dropwise to a stirred solution of the appropriate diaminoalkane (50 mmol). The reaction mixture was stirred at room temperature for 3 h. The solvent was removed under reduced pressure, and the residue flash chromatographed on silica gel, eluting first with a mixture of dichloromethane/methanol 9:1 and then with a mixture of dichloromethane/methanol:32% ammonia 8:2:0.3. The products were obtained as oils and were pure enough for further reactions (**6**,**7** yield 75%; **8** yield 60%). Analytical samples were obtained as oxalates and recrystallized from methanol/isopropanol. **6**·$2H_2C_2O_4$·H_2O, mp 158–159 °C dec.; **7**·$2H_2C_2O_4$·$0.5H_2O$, mp 184–185 °C dec.; **8**·$2H_2C_2O_4$·$0.5H_2O$, mp 174–175 °C dec.

***N*-Cyano-*N′*-(4-(cyano-NNO-azoxy)benzamido)alkyl-*N″*-(3-(3-(1-piperidylmethyl)phenoxy)propyl)guanidines 2–4.** *N*-Hydroxysuccinimide (1.05 g, 9.10 mmol) and DCC (1.87 g, 9.10 mmol) were added to a stirred solution of **1** (1.34 g, 7.00 mmol) in dry THF (75 mL) at room temperature. After 1 h of stirring the residue of dicyclohexylurea was filtered off and the appropriate amine **6**–**8** (7.00 mmol) dissolved in dry THF (20 mL) was added to the solution of the active ester **1a**. The reaction mixture was stirred for 2 h at room temperature and filtered. The solvent was then removed under reduced pressure and the residue dissolved in 100 mL of dichloromethane containing 1 mL of methanol. The organic solution was washed with a saturated solution of sodium bicarbonate, with brine, with water and then dried on anhydrous magnesium sulphate. The residue obtained after solvent removal was purified by flash chromatography first eluting with a mixture of ethyl acetate/methanol

95:5, and then with a mixture of ethyl acetate/methanol 80:20. The obtained products (yields: **2**, 40%; **3**, 60%; **4**, 50%) were transformed into the corresponding oxalates, recrystallized from methanol/ether and dried for 4 days in vacuo at 60 °C. Decomposition maxima were recorded by DSC analysis (Perkin–Elmer DSC 7). **2**·$H_2C_2O_4$·0.5H_2O, decomposition maximum 161 °C, ^{13}C NMR (DMSO-d_6): 21.8, 22.7, 28.6, 38.5, 39.3, 40.6, 52.0, 59.4, 65.4, 111.0, 115.4, 116.8, 118.2, 123.2, 123.3, 128.9, 129.9, 132.3, 140.6, 146.3, 158.7, 159.6, 165.0, 165.2; **3**·$H_2C_2O_4$·H_2O, decomposition maximum 135 °C, ^{13}C NMR (DMSO-d_6): 22.1, 23.1, 26.3, 26.6, 28.8, 38.4, 39.2, 40.9, 52.3, 59.9, 65.3, 111.0, 114.9, 116.5, 118.3, 123.3, 122.8, 128.8, 129.7, 133.5, 140.9, 146.2, 158.7, 159.4, 164.5, 165.0; **4**·$H_2C_2O_4$·0.5H_2O decomposition maximum 137 °C, ^{13}C NMR (DMSO-d_6): 22.2, 23.2, 26.0, 26.3, 28.8, 28.8, 28.9, 38.5, 39.1, 41.0, 52.4, 60.1, 65.3, 111.0, 114.8, 116.5, 118.3, 122.7, 123.3, 128.8, 129.7, 133.8, 140.9, 146.2, 158.7, 159.4, 164.4, 164.8.

Biological Assays

Guinea pig right atria

Guinea pigs (350–400 g) were sacrificed by cervical dislocation followed by exanguination. The right atria were rapidly dissected from the ventricles, cleaned of excess tissue and hung vertically in the organ bath containing oxygenated Ringer–Locke solution of the following composition (mM): NaCl 154; KCl 5.4; $CaCl_2$·$2H_2O$ 1.5; NaH_2PO_4·H_2O 0.25; $NaHCO_3$ 4.3; glucose 8.3 (31 °C). A stabilization period of 60 min was allowed before a cumulative concentration–response curve to histamine was performed. During this equilibration period the bathing solution was changed every 30 min. The preparations were incubated with antagonists for period lengths of time, then washed for 60 min and the concentration–response curves for histamine repeated.

Antimicrobial Activity

Antibacterial activity

Strains. Nineteen clinical *Helicobacter pylori* isolates and NCTC 11637 were used. Two of those (NCTC 11637, 102R) were metronidazole resistant. The strains were maintained at −80 °C in Wilkins Chalgren with 10% (v/v) horse serum (Seromed) and 20% (v/v) glycerol (Merck) until used for the experiments. The bacteria were grown on Columbia agar base (Difco Laboratories) supplemented with 10% horse serum (Seromed) and 0.25% Bacto yeast extract (Difco) incubated for 72 h at 37 °C under microaerobic conditions (10% CO_2) in a gas incubator (Haereus). Before use the media were always preincubated under the same microaerobic conditions for a minimum of 2 h in order to allow them to equilibrate, and none of the cultures were kept in the air for more than 15 min.

Minimal inhibitory concentration (MIC) determination. MICs of all the compounds and metronidazole, taken as reference, were determined using the agar dilution method. In brief, all the substances were dissolved in dimethylsulphoxide (DMSO) and serial double dilutions were performed for calvatic acid **1** ranging from 128 to 0.0039 μg/mL. All remaining compounds including metronidazole, employed in equimolar concentration to **1**, were diluted in agar medium in serial double dilution. The plates of Columbia agar with horse serum and yeast extract containing antimicrobial agents were prepared on the day of use. The inoculum was prepared as follows: a suspension of 72 h growth of each strain on agar plates was made in Wilkins Chalgren broth (Difco) at a turbidity equivalent to N° 0.5 Mac Farland standard. The plates were inoculated using a multipoint inoculator (Denley A 400 PBI) dispensing 5 μL and incubated at 37 °C for 72 h under microareobic conditions (10% CO_2 in gas incubator). The MIC was defined as the lowest concentration capable of inhibiting any visible bacterial growth.

Acknowledgements

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8. Satisfactory analyses (±0.4%) were obtained for all new compounds; ^{1}H NMR spectra are in keeping with the proposed structures.