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Urease from *Helicobacter pylori* is inactivated by sulforaphane and other isothiocyanates

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ABSTRACT

Infections by *Helicobacter pylori* are very common, causing gastroduodenal inflammation including peptic ulcers, and increasing the risk of gastric neoplasia. The isothiocyanate (ITC) sulforaphane [SF; 1-isothiocyanato-4-(methylsulfinyl)butane] derived from edible crucifers such as broccoli is potently bactericidal against *Helicobacter*, including antibiotic-resistant strains, suggesting a possible dietary therapy. Gastric *H. pylori* infections express high urease activity which generates ammonia, neutralizes gastric acidity, and promotes inflammation. The finding that SF inhibits (inactivates) urease (jack bean and *Helicobacter*) raised the issue of whether these properties might be functionally related. The rates of inactivation of urease activity depend on enzyme and SF concentrations and show first order kinetics. Treatment with SF results in time-dependent increases in the ultraviolet absorption of partially purified *Helicobacter* urease in the 260–320 nm region. This provides direct spectroscopic evidence for the formation of dithiocarbamates between the ITC group of SF and cysteine thiols of urease. The potencies of inactivation of *Helicobacter* urease by isothiocyanates structurally related to SF were surprisingly variable. Natural isothiocyanates closely related to SF, previously shown to be bactericidal (berteroin, hirsutin, phenethyl isothiocyanate, alyssin, and erucin), did not inactivate urease activity. Furthermore, SF is bactericidal against both urease positive and negative *H. pylori* strains. In contrast, some isothiocyanates such as benzoyl-ITC, are very potent urease inactivators, but are not bactericidal. The bactericidal effects of SF and other ITC against *Helicobacter* are therefore not obligatorily linked to urease inactivation, but may reduce the inflammatory component of *Helicobacter* infections.

1. Introduction

Colonization of the gastric mucosa by *Helicobacter pylori* is probably the most prevalent infection afflicting more than one-half of all humans and significantly increasing their risk for developing peptic ulcer, gastric malignancy and lymphoma [1,2]. The ability of *H. pylori* to thrive in the unfavorable acidic environment of the stomach depends on the generation of large amounts (10–15% of total protein) of the enzyme urease (urea aminohydrolase; EC 3.5.1.5) [3]. Although widely distributed in nature, this enzyme does not occur in mammalian tissues. By producing ammonia from host urea, urease neutralizes gastric acidity and permits *Helicobacter* to proliferate. Urease-deficient mutant strains of *Helicobacter* have never been isolated from patients and are presumed not to be infectious [4,5]. Interestingly, urease promotes mucosal inflammation and also contributes to the pathogenicity of several other important human infections: *Mycobacterium tuberculosis*, *Cryptococcus neoformans* (associated with lung infections), and *Proteus* spp. (associated with urinary tract infections) [6].

Ten years ago, we made the totally unexpected observation that sulforaphane [SF; $CH_3S(O)(CH_2)_4NCS$], an isothiocyanate derived from its cognate glucosinolate (glucoraphanin) that is abundant in broccoli and other edible crucifers, is very potently and quite specifically bactericidal for *H. pylori* [7]. Moreover, SF was very active against a large number of clinical isolates of *H. pylori*, many of which were resistant to conventional antibiotics, such as clarithromycin and metronidazole. This immediately posed the question whether dietary administration of SF might be a practical and economically accessible therapeutic strategy to combat *H. pylori* infections globally. Both clinical cases and murine infections of *H. pylori* have responded [8], and while not curative, SF has reduced colonization and inflammation significantly [8].

Abbreviations: ITC, isothiocyanate(s); MBC, minimum bactericidal concentration; SF, sulforaphane.

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Although urease was crystallized from jack beans in 1926 [9], its molecular structure was only elucidated much more recently [3,10]. Ureases from plants and bacteria are very large (1.1 million Da) and highly homologous molecules, comprising 12 thiol-rich catalytic subunits (12 cysteine residues per subunit) with two nickel ions (Ni^{2+}) present at each active site. The reactivity of these cysteine thiols and their modification by both reversible inhibitors and irreversible inactivators has been extensively studied [11–20] and recently reviewed [21]. Many of the cysteine residues are susceptible to inhibition by Michael reaction acceptors such as α,β-unsaturated ketones [22]. It is therefore not surprising that isothiocyanates such as SF are efficient inactivators of urease.

This paper analyzes the mechanisms of the inhibitory effects of SF and related isothiocyanates on the urease of *H. pylori*, and how this process relates to the bactericidal activity of SF. Since SF and other ITC are derived from widely-consumed cruciferous plants, and have been administered orally and are well tolerated in a number of clinical trials [23], it is hoped that they can be developed as a global strategy to ameliorate *H. pylori* infections.

2. Materials and methods

2.1. Materials

Jack bean (*Canavalia ensiformis*) urease and other reagents were purchased from Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). All work with live *Helicobacter* cultures was done in a biosafety level 2 laboratory.

2.2. Cell cultures

Five *H. pylori* strains were used in this study. All strains except SS-1 (kindly provided by Dr. James Fox, MIT) were obtained from the American Type Culture Collection: J99 (ATCC 700824), 26695 (ATCC 700392), 60190 (ATCC 49503); urease-negative variant of 60190 (ATCC 51110). All *H. pylori* cultures were maintained on tryptic soy agar (Difco) supplemented with 5% defibrinated sheep blood (Hemostat Laboratories, Dixon, CA) and Difco Brucella Broth with 5% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA). All *H. pylori* cultures were maintained at 37 °C under microaerophillic conditions in the BBL Campy Pack Plus Systems (Becton Dickinson, Franklin Lakes, NJ), using 3 oxygen scavenging sachets per box, replaced every 2–3 days, or in an incubator supplied with 10% CO_2.

2.3. Assay of urease activity

Assay mixtures were prepared in a 96-well microtiter plate by combining 25 μl/well 100 mM potassium phosphate buffer, pH 6.8, containing between 1 and 4 I.U. of urease and 25 μL/well of the potential inhibitor, and incubated at 25 °C for specified periods. Following the inactivation period, enzyme assay mixtures were added containing 100 mM potassium phosphate buffer, pH 6.8, up to 150 mM urea, and 0.002% phenol red in a volume of 200 μL [17]. Linear changes in absorbance at 570 nm were measured over a 3 min period and expressed as milli-absorbance units per minute (mAU/min). For specific activity determinations, these velocities were normalized for protein concentrations.

2.4. Isolation and purification of H. pylori urease

2.4.1. Crude urease extraction

One-hundred 10 cm Petri plates (see above), were inoculated with *H. pylori*, and incubated for 4 days; bacterial lawns were scraped directly into 20 mM potassium phosphate buffer, pH 7.0, and frozen at −80 °C for >8 h. Cold distilled water (1–4 °C) was added to the frozen cell pellet, which was vortexed to thaw, and centrifuged (5600*g*) for 10 min. The supernatant fraction was removed, pellets re-extracted with 5 mL of cold water, and pooled supernatants were filtered (0.22 μm). Filtrate (total of ~35 mL) was centrifugally concentrated (100,000 MWCO Centricon) to a volume of 11 mL, mixed with glycerol to give a final concentration of 20% (v/v) in a volume of 13.2 mL, and stored at −20 °C in 0.5 mL aliquots.

2.4.2. Purification of H. pylori urease

A portion of the crude *H. pylori* urease preparation was further purified by FPLC, employing a sizing column, followed by anion exchange in a modification of published techniques [24]. Briefly, urease activity in fractions was measured using the phenol red assay, and protein content was measured using the bicinchoninic acid assay [25]. Fractions containing urease activity were pooled, desalted and concentrated by dialysis (10,000 MWCO Slide-A-Lyzer cassettes; Thermo Scientific). The final set of pooled fractions was concentrated in a 10,000 MWCO Amicon "Ultracell" (Millipore, Billerica, MA). Fractions were assessed for purity by electrophoresis on SDS PAGE 12% separation gels (BioRad, Hercules, CA) [24]. The first FPLC separation utilized a Sephacryl S-300 HR 26/60 column (60 × 2.6 cm, Pharmacia) equilibrated with gel-permeation buffer; flow rate was 1 mL/min, and 4 mL fractions were collected continuously after elution of V_o volume. Pooled urease-containing fractions were dialyzed against ion-exchange loading Bis–Tris propane buffer (120 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane) adjusted to pH 6.9 with HCl. Pooled urease fractions were concentrated in a 100K MWCO Amicon Centricon device to minimal volume. Final purification was performed on a Mono Q HR 5/5 anion exchange column (5 × 0.5 cm, Pharmacia) equilibrated with the same buffer (flow rate 1 mL/min, UV peaks collected using a linear 0–500 mM NaCl-gradient over 18 mL). Fractions containing urease were pooled, dialyzed against gel-permeation buffer, and concentrated to 500 μL.

2.5. Spectroscopic studies of urease

The time course of the spectral change induced by a single addition of sulforaphane to *H. pylori* urease was measured (0–120 min) essentially as described previously [26–28]. Briefly, partially purified *H. pylori* urease (3 mg protein/mL) was diluted into 10 mM potassium phosphate buffer, pH 7.2, and 0.74 mM sulforaphane (LKT Laboratories, St. Paul, MN); (9 μg in final volume of 300 μL). Spectral measurements were made with a Varian Cary 1E UV/Visible Spectrophotometer (Varian, Walnut Creek, CA) using a matched pair of quartz cuvettes (4 mm window).

2.6. Determination of minimum bactericidal concentrations (MBC)

H. pylori strains were grown as stock cultures on tryptic soy agar containing 5% defibrinated sheep's blood and cultured at 37 °C under micro-aerophilic conditions (10% CO_2) for 3–4 days. Determination of minimum bactericidal concentrations (MBC) were performed in 96-well microtiter plates with test compounds serially diluted in tryptic soy broth containing 5% (v/v) fetal bovine serum (100 μL per well final volume) [29]. Each test well was inoculated with 5 μL of a suspension of *H. pylori* adjusted to ~10^7 CFU/mL final concentration (A_{600nm} = 0.12). Assay plates were incubated for 3 days, then samples from each treatment well were plated on solid medium for an additional 3–4 days. The MBC was scored as the lowest concentration of test compound that inhibited *H. pylori* colony formation.

3. Results and discussion

3.1. Measurements of urease activity

$$NH_2{-}CO{-}NH_2 + H_2O \rightarrow H_2N{-}COOH + NH_3 \rightarrow H_2CO_3 + 2NH_3$$

Urease activity was determined in 96-well microtiter plates in weakly buffered (pH 6.8) assay systems containing phenol red. Ionization of the phenolic hydroxyl groups of phenol red (pK_a 8.2) by the generated NH_3 resulted in increases in absorption at 570 nm. These absorption increases during the initial 3 min reaction period were used as a measure of enzyme activity as originally described by Van Slyke and Archibald [30]. This assay was adapted for a microtiter plate format. Since linearity of absorption change at 570 nm with respect to NH_3 generation and pH was not demonstrated previously [22,30], we established that reaction velocity determined in this way was reasonably linearly related to enzyme quantity over a sufficiently wide range (Fig. 1) to provide a simple quantitative assay that is adequate for these studies.

By use of linear double reciprocal plots of velocity with respect to urea concentration, the Michaelis constants for urea were 20 mM for jack bean urease and 4.0–6.25 mM for several preparations of partially purified urease from *H. pylori* in 80 mM potassium phosphate buffer at pH 6.8 and 25 °C.

3.2. Kinetics of inactivation of Helicobacter urease by sulforaphane

Direct addition of sulforaphane or of boric acid (a well-established inhibitor of urease) [31] to *Helicobacter* or jack bean urease in the standard assay system was without effect on the urease activity when measured in the presence of 20–120 mM urea. In contrast, incubation of these enzymes with inhibitors in the absence of urea resulted in loss of enzyme activity. This rate and extent of loss of urease activity depended on three factors: the concentrations of inhibitors, the duration of incubation with inhibitors, and the concentration of enzyme. With use of SF as inhibitor, the residual enzyme activity was completely unaffected by the

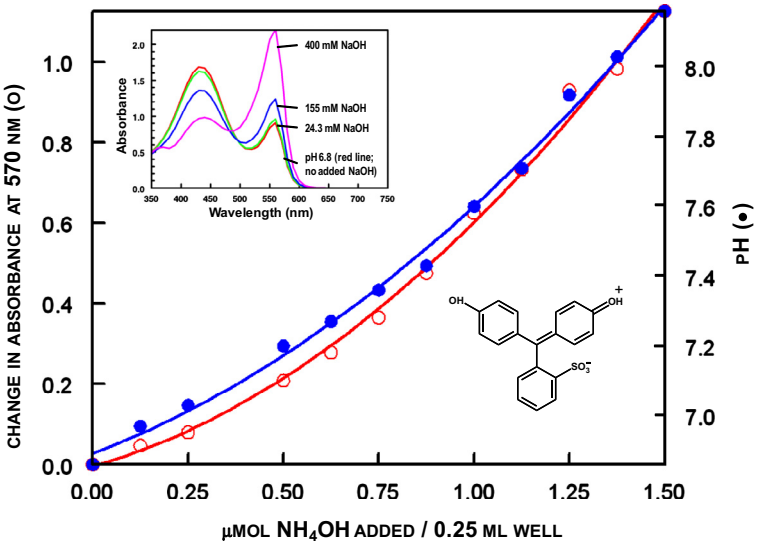


Fig. 1. Microtiter well calibration curve for measurement of ammonia by change in absorption of phenol red. Aliquots of NH_4OH were added to 200 μL of 100 mM potassium phosphate, pH 6.8, containing 0.002% phenol red, and the changes in absorption at 570 nm (○) and the pH (●) determined. The insets show the absorption spectra of phenol red upon addition of increasing quantities of NaOH, and the structure of the chromophore. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

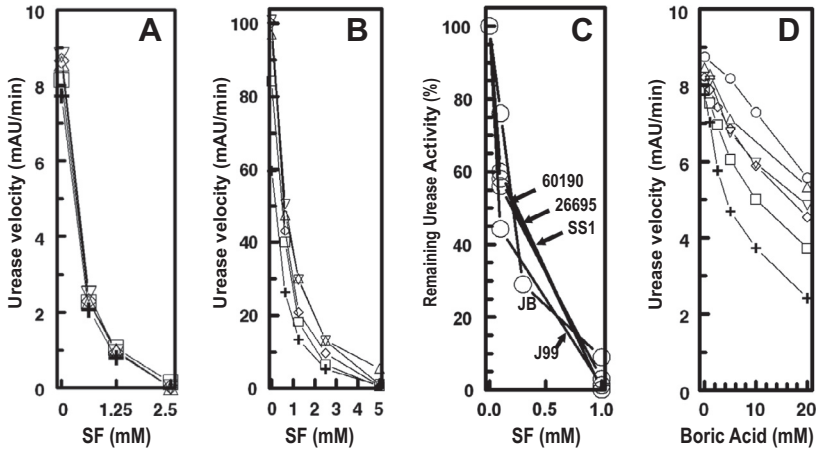


Fig. 2. Inactivation of ureases by a series of concentrations of sulforaphane (A–C) and boric acid (D). The ureases from (A) *H. pylori* strain J99 and (B) jack bean were incubated for 1 h with inactivator prior to assay. The ureases from (C) 4 strains of *H. pylori* (60190, 26695, SS1, J99) and jack bean (JB), were incubated for 2 h with sulforaphane (SF) prior to assay. (D) Urease from *H. pylori* strain J99 was incubated with boric acid (BA) for 1 h prior to assay. Urease activities were measured in the presence of urea 120 (○), 100 (△), 80 (▽), 60 (◇), 40 (□), and 20 (+) mM urea.

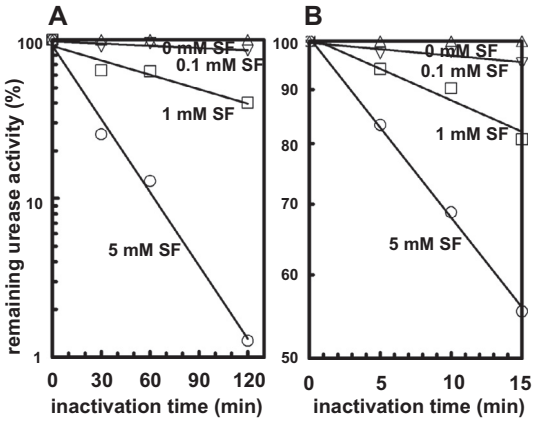


Fig. 3. Time-course of inactivation of partially purified *H. pylori* urease by a series of concentrations of sulforaphane (SF). The urease concentrations were 26.7 μg/mL (A) and 80 μg/mL (B) and the remaining urease activities were measured in the presence of 20 mM urea. Semi-log plots of residual activity with respect to time of inactivation are linear, consistent with a first-order inactivation process.

concentration of urea (20–120 mM) used in the assay system, and once inactivation had occurred, the activity could not be restored by increasing the concentration of urea in the assay system (Fig. 2A and B). In this respect, urease from several strains of *Helicobacter* (Hp J99, Hp 26695, Hp SS-1, and Hp 60190) and from jack bean behaved similarly (Fig. 2C). In sharp contrast, prior incubation of urease with boric acid also reduced the enzymatic activity, in a time- and concentration-dependent manner, but increasing the urea concentration in the assay system at least partially reversed the inhibition (Fig. 2D). This behavior is consistent with an essentially irreversible *inactivation* of *H. pylori* urease by SF, suggesting a covalent reaction between enzyme and inhibitor, and the conclusion that boric acid is at least in part a *reversible* inhibitor.

More detailed examination of the time-course of inactivation of *H. pylori* urease by a range of SF concentrations showed that the inactivation is a first order process whose rate depended both on the concentration of SF and of the enzyme (Fig. 3). Furthermore, double reciprocal plots of the pseudo-first-order rate constants of inactivation and the inactivator concentration were linear, consistent with the analysis of Kitz and Wilson [32] for another system.

3.3. Spectroscopic analysis of sulforaphane interaction with urease

Consistent with the suggestion that SF inactivates urease by covalent interaction, incubation of purified *H. pylori* urease with SF resulted in a time-dependent increase in ultraviolet absorption in the 260–320 nm region (Fig. 4). The magnitude of changes in the absorption increase depended on the concentration of SF. Fig. 4B shows the absorption difference at 280 nm observed after 60 min for SF concentrations ranging from 7.4 to 740 μM. The observed absorption changes are consistent with the interaction of the isothiocyanate (—N=C=S) group of SF with one or more cysteine thiol group(s) of urease with the formation of dithiocarbamates that absorb more strongly in this wavelength region [33,34]:

$$-N{=}C{=}S + HS\text{-}R \rightarrow -NH{-}C(SR){=}S$$

The difference spectrum between urease and the urease-SF complex has two absorption maxima (at 280 and near 320 nm). The latter is consistent with the formation of a dithiocarbamate. The presence of two isosbestic points (near 250 and 330 nm) in the difference spectra suggests that the reactions responsible for these spectral changes involve relatively specific chemical reactions (Fig. 4A).

3.4. Inactivation potencies of other isothiocyanates on H. pylori urease

Treatment of *H. pylori* urease for 2 h with 5 mM concentrations of a number of SF analogs disclosed a wide range of inhibitory (inactivational) potencies (Table 1). Thus, whereas the natural product iberin (methylsulfinylpropyl-NCS) was equally potent to SF, methylthiopentyl-NCS, methylthiobutyl-NCS, methylsulfinyloctyl-NCS, and methylsulfinylpentyl-NCS were inactive, as were *n*-hexyl-NCS, benzyl-NCS, phenylethyl-NCS, and the rhamnosyloxybenzyl-NCS derived from the Moringa tree. An unexpected finding was the very high inactivator potency of benzoyl-ITC which has not been isolated as a natural product, but which bears substantial structural similarities to the clinically tested urease inhibitor *N*-(diaminophosphinyl)-4-fluorobenzamide [31].

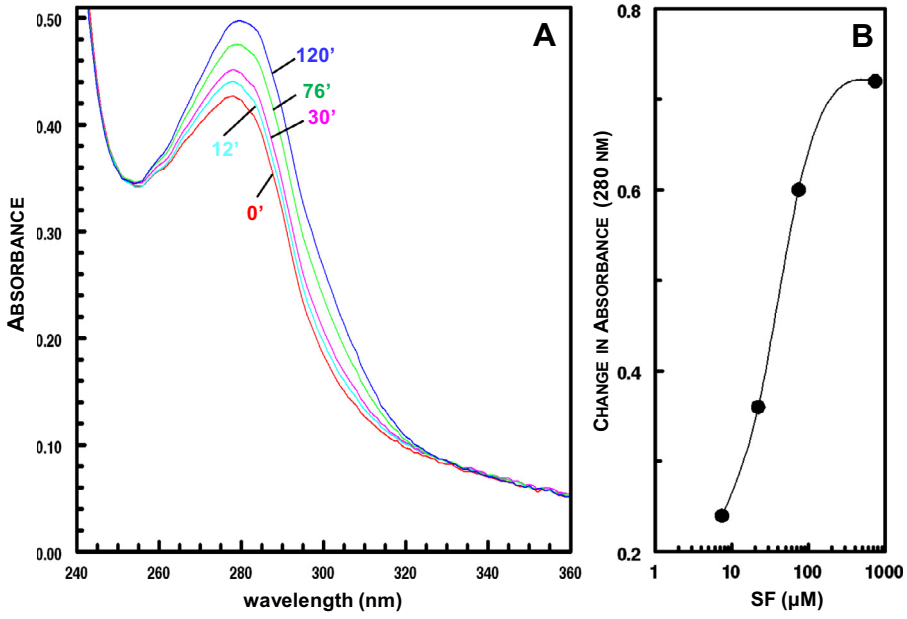


Fig. 4. (A) Time-course of difference spectra of partially purified *H. pylori* urease (9 μg/mL) treated with 740 μM SF for 12, 30, 76, and 120 min. The control cuvette contained the equivalent concentration of sulforaphane. (B) Change in absorption at 280 nm in 60 min when sulforaphane (7.4–740 μM) was added to partially purified *H. pylori* urease (30 μg/mL).

Table 1
Inactivation of partially purified *H. pylori* urease by various isothiocyanates (ITC). Substrate concentration was 20 mM urea, and 80 μg urease was used for each test.

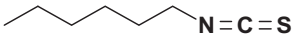
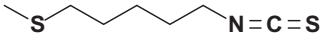
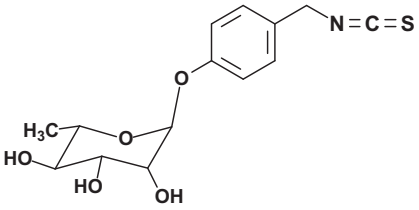
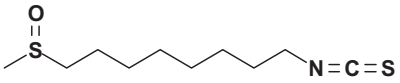
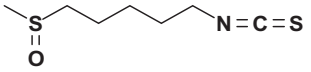
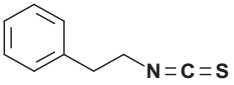
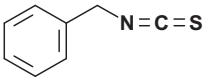
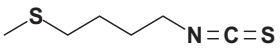
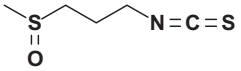
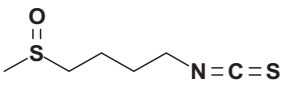
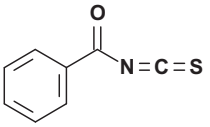
Isothiocyanate	Common Name	Structure	Conc (mM)	Remaining activity (%) at:	
				30'	120'
hexyl-NCS	–		5	100	100
5-(methylthio)pentyl-NCS	berteroin		5	100	100
4-rhamnopyranosyl-oxy(benzyl)-NCS	–		5	100	100
8-(methylsulfinyl)octyl-NCS	hirsutin		5	–	100
5-(methylsulfinyl)pentyl-NCS	alyssin		5	–	100
2-phenylethyl-NCS	phenethyl		5	–	100
benzyl-NCS	tropaeolin		5	–	100
4-(methylthio)butyl-NCS	erucin		5	100	–
3-(methylsulfinyl)propyl-NCS	iberin		5	43	5
4-(methylsulfinyl)butyl-NCS	sulforaphane		5	36	0
benzoyl-NCS	–		5 1 0.1 0.01	0 12 77 100	0 – – –

Table 2
Minimum bactericidal concentration (MBC) of sulforaphane (SF) against several *H. pylori* strains.

ATCC Strain no.	Common designation	Properties	MBC (μg/mL)
49503	60190	A cytotoxin-producing strain	2.8
51110	*ure*⁻60190 variant	Near-isogenic urease-minus variant of 60190	5.6
700824	J99	Genome-sequenced type-strain; cagA+, vacA+	2.8
700392	26695	Genome-sequenced type-strain	5.6
–	SS-1	Mouse-adapted human strain	2.8

3.5. Relation of bactericidal activity of isothiocyanates to urease inhibition potency

The minimum bactericidal concentration (MBC) of SF for various strains of *H. pylori* has been previously reported to be in the low microgram per mL range [7]. A more detailed examination of the MBC for a number of commonly-used *H. pylori* strains is shown in Table 2. The values are relatively uniform (2.8–5.6 μg/mL) and comparable to our previous measurements obtained by somewhat different methods (mean 2–4 μg/mL; [7]).

Very importantly, SF is potently bactericidal against the urease-negative and therefore noninfective strain (ATCC 51110), whereas the potent urease inhibitor benzoyl-ITC does not have bactericidal activity against several strains of *H. pylori* that we tested (data not shown). Furthermore, a number of ITC inducers of cytoprotective enzymes which were previously shown [35] to be bactericidal to *H. pylori* (e.g., 5-(methylthio)pentyl-ITC (berteroin), benzyl-ITC 8-(methylsulfinyl)octyl-ITC (hirsutin), phenethyl-ITC, 5-(methylsulfinyl)pentyl-ITC (alyssin), and 4-(methylthio)butyl-ITC (erucin) were not inhibitors of *H. pylori* urease when tested under identical conditions to SF, benzoyl-ITC, and iberin (Table 1). Similar observations were made with the ellagitannin tellimagrandin and the triterpenoid TP-225 in that they had antibiotic activity against *H. pylori*, but were inactive as urease inhibitors (data not shown).

Interestingly, and in marked contrast to the very high levels of SF accumulation in cultured mammalian cells [36], *H. pylori* did

not concentrate SF appreciably, from culture medium (data not shown). Clearly, since SF kills both virulent (*ure*$^{+}$; urease-containing) *H. pylori* and a mutant strain that lacks a functional urease (*ure*$^{-}$), the bactericidal/bacteriostatic effects of SF are not due primarily to urease inactivation.

Urease is involved in directly increasing the inflammation associated with *H. pylori* infections in a manner that has been postulated to increase tight junction permeability, and is not associated with the catalytic activity of the enzyme [37,38]. Whether inhibition of this mode of urease action by ITCs could alter the pathogenicity of *H. pylori* without killing or inhibiting the organism directly, requires elucidation in appropriate model systems.

In conclusion, it is intriguing to reflect on the unforeseen roles that urease has played in the history of biological science: (i) urease was the first enzyme to be crystallized (from jack bean) leading J.B. Sumner to attribute a specific biological activity to a pure protein, and thereby setting off one of the bitterest scientific polemics of the twentieth century; (ii) urease was the first enzyme shown to require two nickel atoms at the active site; (iii) urease is synthesized in large quantities by several important human infective agents in addition to *H. pylori,* including *Proteus mirabilis*, *Mycobacterium tuberculosis*, *and Cryptococcus neoformans*, and contributes in important ways to the inflammatory consequences of these infections; (iv) a large number of urease inhibitors have been developed for agricultural purposes to prevent soil nitrogen depletion by denitrification (and subsequent evaporation of ammonia). The present study raises the possibility that in addition to-, or as an alternative mode of action-ITC inactivation of *H. pylori* urease may reduce gastric colonization by this organism and/or the inflammation associated with it. Whether its inhibition can play a significant role in reducing the consequences of *H. pylori* colonization of half of the world's population warrants further exploration.

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