Evaluation of the Antimicrobial Effects of Several Isothiocyanates on *Helicobacter pylori*

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Original Paper

Abstract

The antibacterial activity of sulforaphane [4-(methylsulfinyl)butyl isothiocyanate] has been previously described. We have analysed the activities of 12 isothiocyanates (ITC) including sulphoraphane on 25 strains of *Helicobacter pylori* using an agar dilution assay. In addition, bactericidal effects against *H. pylori* were determined for the 6 most active ITCs, both directly and against intracellular bacteria in cultured human epithelial (HEp-2) cells. The MIC_{90} values for these ITCs ranged between 4 and 32 μg/mL and four of the most potent compounds exhibited bactericidal activity against both extra- and intracellular bacteria. Overall, our data indicate that ITCs have a potent antibacterial effect against *H. pylori* and these naturally occurring phytochemicals might have potential as novel therapeutic agents for *H. pylori* eradication.

Key words
Helicobacter pylori · isothiocyanate · time-to-kill assay · minimal inhibitory concentration

Introduction

Helicobacter pylori, which causes chronic gastritis as well as gastric and duodenal ulcers, is also an important risk factor for development of gastric cancer and mucosa-associated lymphoid tissue lymphoma. About half of the world's population is supposed to be infected, particularly in developing countries where the prevalence of the infection may reach 90% [1]. *H. pylori* is difficult to eradicate in 15 – 20% of patients even if multidrug therapies consisting of two or more antibiotics (e.g., amoxicillin, clarithromycin or metronidazole) with an inhibitor of acid secretion are used. This level of treatment failure appears to be due to the development of resistance to these antibiotics and poor patient compliance [1]. It may also be related to the persistence of organisms within gastric epithelial cells [2]. Therefore, there is a need for new antimicrobial agents that can be used to treat *H. pylori* infection.

The isothiocyanate sulforaphane [4-(methylsulfinyl)butyl isothiocyanate; (–)-1-isothiocyanato-(4*R*)-(methylsulfinyl)butane], a naturally occurring inducer of chemoprotective and antioxidant enzymes and a potent inhibitor of experimental carcinogenesis [3], [4], [5], [6], [7] was recently shown to be active against *H. pylori* [3], [8].

Broccoli sprouts, young plants of the family Cruciferae, are a rich source of glucoraphanin, the naturally occurring glucosinolate precursor of sulforaphane [4], [7]. Others plants, especially Cruciferae, contain glucosinolates that are converted into isothiocyanates by the action of myrosinase (E.C 3.2.3.1). Glucosinolates

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may act locally [9], or may be absorbed after conversion into isothiocyanates [4], [10]. It has been reported than isothiocyanates other than sulforaphane have antibacterial or chemoprotective activities [11], [12], [13]. They, too, may be of interest for the prevention and/or the treatment of *H. pylori* infection. However, the activities of these compounds against *H. pylori* are unknown at the present time.

In this study, we have evaluated *in vitro* the activity of the naturally occurring L-sulforaphane, its synthetic enantiomer (D-sulforaphane) and a synthetic chiral mixture (D,L-sulforaphane) against *H. pylori* both in pure culture and in cultured mammalian epithelial (HEp-2) cells, compared to 11 other isothiocyanates (Fig. **1**).

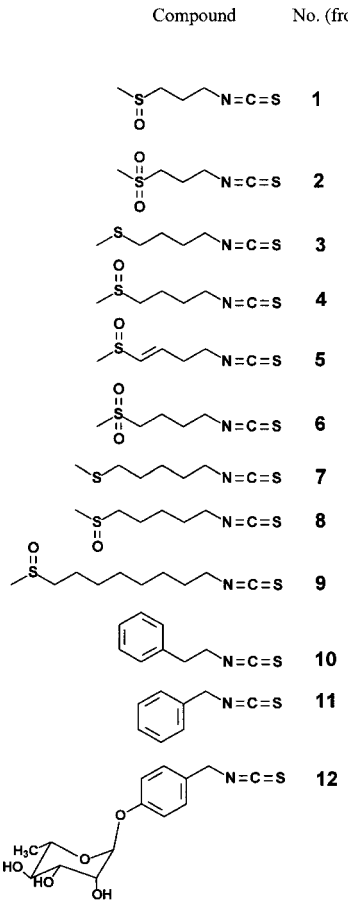

Fig. **1** Structures of isothiocyanates (ITCs) tested (see Table **1** for compound names).

Materials and Methods

Bacterial strains

Two reference strains (26 695 and ATCC 43 504) and 23 clinical strains (LBN 202 to LBN 224) of *H. pylori* were used in this study. The clinical isolates were obtained in 2000 and 2001 at the University Hospital Center of Nancy, France, from individual patients with gastritis and gastric or duodenal ulcers. Identity as *H. pylori* was established by Gram stain, and oxidase, catalase and urease production. All strains were stored in *Brucella* broth (Oxoid, Basingstoke, England) containing 15% (wt/vol) glycerol at -80 °C until use. Experiments were all performed at 37 °C in a microaerophilic (5% O_2, 15% CO_2, 80% N_2) atmosphere and the culture medium was Columbia agar containing 10% horse blood.

Chemicals

Iberin (**1**), cheirolin (**2**), erucin (**3**), D,L-sulforaphane (**4**), D-sulforaphane (**4**), L-sulforaphane (**4**), L-sulforaphene (**5**), erysolin (**6**), berteroin (**7**), alyssin (**8**), hirsutin (**9**), phenylethyl isothiocyanate (PEITC) (**10**) and benzyl isothiocyanate (BITC) (**11**) were obtained from Coger (Paris, France). 4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate (4RBITC) (**12**) was produced by myrosinase hydrolysis of the cognate glucosinolate isolated from seeds of the horseradish tree, *Moringa oleifera* as described previously [14]. The structures of all isothiocyanates used in this study are shown in Fig. **1**. Purity, common and chemical names, and botanical sources are included in Table **1**. Amoxicillin was supplied by GlaxoSmith-Kline and clarithromycin by Sanofi-Synthelabo (Paris). Metronidazole was purchased from Sigma-Aldrich (St Quentin, France). Stock solutions of isothiocyanates were prepared in acetonitrile, and the other antibiotics were prepared as recommended by their manufacturers. Subsequent dilution was made into sterile water.

Determination of minimal inhibitory concentrations

Minimal inhibitory concentrations (MICs) of isothiocyanates and conventional antibiotics were determined by using the agar dilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [15]. Two-fold dilutions of each drug ranging from 64 to 0.03 μg/mL were tested. The MIC was defined as the lowest concentration of each compound that resulted in no visible growth after 3 days of incubation. Clarithromycin resistance was defined by an MIC ≥ 1 μg/mL, as recommended by NCCLS [15]. Resistance breakpoints for metronidazole and amoxicillin were defined as > 8 μg/mL and > 0.5 μg/mL, respectively, as described in [3].

Determination of bactericidal activity

The bactericidal activities of six isothiocyanates (**2**, **5**, **6**, **7**, **9**, and **12**) showing the lowest modal MICs (Table **2**) were determined against *H. pylori* strains 26 695 and LBN 211. This evaluation was performed by monitoring, as previously described [3], the viability of *H. pylori* in extracellular (broth) or intracellular (human epithelial cells) locations after variable incubation times with each isothiocyanate at 0.5×, 1×, and 5× MIC. To evaluate the extracellular bactericidal activity, time-to-kill studies were performed in *Brucella* broth supplemented with 10% fetal calf serum with a final inoculum of approximately 5×10^6 CFU/mL. After 0, 8, 16, and 24 h of incubation at 37 °C under microaerophilic conditions and gentle shaking, samples were serially diluted and plated onto Columbia blood agar. After 5 days of incubation under microaerophilic conditions at 37 °C, colonies were counted. The determination of intracellular bactericidal activity was performed in a human epithelial cell line (HEp-2; ATCC CCL 23). Briefly, 10^6 HEp-2 cells were exposed to 10^8 CFU of *H. pylori* strains in EMEM with FCS for 12 h at 37 °C in 5% CO_2. Cells were gently washed with Hank's balanced salt solution (HBSS) to remove any non-adherent bacteria, and then incubated with a solution containing 100 μg/mL of gentamicin for 1.5 h to kill extracellular bacteria. Monolayers were then washed 6 times with HBSS, and incubated in culture medium containing one of the compounds tested at 37 °C in 5% CO_2. After 0, 4, 8, 12, and 24 h, wells were washed 6 times with HBSS, and cells were lysed with distilled water. At each time, samples were serially diluted and plated onto Columbia blood agar. After 5 days of incubation under microaerophilic conditions at 37 °C, colonies were counted. For all assays, tests were performed in duplicate and the limit

Table **1** Isothiocyanates (ITCs) tested for antibiotic efficacy against *H. pylori*

Compound No	*Common name (% purity)*	*Chemical name (isothiocyanate, ITC)*	*Botanical source(s)*
1	Iberin (97)	3-(methylsulfinyl)propyl	*Iberis* sp. L. (Cruciferae)
2	Cheirolin (97)	3-(methylsulfonyl)propyl	*Cheiranthus cheiri* L. (Cruciferae)
3	Erucin (97)	4-(methylthio)butyl	*Eruca sativa* (Miller) Thell. (Cruciferae)
4	D,L-Sulforaphane (99) D-Sulforaphane (99) L-Sulforaphane (99)	4-(methylsulfinyl)butyl 4-(methylsulfinyl)butyl 4-(methylsulfinyl)butyl	[synthetic] [synthetic] *Cardaria draba* (L.) Desv. (Cruciferae), *Brassica oleracea italica* L. (Cruciferae)
5	L-Sulforaphene (99)	4-methylsulfinyl-3-butenyl	*Raphanus sativus* L. (Cruciferae)
6	Erysolin (97)	4-(methylsulfonyl)butyl	*Erysimum* sp. L. (Cruciferae)
7	Berteroin (97)	5-(methylthio)pentyl	*Berteroa incana* DC. (Cruciferae)
8	Alyssin (97)	5-(methylsulfinyl)pentyl	*Alyssum* sp. L. (Cruciferae)
9	Hirsutin (97.3)	8-(methylsulfinyl)octyl	*Rorippa* sp. Scop. (Cruciferae), *Nasturnium officinale* R. Br. (Cruciferae), *Lepidium sativum* L. (Cruciferae), *Arabis* sp. L. (Cruciferae), *Bisutella* sp. L. (Cruciferae), *Sibara virginica* E. Greene (Cruciferae)
10	PEITC (96)	2-phenylethyl	*Nasturtium officinale* R. Br. (Cruciferae), *Raphanus* sp. L. (Cruciferae), *Rorripa* sp. Scop. (Cruciferae)
11	BITC (99.8)	benzyl	*Nasturtium officinalis* R. Br. (Cruciferae), *Tropaeolum majus* L. (Cruciferae)
12	4RBITC (95)	4-(α-L-rhamnopyranosyloxy)benzyl	*Moringa oleifera* Lam. Moringaceae

Table **2** Susceptibility of 25 *H. pylori* strains to isothiocyanates (ITCs) and to other antibiotics

ITCs (compound no)	*MIC(s) (μg/mL)*			
	Range	*MIC_{50}[a]*	*MIC_{90}[b]*	*Modal MIC*
Iberin (**1**)	16 – 32	16	32	16
Cheirolin (**2**)	4 – 32	8	16	8
Erucin (**3**)	8 – 32	16	32	32
D,L-Sulforaphane (**4**)	0.06 – 8	2	4	4
D-Sulforaphane (**4**)	0.125 – 8	2	4	4
L-Sulforaphane (**4**)	0.06 – 8	2	4	4
L-Sulforaphene (**5**)	4 – 16	8	16	8
Erysolin (**6**)	4 – 32	8	32	8
Berteroin (**7**)	1 – 16	8	16	4
Alyssin (**8**)	8 – 16	8	16	16
Hirsutin (**9**)	2 – 8	4	8	4
PEITC (**10**)	8 – 32	16	32	16
BITC (**11**)	8 – 16	8	16	16
4RBITC (**12**)	0.125 – 8	2	4	4
Amoxicillin	0.06 – 0.5	0.06	0.5	0.06
Clarithromycin	0.06 – 32	0.06	32	0.06
Metronidazole	0.06 – 64	1	64	1

[a] MIC_{50}: MIC at which growth of 50% of strains is inhibited.
[b] MIC_{90}: MIC at which growth of 90% of strains is inhibited.

of detection was 20 CFU/mL. Results are expressed as mean $\log_{10}$ CFU/mL. Bactericidal activity was defined as a greater than 1,000-fold reduction in viable CFU compared to the inoculum.

Results

D,L-Sulforaphane, D-sulforaphane, and L-sulforaphane (compound **4**) exhibited similar and high antibacterial activities (overall MIC range: 0.06 – 8 μg/mL) against all strains tested. For these three compounds, MICs varied by less than one single serial dilution step (Table **2**). The activities of **4** and **12** were greater (4- to 8-fold lower MICs) than those of the other compounds tested. Among the latter ITCs, **9** exhibited the highest activity (MIC_{50}: 4 μg/mL – MIC_{90}: 8 μg/mL). For all isothiocyanates tested, no differences in MIC_{90} were found between clarithromycin- and metronidazole-susceptible strains and either clarithromycin-resistant strains (4 strains), metronidazole-resistant strains (4 strains) or clarithromycin- and metronidazole-resistant strains (4 strains) (data not shown).

At a concentration of 5 × MIC, all ITCs tested except **5** exhibited a significant bactericidal activity against the two strains tested after 8 h (**2**, **9** and **12**) or 16 h (**6** and **7**) of incubation (Table **3**). Compound **5** only exhibited a bactericidal effect against *H. pylori* LBN 211 at supra-inhibitory concentrations after 16 h of incubation. At a concentration of 1 × MIC, **12** was bactericidal only against *H. pylori* LBN 211 after 24 h of incubation, whereas **9** was only bactericidal against *H. pylori* 26 695 after 16 h of incubation (Fig. **2**). The other ITCs studied did not exhibit any significant bactericidal activities at a concentration of 1 × MIC.

Against intracellular bacteria, **2**, **6**, **9** and **12** exhibited a significant bactericidal effect after 24 h at a concentration of 5 × MIC (Table **3**). For these compounds, no significant bactericidal effect was observed at the lower concentrations tested while **5** and **7** did not exhibit any intracellular bactericidal effect at any concentration tested.

Discussion

In the present work, we showed for the first time that ITCs other than sulforaphane also exhibit a potent effect against *H. pylori*. It is noteworthy that, as for sulforaphane, this inhibitory potency was not related to the resistance of strains to clarithromycin and/or metronidazole. Among the compounds tested in the present study, 4RBITC (**12**) and sulforaphane (**4**) exhibited the highest inhibitory activity against *H. pylori*. However, the less active molecules (**1**, **3**, **6** and **10**) were as potent as other plant-derived or phytochemicals such as epigallocatechin gallate from green tea, MIC_{90} = 32 μg/mL [16]; resveratrol from red wine, MIC_{90} = 25 μg/mL [17]; or allixin from garlic bulb, MIC_{90} = 25 μg/mL [18]. The fact that all ITCs tested were active against *H. pylori* suggests that this potency may be related to the presence of the ITC group. This activity was not dependent upon chirality as similar antibacterial effects were observed with optical enantiomers of **4**. This compound and its homologues (**1**, **8** and **9**) exhibited variable antibacterial activity while the desaturation of the side chain, in the case of **5**, was associated with a slight decrease of the inhibitory activity. These findings indicate that the number of carbon atoms in the side chain may be important for activity. Our results also suggest that the presence of an aromatic group in the side chain reduces antibacterial activity, since **10** and **11** were less active than **4**. However **12**, with a rhamnose moiety esterified to its benzyl side chain, was as active as **4** against *H. pylori*. The quinone reductase induction potential of **12** was recently shown to be considerably higher than that of **4** in cultured murine hepatoma cells, but lower in cultured mammalian cells [19]. The differential activities may be related to the facility with which these ITCs are taken up into cells, an effect that would be dramatically modified by the introduction of a side chain sugar substitution (e.g., in **12**). Clearly, this effect has been known to be important in the quinone reductase inducer potency of ITCs on mammalian cells [6]. Whether bacterial uptake is related to antibacterial potency is currently under study in our laboratories. In the present study, we also found that, in addition to sulforaphane (**4**), four of the most inhibitory ITCs tested (**2**, **6**, **9**, and **12**) were highly bactericidal against extra- and intracellular forms of *H. pylori* at concentrations of 5 × MIC. These results are similar to those previously obtained with sulforaphane [3]. Concentrations which are necessary to achieve such an effect could easily be achieved *in vivo.* For example, a person consuming a small portion (30 g) of broccoli sprouts consumes in the order of 180 μmoles of glucoraphanin, which when converted to sulforaphane accounts for ca. 32 mg of intake. Distributed across one litre of fluid in the stomach, this is equivalent to 32 μg/mL, for a short period of direct exposure. Consumption of a small portion of edible plant seeds in many cases provides an even richer source of glucosinolates than older plants or sprouts grown from these seeds [4], and

Table **3** Bactericidal effect of cheirolin, sulforaphene, erysolin, berteroin, hirsutin, 4RBITC, amoxicillin, clarithromycin, and metronidazole at 5 × MIC on intracellular *H. pylori*. Reduction (–$\log_{10}$ CFU/mL) of viable intracellular bacteria compared to the inoculum

Compound no	*H. pylori strain*	*4 h*	*8 h*	*12 h*	*24 h*
Cheirolin (**2**)	26695	–0.3	–0.9	–1.4	–4.4
	LBN 211	–0.2	–0.9	–1	–4.5
Sulforaphene (**5**)	26695	0	0	–0.1	–0.1
	LBN 211	0	–0.2	–0.3	–0.8
Erysolin (**6**)	26695	0	0	0	–4
	LBN 211	0	0	–0.9	–4.5
Berteroin (**7**)	26695	–0.1	–0.3	–0.5	–0.5
	LBN 211	–0.3	–0.3	–0.4	–0.4
Hirsutin (**9**)	26695	–0.1	–0.2	–0.8	–4.6
	LBN 211	–0.3	–0.7	–0.8	–4.5
4RBITC (**12**)	26695	0	–2.3	–3.5	–4.6
	LBN 211	0	–1.3	–1.4	–4.5
Amoxicillin	26695	0	0	0	–0.2
	LBN 211	0	0	0	–0.3
Clarithromycin	26695	0	–0.3	–0.5	–0.6
	LBN 211	0	–0.3	–0.4	–0.5
Metronidazole	26695	0	0	–0.3	–0.8
	LBN 211	0	0	–0.3	–0.6

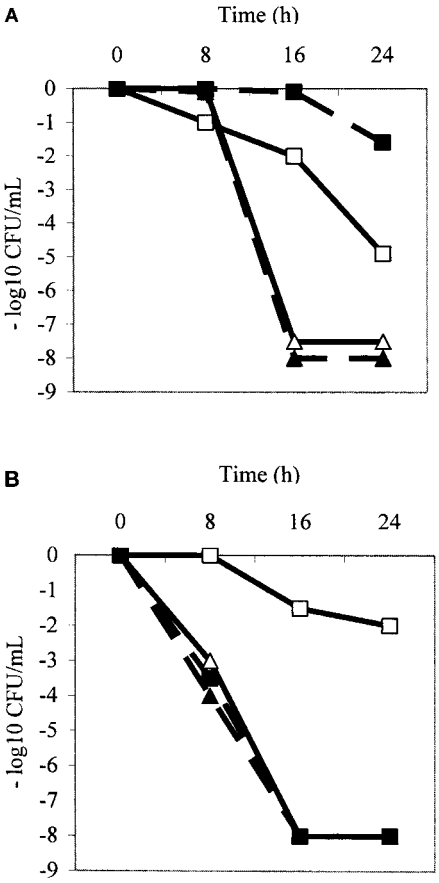

Fig. **2** Bactericidal activity of 4RBITC (**12**) [**A**] and hirsutin (**9**) [**B**] against *H. pylori* LBN 211 at 1×MIC [open squares] and 5×MIC [open triangles] and *H. pylori* 26695 at 1×MIC [filled squares] and 5×MIC [filled triangles].

transient levels in the stomach could reach as high as a few hundreds μg/mL. Exposure of *H. pylori* to these compounds may be equally, or even more, important via absorption and redistribution through the enterohepatic circulation. It has also been shown that the consumption of vegetables, especially cruciferous vegetables, and fruits reduces the incidence of cancer [20]. Some of the chemopreventive effects of fruits and vegetable are linked to their content of components such as glucosinolates/isothiocyanates. Among ITCs tested in this study, **4** (highest content in broccoli sprouts, *Brassica oleracea* L. var. *italica*, Cruciferae), **5** (highest content in radish seeds, *Raphanus sativus* L., Cruciferae), **9** (high content in cress, *Lepidium sativum*, and other species), **10** (high content in watercress and radish), **11** (high content in nasturtium, *Tropaeolum majus* L., Tropaeolacae, and watercress, *Nasturtium officinale* R.Br, Cruciferae), and **12** (highest content in *Moringa oleifera* Lam, Moringacae) (see Table **1**) have all been shown to exhibit cancer-preventive effects [11], [13], [21], [22].

Thus, the parallel findings that **4**, **9** and **12** exhibit strong direct antibacterial activity against *H. pylori,* and are also chemoprotective by indirect measures, offers suggestive evidence that these effects might operate synergistically to treat *H. pylori* infection and to prevent gastric cancer. It has been recently shown in animal models that sulforaphane contributes *in vivo* to the eradication of *H. pylori* and to the prevention of chemically induced stomach tumors [3]. Similar evidence should be sought for the other two isothiocyanates (**9** and **12**) highlighted herein before investigating the activity of these compounds in humans.

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