The Journal of Antibiotics (2018) 71:543–548
https://doi.org/10.1038/s41429-018-0027-1

JARA Japan Antibiotics Research Association
The Society for Actinomycetes Japan

BRIEF COMMUNICATION

Anti-*Helicobacter pylori* activities of selected *N*-substituted cinnamamide derivatives evaluated on reference and clinical bacterial strains

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Received: 24 August 2017 / Revised: 19 December 2017 / Accepted: 28 December 2017 / Published online: 13 February 2018

Abstract

In this study, thirty-five *N*-substituted derivatives of cinnamic acid amide (cinnamamide) were evaluated for anti-*Helicobacter pylori* activity using an agar disc-diffusion method. Qualitative screening was performed on a reference *H. pylori* strain (ATCC 43504), resulting in the identification of the three most active compounds, **8** (*R,S*-(2*E*)-3-(4-chlorophenyl)-*N*-(2-hydroxypropyl)prop-2-enamide, minimal inhibitory concentration, MIC = 7.5 μg/mL), **23** ((2*E*)-3-(4-chlorophenyl)-*N*-(2-hydroxycyclohexyl)prop-2-enamide, MIC = 10 μg/mL), and **28** ((2*E*)-3-(4-chlorophenyl)-*N*-(4-oxocyclohexyl)prop-2-enamide, MIC = 10 μg/mL). These compounds were further tested on twelve well-characterized clinical strains, yielding MIC values that ranged from 10 to 1000 μg/mL. Preliminary safety assessments of the compounds were made using the MTT viability test for cytotoxicity and Ames test for mutagenicity, which showed them to be generally safe, although compounds **8** and **28** showed mutagenic activity at some of the tested concentrations. The results of this study showed the anti-*H. pylori* potential of cinnamamide derivatives.

Helicobacter pylori is a gram-negative, spiral shaped bacterium that inhabits the human gastric mucosa. *H. pylori* is widespread in humans, with an estimated 50% of the world's population infected [1]. *H. pylori* is an etiological agent of type B gastritis and is one of the pathogenic factors that causes a predisposition to the development peptic ulcer disease [2]. Moreover, according to The International Agency for Research on Cancer of the World Health Organization (IARC/WHO), since 1994, *H. pylori* has been classified as a first class carcinogen, promoting the development of gastric cancer and mucosa associated lymphoid tissue (MALT) lymphoma [3].

Eradication of *H. pylori* infections plays an essential role in overcoming gastric diseases. The recommendations for the treatment of *H. pylori* infection, which were issued in 2016, include the use of three types of drugs, including antisecretory (proton pump inhibitors (PPI)), cytoprotectant (bismuth salts) and antibacterial drugs (clarithromycin, amoxicillin, metronidazole, tetracycline, levofloxacin, and rifabutin). Treatment regimens include triple therapy, consisting of PPI and two antibiotics/chemotherapeutics mentioned above, or quadruple therapy, consisting of PPI, metronidazole, tetracycline, and bismuth salt. Quadruple therapy is especially recommended in regions with high clarithromycin resistance [4].

H. pylori eradication therapy failure is caused by resistance of *H. pylori* to antibiotics and chemotherapeutics used in empirical treatment, especially to clarithromycin, which is conferred by the presence of point mutations in domain V of the 23S rRNA gene, primarily A2143G, A2142G, A2142C [5]. Thus, clarithromycin is not recommended if

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the resistance level is above 15%, and its use is only allowed after the exclusion of resistance using microbiological or genetic diagnostic methods [4]. For example, in Poland, where the *H. pylori* clarithromycin resistance is higher than 20%, this drug should not be used in empirical treatments [6]. The 'Kyoto global consensus report on *Helicobacter pylori* gastritis' recommended eradication therapy in cases of confirmed *H. pylori* infection (especially *cagA* positive strains), despite the absence of inflammatory changes in the gastric mucosa to avoid the development of inflammatory diseases and gastric cancer [7].

Currently, *H. pylori* eradications have diminished because of the increasing resistance of *H. pylori* strains to currently available antibiotics. The identification of novel compounds that are active against *H. pylori* can lead to the development of new alternative drugs to currently used antibiotics and chemotherapeutics. Therefore, the search for new anti-*H. pylori* agents is crucial to be able to conduct effective therapy in the future.

Cinnamic acid and its derivatives, such as cinnamamides and cinnamates, have been previously identified as potent antimicrobial agents. Multiple studies have reported on their antibacterial, antifungal, and antimycobacterial activities [8]. However, there are only few published studies on the anti-*H. pylori* activity of this group of compounds, and moreover, the results primarily concerned plant extracts or cinnamic acid itself rather than its synthetic derivatives [9]. Knowing their antimicrobial potential, we synthesized and evaluated the anti-*H. pylori* activities of a series of *N*-substituted amide derivatives of cinnamic acid (cinnamamides).

The chemical structures of the tested compounds are shown in Table 1. The compounds were synthesized by *N*-acylation of the appropriate amine using (*E*)-cinnamoyl chloride or (*E*)-4-chlorocinnamic acid chloride. The reactions were carried out in a two-phase system (toluene/water/K_2CO_3) or reagents were refluxed in toluene with anh. K_2CO_3. Some of the obtained derivatives were subjected to further oxidation of the hydroxyl group in *N*-substituent using Dess-Martin periodinane. The purity and chemical structures of the final compounds were confirmed by means of high-performance liquid chromatography, elemental analysis, and spectral methods (LC-MS, ^{1}H NMR). All synthesized compounds had the *E* configuration of the double bond. The physicochemical properties of compounds **1-22**, **24-26**, **29**, and **31** were published previously [10–13], while new compounds (**23**, **27**, **28**, **30**, **32–35**) were characterized for the purpose of current study.

The efficacy of the new potential antibacterial compounds was assessed by determining minimal inhibitory concentration (MIC) values using the disc-diffusion method [14]. Tested compound were diluted in DMSO to obtain a stock solution (1000 mg/L, 1%), from which 12 dilutions were prepared in water. The first step of the screening involved using the stock solution (1%) against the reference *H. pylori* strain ATCC 43504. An inoculum of 3.0 McFarland standards in a sterile 0.85% NaCl solution was prepared from a pure bacterial culture grown for 72 h. The inoculum was spread onto Schaedler agar with 5% sheep blood and then disc that was impregnated with 15 µL of the stock solution of the compound being tested was placed onto the plate. After 72 h of incubation under a microaerophilic atmosphere at 37 °C, the diameter of the zone of growth inhibition was measured. If the zone of growth inhibition was ≤8 mm, the compound was recorded as having no antibacterial activity. If the zone was >8 mm, the compound was recorded as possessing antibacterial activity (Table 1).

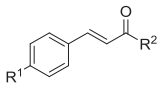
In the second step, nine compounds with zones of growth inhibition of >27 mm were observed as the most active compounds against the reference *H. pylori* strain ATCC 43504 and were further quantitatively evaluated. To obtain MIC values, the disc diffusion method was performed using 12 discs that were impregnated with decreasing concentrations of a given compound. The MIC value was determined as the lowest concentration of the tested compound that inhibited bacterial growth for zones of inhibition that were >8 mm (Table 1).

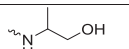
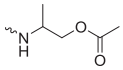
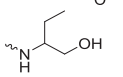
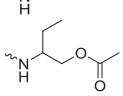
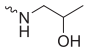
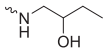
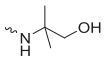
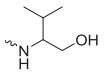
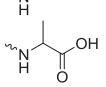
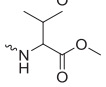
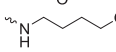
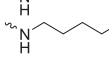
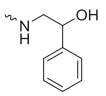
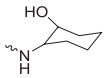
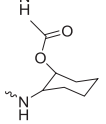
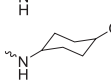
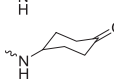
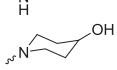
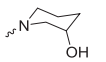
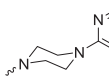
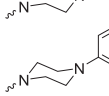
To ensure quality control of the disc diffusion method, we conducted a quantitative assay to obtain the MIC value for metronidazole against an *H. pylori* reference strain. Additionally, the MIC value for metronidazole was assayed by a reference method using strips impregnated with an antibiotic gradient (*E*-test).

The investigated group of cinnamamide derivatives possessed various *N*-substituents, including hydroxyalkyl, hydroxycycloalkyl, carboxyalkyl, metoxycarbonylalkyl, oxocycloalkyl, acetylhydroxyalkyl or arylalkyl moieties. Moreover, in some compounds, the nitrogen atom of the amide group was incorporated into a ring (piperidine or piperazine). Another assayed modification was a 4-chloro substitution in the phenyl ring.

The most active compounds identified were *R,S*-(2*E*)-3-(4-chlorophenyl)-*N*-(2-hydroxypropyl)prop-2-enamide (**8**, MIC = 7.5 µg/mL), (2*E*)-3-(4-chlorophenyl)-*N*-(2-hydroxycyclohexyl)prop-2-enamide (**23**, MIC = 10 µg/mL), and (2*E*)-3-(4-chlorophenyl)-*N*-(4-oxocyclohexyl)prop-2-enamide (**28**, MIC = 10 µg/mL), which significantly differed in their *N*-substituent but all possessed a chlorine atom in their phenyl rings. Indeed, for the majority of the compounds tested, the introduction of a 4-chloro substituent in the phenyl ring increased the anti-*H. pylori* activity, although not in every case. Thus, the relationship between the 4-chloro substitution and activity is unclear, as the obtained results were surprising and a structure-activity relationship could not be identified. Meaningful zones of bacterial growth inhibition were observed for various derivatives,

Table 1 Chemical structures of the evaluated compounds and the results of their anti-*H. pylori* activity screening using the reference strain ATCC 43504



Compound	R^1	R^2	Configuration	Diameter of growth inhibition (mm)	MIC value (μg/mL)
1	H		*R,S*	12	
2	Cl		*R,S*	27	1000
3	H		*R,S*	13	
4	H		*R,S*	12	
5	Cl		*R,S*	54	200
6	H		*R,S*	11	
7	H		*R,S*	11	
8	Cl		*R,S*	27	7.5
9	H		*R,S*	13	
10	Cl		*R,S*	37	75
11	H		-	13	
12	Cl		-	17	
13	H		*R,S*	13	
14	Cl		*R,S*	31	
15	H		*R,S*	12	
16	H		*R,S*	13	
17	H		-	13	
18	H		-	15	
19	Cl		-	26	
20	H		*R,S*	13	
21	Cl		*R,S*	13	
22	H		*trans, D,L*	19	
23	Cl		*trans, D,L*	34	10
24	H		*trans, D,L*	38	200
25	H		*trans*	13	
26	Cl		*trans*	16	
27	H		-	13	
28	Cl		-	27	10
29	H		-	14	
30	Cl		-	25	
31	H		*R,S*	11	
32	Cl		*R,S*	43	150
33	H		-	18	
34	Cl		-	8	
35	H		-	44	500

The anti-*H. pylori* activity results are expressed as the diameter of the zone of bacterial growth inhibition and MIC values

including those possessing in *N*-substituent moieties such as hydroxyalkyl (compounds **2**, **5**, **8**, **10**, **14**, **19**), hydroxycycloalkyl (compound **23**), and oxocycloalkyl (compound **28**), as well as compounds with the nitrogen atom of the amide group incorporated into a ring (**32**, **35**). One of the three *O*-acetylated derivative (**24**) was more active than the parent compound (**22**), while the remaining two were not active (**3** and **6**), similar to their parental compounds (**1** and **4**, respectively). Interestingly, some similar compounds significantly differed in their activities, e.g., **12** vs. other hydroxyalkyl derivatives, **26** vs. **28**, and **33** vs. **35**. To summarize, the described screening procedure is a useful method to identify anti-*Helicobacter pylori* compounds of various chemical compositions.

Among the assayed compounds, the three with the lowest MIC values (**8**, **23**, and **28**) were chosen for further evaluation using two additional *H. pylori* reference strains and twelve clinical strains isolated from infected patients who underwent gastroscopies at the Falck Outpatient Clinic in Krakow, Poland. This study was approved by the Bioethical Commission of the Jagiellonian University (Krakow, Poland) (Approval no. 122.6120.273.2015), and each patient signed an informed consent document. The clinical strains were characterized by phenotypic and genotypic studies. The presence of *H. pylori* in the collected samples was confirmed by a positive test for urease, catalase, and oxidase and the presence of spiral-shaped bacteria in gram-stain slides. Antimicrobial susceptibility testing was conducted according to the EUCAST recommendation for clarithromycin, metronidazole, levofloxacin, tetracycline, and amoxicillin using strips impregnated with the antibiotic/chemotherapeutic gradient to obtain MIC values [15]. Quality control was ensured using the *H. pylori* reference strain ATCC 43504. For the genotypic studies, isolation of genomic DNA was performed using a Sherlock AX isolation kit according to the manufacturer's protocol. In the 16 S rRNA-based identification, PCR reaction contained 2 μL of bacterial DNA, 2 μL of each primer (HP-1 and HP-2, 5 μL of GoTaq® Flexi Buffer, 1.5 μL of $MgCl_2$, 0.5 μL of PCR Nucleotide Mix, 0.125 μL of GoTaq® Flexi DNA Polymerase, and 25 μL of Nuclease-Free Water [16]. Genomic DNA extracted from *H. pylori* strains was used for PCR-based genotyping of the *ureB, cagA*, and *vacA* genes [17], using HPU 50 and HPU 25 primers, D008 and R008 primers, and VAC3624F and VAC3853R primers, respectively. Finally, to detect the most frequently occurring point mutations that account for *H. pylori* clarithromycin resistance (A2143G and A2142G), PCR followed by an RFLP analysis was performed. The 425-bp fragment of the peptidyl transferase region of the 23 S rRNA was amplified with the primers K1 and K2 [18].

The results of the phenotypic and genotypic identification of clinical strains are presented in Table 2, together

Table 2 Phenotypic and genotypic characterization of 12 clinical *H. pylori* strains and the activities of compounds **8**, **23**, and **28** against 12 clinical and 3 reference *H. pylori* strains expressed by MIC values

Strain characterization		Genetic characterization		MIC value (μg/mL)		
Strain	Resistance to antimicrobial agents	Type of mutation conferring clarithromycin resistance	Virulence factors	Compd 8	Compd 23	Compd 28
HP 34	Clarithromycin resistant	A2143G	*cagA*+, *vacA*+	1000	1000	1000
HP 194	Clarithromycin resistant	not detected	*cagA*+, *vacA*+	200	10	10
HP 316	Clarithromycin resistant	A2143G	*cagA*+, *vacA*+	150	150	200
HP 181	Clarithromycin and metronidazole resistant	A2143G	*cagA*+, *vacA*+	150	200	500
HP 217	Clarithromycin and metronidazole resistant	A2142G	*cagA*+, *vacA*+	200	200	1000
HP 234	Clarithromycin and metronidazole resistant	A2142G	*cagA*+, *vacA*+	200	200	1000
HP 234	Clarithromycin and metronidazole resistant	A2143G	*cagA*+, *vacA*+	200	50	200
HP 302	Clarithromycin and metronidazole resistant	A2143G	*cagA*-, *vacA*+	200	200	200
HP 148	Clarithromycin and levofloxacin resistant	A2143G	*cagA*+, *vacA*+	75	150	75
HP 304	Metronidazole and levofloxacin resistant	–	*cagA*+, *vacA*+	150	500	100
HP 198	Levofloixacin resistant	–	*cagA*+, *vacA*+	150	100	150
HP 335	Metronidazole resistant	–	*cagA*-, *vacA*+	150	150	150
ATCC 43504	Metronidazole resistant	–	*cagA*+, *vacA*+	7.5	10	10
J99	Clarithromycin, metronidazole and levofloxacin susceptible	–	*cagA*+, *vacA*+	200	150	200
ATCC 700684	Clarithromycin resistant	A2143G	*cagA*+, *vacA*+	50	100	50

with the anti-*H. pylori* activity results for the investigated compounds. The MIC values obtained for the clinical strains ranged from 10 to 1000 μg/mL. To compare the activities of the tested compounds, we calculated MIC_{50} and MIC_{90} values, which are the minimal inhibitory concentrations observed for 50 and 90% of the tested strains, respectively. For compound **8**, the $MIC_{50} = 150$ μg/mL and the $MIC_{90} = 200$ μg/mL. For compound **23**, the $MIC_{50} = 150$ μg/mL and the $MIC_{90} = 500$ μg/mL. Lastly, for compound **28**, the $MIC_{50} = 200$ μg/mL and the $MIC_{90} = 1000$ μg/mL. These values suggest that compound **8** possesses the highest anti-*H. pylori* potential among the tested derivatives.

For compounds **8**, **23**, and **28**, which were identified from the screening procedure as potential anti-*H. pylori* agents, cytotoxicity was determined in three cell lines (human liver cancer cells, Hep G2, ATCC® 59195™, human neuroblastoma cancer cells SH-SY5Y, ATCC® CRL-2266™, and human normal skin fibroblasts BJ, ATCC® CRL-2522™) using an MTT assay [19]. Our results showed that at concentrations of 10–50 μM, all analyzed compounds did not exhibit a cytotoxic activity against the assayed cell lines. For comparison, doxorubicin, a highly toxic chemotherapeutic agent, was used as a positive control.

Compounds **8**, **23**, and **28** were evaluated for mutagenicity using the Ames test, involving the use of *Salmonella typhimurium* strains TA100, TA98 and TA102, which detect base-pair substitution at GC pair, frameshift mutations and transitions/transversions at AT pair, respectively [20]. Each compound was tested in triplicate at 5 different concentrations, ranging from 10 to 500 μg/plate. All compounds were dissolved in DMSO, and DMSO alone served as a negative control. Sodium azide (5 μg/plate), 4-nitro-*O*-phenylenediamine (2.5 μg/plate), and mitomycin C (0.5 μg/plate) served as positive controls in tests involving the TA100, TA98, and TA102 strains, respectively. The experiment was conducted according to a preincubation assay. According to the adopted criteria (mutagen produces a reproducible, dose-related increase in the number of revertants in at least one strain [20]), none of tested compounds exhibited mutagenic activity. However, it should be noted that compounds **8** and **28** showed a significant increase in the number of revertants for strain TA98, which were classified as non-mutagenic due to an absence of a clear dose-dependent relationship for the observed effect over the range of tested concentrations.

Based on the results of the anti-*H. pylori* activity and safety evaluations, compound **23** ((2*E*)-3-(4-chlorophenyl)-*N*-(2-hydroxycyclohexyl)prop-2-enamide) represents the most promising derivative of the series of compounds tested in this study.

Acknowledgements This work was supported by Jagiellonian University Medical College in Kraków (Grants K/DSC/004292, K/ZDS/005487, K/DSC/003549), the Polish National Science Center, decision on grant no. DEC-2013/11/B/NZ7/04834, and by a research grant funded by the Polish Government in the years 2011-2013 (NN404547640). *Salmonella typhimurium* strains TA98 and TA102 were kindly provided by Dr T Nohmi (Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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