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Identification of 3′,4′,5′-trimethoxychalcone analogues as potent inhibitors of *Helicobacter pylori*-induced inflammation in human gastric epithelial cells

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ABSTRACT

Efforts to identify potent small molecule inhibitors of *Helicobacter pylori* led to the evaluation of 23 3′,4′,5′-trimethoxychalcone analogues. Some of the compounds displayed potent antibacterial activity against *H. pylori*. Three most active and selective compounds **1**, **7**, and **13** also showed the bactericide activity against the reference as well as multidrug-resistant strains of *H. pylori*. Additionally, the aforementioned three compounds potentially inhibited the *H. pylori* adhesion and invasion to human gastric epithelial (AGS) cells. Furthermore, these selective compounds inhibited the *H. pylori*-induced gastric inflammation by reduced inflammatory mediator's nuclear factor kappa B activation, and the secretion of interleukin-8.

Helicobacter pylori is a microaerophilic Gram-negative bacterium which inhabits approximately one-half of the global human population.[1] *H. pylori* binds to human gastric epithelial (AGS) cells in the stomach via bacterial adhesions causes various gastric diseases such as chronic gastritis, peptic ulcer, and gastric cancer.[2] *H. pylori* induces inflammation-associated gene expression in AGS cells, including activation of nuclear factor kappa B (NF-κB), and production of inflammatory cytokines including interleukin (IL)-8.[3] IL-8 secret by AGS cells is an important host mediator inducing neutrophil migration to the site of infection, and consider to be crucial cytokine in the regulation of inflammation and immune processes.[4] NF-κB plays an important role in expression of IL-8 in *H. pylori*-infected gastric inflammation.[5] Thus in *H. pylori*-infected AGS cells, inhibition of inflammatory mediators NF-κB activation and IL-8 secretion might be a useful therapeutic strategy in chronic gastritis.

Treatment for *H. pylori* stomach infection usually includes two antibiotics clarithromycin (CLR), and amoxicillin (AMX) or metronidazole (MTZ) that help to kill the bacteria, and one proton pump inhibitor which causes the stomach to make less acid and help the ulcer to heal—known as triple therapy. However, the successful rate of *H. pylori* eradication with triple therapy has declined from over 90% to about 60% during the past decade.[6] In addition, increased drug-resistance problems caused by extensive use of antibiotics, adaptive survival mechanisms of pathogenic bacteria to counteract currently used antimicrobials, lack of protection against re-infection, and the necessity of taking a long-term prescription for complete eradication has became a global issue.[7] In addition, the cost of this therapy is significant, and therefore the development of new effective drugs with high selectivity, minimum side effects and low manufacture costs is urgently required.

The economical, facile, and rapid synthesis of chalcones make them attractive as potential drug candidates to fight against *H. pylori*.[8] Although many studies have been reported the wide variety of pharmacological activities of chalcones include anticancer, angiotensin converting enzyme inhibitors, anti-inflammatory, anti-leishmanial,[9] however, there are few reports on chalcones as inhibitors of *H. pylori*-induced inflammation.[8] Chalcones obtained from the Claisen–Schmidt condensation reaction between the corresponding aromatic aldehydes and acetophenones have a similar structure to sofalcone, an oral gastrointestinal medication. Their structural differences exist only in the substituent pattern of two aromatic rings. We have previously reported the 3′,4′,5′-trimethoxychalcone analogues (TMCs) as potent dual inhibitors of nitric oxide production and tumor cell proliferation.[10] Encouraged by the aforementioned information and in an attempt to find more potent compounds against *H. pylori*-induced gastric inflammation, herein we report our results which lead to the identification of potent growth inhibitors of reference as well as multidrug-resistant

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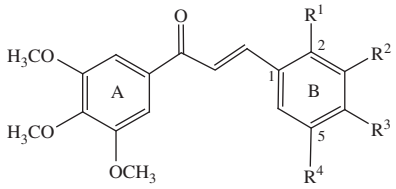
strains of *H. pylori*; the inhibition of *H. pylori* adhesion and invasion to AGS cells; and suppression of inflammatory mediators NF-κB activation and IL-8 release in *H. pylori*-infected AGS cells.

TMCs **1**–**23** are synthesized in accordance with our established synthetic route (Table 1), and the details for synthesis and characterization have been described in our previous report.[10] In this letter, TMCs **1**–**23** at a concentration of 0.5 mg/ml were evaluated for their growth inhibitory effect against *H. pylori* reference strain Hp 26695 by the disk agar diffusion method.[11] As shown in Table 1, tested compounds showed a wide range of potency with inhibition zone in a range from 0 to 26 mm. The chalcone **7**, which substituted with a methoxy group in position R^2 and a hydroxyl group in R^3 showed the most potent inhibitory effect against *H. pylori* growth with inhibition zone of 26 mm. This inhibitory effect was conserved when these substitutions reversed as in **14** (20 mm). A similar inhibitory trend as in **7** was observed for chalcones bearing 3,4,5-tri-OMe (**3**), 3,4-di-OH (**11**), 4-OH (**6**), 4-OMe (**13**), and 4-CHO (**23**) in ring-B with inhibition zone of 23, 23, 22, 22, and 22 mm, respectively. Furthermore, the TMCs substituted in ring-B with 2,3-di-OMe (**1**), 3-OH, 4-OMe (**14**), 3-OMe (**15**), and 4-NO_2 (**18**) also displayed potent anti-*H. pylori* activity with a inhibition zone between 18 and 21 mm (Table 1). At a concentration of 0.05 mg/ml, the commonly used antibiotic drugs CLR and AMX for treatment of *H. pylori* infection inhibited the bacterial growth with inhibition zone of 21 mm and 14 mm, respectively. Previous studies in the infection model have revealed that *H. pylori* could survive and replicate in the intracellular niche.[12] The need for an antibiotic with intracellular activity reflects the ability of *H. pylori* to evade antibiotic treatment. Based on the previous kinetics of antibiotics AMX and MTZ were not administered the bactericide effect on intracellular *H. pylori*.[13,14] Although CLR is known to concentrate in the cells and has antibacterial activity in both extra- or intracellularly, however, *H. pylori* can easily develop resistance during exposure to CLR.[15] Additionally, CLR is more expensive (around sevenfold cost) as compared with AMX and MTZ in the clinical therapy of *H. pylori*-related gastrointestinal diseases. Thus, we used lower concentration of CLR and AMX at 0.05 mg/ml, and higher concentration of MTZ at 0.8 mg/ml as the positive control, and the tested TMCs **1–23** were at 10-fold concentration of authentic antibiotics. In this letter, the TMCs **1–23** were examined at 10-fold lower concentration as compared with positive control (0.5 mg/ml vs 0.05 mg/ml), that is, generally used in the evaluation of chemical compounds to examine the *H. pylori* growth inhibition. At the experimental concentrations, chalcones **3**, **6**, **7**, **11**, **13**, and **23** exhibited potent inhibitory effect than the three positive controls, while **4**, **5**, **10**, **12**, **17**, **19**, and **21** showed nearly similar inhibition zone range 11–16 mm to that of AMX (Table 1). In contrast, the TMCs **8**, **9**, **20**, and **22** had negligible effect on *H. pylori* growth inhibition. Thus, these data indicated that TMCs may useful to develop potential growth inhibitors against *H. pylori*.

Next, the effect of TMCs **1**–**23** on gastric epithelial cell (AGS cells) viability was analyzed using MTT method.[16] At the maximum concentration tested for *H. pylori* growth inhibition (0.5 mg/ml), although some of the TMCs exhibited cytotoxic effect, however, the potent chalcones 2,3,3′,4′,5′-pentamethoxychalcone (**1**), **7**, and 3′,4,4′,5′-tetramethoxychalcone (**13**) had negligible effect on AGS cell viability. Therefore, the three selective and potent compounds **1**, **7**, and **13** were chosen for the further experiments. We subsequently determined the minimum bactericidal concentration (MBC) for the chalcones **1**, **7**, **13** and three standard antibiotics against *H. pylori* reference strain Hp 26695, and multidrug-resistant strains v1254 and v1354.[17] As shown in Table 2, the chalcone **7** showed the highest activity with MBC of 12.5 μM against the reference and multidrug-resistant strains of *H. pylori*. Noticeably, this chalcone has comparable inhibitory effect against the reference as well as multidrug-resistant strains. The MBC of chalcone **1** against Hp 26695, v1254, and v1354 strains was 25.0, 50.0, and 25.0 μM, respectively, while the same for **13** was 100 μM against each strain (Table 2). Noticeably, the MBCs of chalcones **1** and **7** were comparable with standard antibiotics CLR and MTZ (Table 2). These results suggest that chalcones **1**, **7**, and **13** were not only having the bactericide activity against the antibiotic-susceptible *H. pylori* strain but also inhibited the multidrug-resistant strains. The results of this study was in parallel to our previous report,[10] that chalcones **1**, **7**, and **13** are potent inhibitors of nitric oxide produc-

Table 1
Chemical structures of 3′,4′,5′-trimethoxychalcone analogues **1**–**23**, and their inhibitory effect on *H. pylori* growth using disk agar diffusion test



Compound	R^1	R^2	R^3	R^4	Inhibition zone[a] (mm)
1	OMe	OMe	H	H	21
2	H	OMe	OMe	H	16
3	H	OMe	OMe	OMe	23
4	H	H	$N(CH_3)_2$	H	12
5	OMe	H	OMe	OMe	16
6	H	H	OH	H	22
7	H	OMe	OH	H	26
8	OMe	H	OMe	H	0
9	OH	H	H	OH	0
10	OH	OMe	H	H	13
11	H	OH	OH	H	23
12	H	OH	H	H	11
13	H	H	OMe	H	22
14	H	OH	OMe	H	20
15	H	OMe	H	H	19
16	H	H	F	H	17
17	H	H	Br	H	11
18	H	H	NO_2	H	18
19	H	H	Me	H	15
20	OH	H	H	NO_2	0
21	CHO	H	H	H	14
22	H	CHO	H	H	0
23	H	H	CHO	H	22
AMX					14
CLR					21
MTZ					7

AMX: amoxicillin, CLR: clarithromycin, and MTZ: metronidazole.
[a] Concentration of compounds **1**–**23** was at 0.5 mg/ml and AMX at 0.05 mg/ml, CLR at 0.05 mg/ml and MTZ at 0.8 mg/ml. DMSO was used as a negative control.

Table 2
Minimum bactericidal concentration (MBC) of chalcones (**1**, **7** and **13**) and standard antimicrobial agents (AMX, CLR and MTZ) against *H. pylori* reference strain Hp 26695, and multidrug-resistant strains v1254 and v1354

Chalcone or antibiotic[a]	MBC[b]		
	Hp 26695[c]	v1254[c]	v1354[c]
1	25.0	50.0	25.0
7	12.5	12.5	12.5
13	100.0	100.0	100.0
AMX	12.5	12.5	12.5
CLR	1.6	50.0	25.0
MTZ	25.0	400.0	800.0

[a] AMX: amoxicillin, CLR: clarithromycin, and MTZ: metronidazole.
[b] Concentrations of chalcones **1**, **7**, and **13**, and standard antimicrobial agents were in μM.
[c] Hp 26695 was a reference strain. Strains v1254 and v1354 were clinical isolates, which show resistant to both metronidazole and clarithromycin

tion from peritoneal macrophage cells.[10] In addition, the potential of chalcones **7** to eradicate *H. pylori* is also supported by previously reported antibacterial activity against other bacteria strains, including *Staphylococcus aureus* and *Escherichia coli*.[18] To the best of our knowledge, this was the first report to demonstrate that the TMCs could significantly inhibited the *H. pylori*.

H. pylori adhesion to gastric mucosa of epithelial cells is an important initial event in the pathogenesis of gastric malignancies, and inhibition of bacterial adhesion may prevent certain pathogen-related diseases, such as duodenal ulcer and gastric cancer.[1] In this letter, chalcones **1**, **7**, and **13** were further assayed with regard to their ability to inhibit the adhesion of *H. pylori* to AGS cells.[11] As shown in Figure 1A, chalcone **7** displayed most potent anti-adhesion activity with a reduction of 88.2% and 98.8%, at concentrations of 2.5 and 5.0 μM, respectively. The chalcone **1** has such anti-adhesion effect with a reduction of 66.6%, 73.3%, and 99.9% at 2.5, 5.0, and 10.0 μM, respectively. On the other hand, it is interesting to observe that chalcone **13** had a minor inhibitory effect at relatively higher concentrations 5.0 and 10.0 μM, compared to the control (Fig. 1A). In addition, we further assayed chalcones **1**, **7**, and **13** abilities to inhibit the invasion of *H. pylori* to AGS cells.[11] As expected, chalcone **7** showed the highest inhibition of *H. pylori* invasion into AGS cells by 57.6%, 91.0%, and 100% at the concentrations of 2.5 μm, 5.0 μm, and 10.0 μM, respectively (Fig. 1B). Comparing the effect of chalcone **7** on *H. pylori* invasion into AGS cells, we observed a similar pattern of inhibitory effects on adhesion of bacteria to AGS cells. A low concentration of 2.5 μM, compounds **1** and **13** did not significantly affect *H. pylori* invasion into AGS cells, however, treatment with higher concentrations of 5.0 and 10.0 μM resulted a moderate inhibition of bacterial invasion into AGS cells (Fig. 1B). When treated with AMX and CLR at a concentration of 2.5 μM, the inhibitory effects of *H. pylori* invasion and adhesion to AGS cells were more than 90%, while only slight inhibitory effect (around 20%) of MTZ was observed (Fig. 1). DMSO vehicle had no effect on adhesion and invasion assays. Results from this study demonstrated that chalcones **7** followed by **1** have the potential to inhibit the adhesion and invasion of *H. pylori* into AGS cells.

We further examine the effects of chalcones **1**, **7**, and **13** on *H. pylori*-induced inflammation by the determination inflammatory mediators production NF-κB activation and IL-8 secretion from the *H. pylori*-infected AGS cells.[17] We transfected AGS cells with an NF-κB reporter construct (NF-κB-luc), and used to determine luciferase expression following treatment of tested compounds in a concentration range between 2.5 and 10 μM. Treatment of transfected AGS cells with chalcones **1**, **7**, and **13** and *H. pylori* infection led to a dose-dependent reduction in the stimulation of luciferase activity (Fig. 2A). The chalcone **7** displayed a dramatic luciferase inhibitory activity by 86.1%, 98.6%, and 99.9% at concentrations of 2.5, 5.0, and 10.0 μM, respectively, compared with that of control DMSO. However, three antimicrobial agents had no effect on induction of NF-κB activity assay at a concentration of 2.5 μM (Fig. 2A). At the concentrations of 2.5, 5.0, and 10.0 μM, chalcones

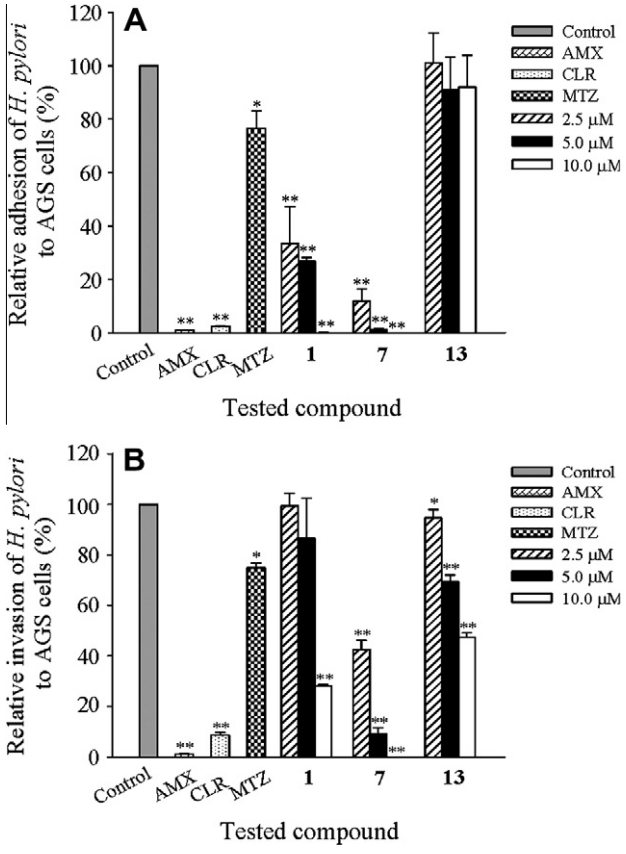


Figure 1. Effect of 3′,4′,5′-trimethoxychalcone analogues **1**, **7**, and **13** on *H. pylori* adhesion (A), and invasion (B), to human gastric epithelial cells. The bacteria to gastric epithelial cells that was treated or untreated with chalcones **1**, **7**, and **13** prior infection with *H. pylori* at a MOI of 50 for 6 h. The standard antibiotics amoxicillin (AMX), clarithromycin (CLR), and metronidazole (MTZ) were used as positive control at a concentration of 2.5 μM. Each experiment was shown represent mean values ± SD of at least six independent experiments. Statistical significance was calculated using Student's *t*-test when compared to DMSO treated cells. *P*-values were determined by Student's *t*-test. The significant difference was set at $^{*}P$ <0.05; $^{**}P$ <0.01.

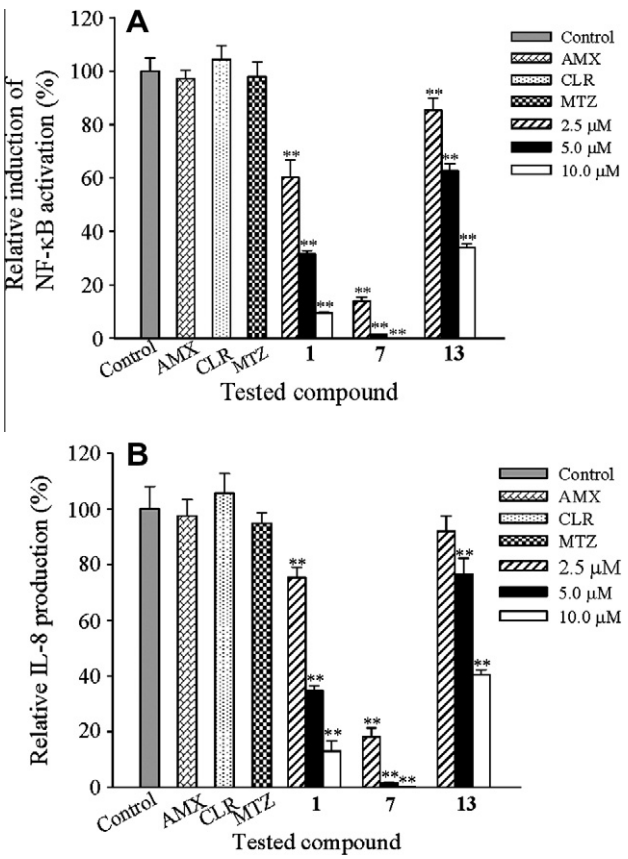


Figure 2. Inhibitory effects of 3′,4′,5′-trimethoxychalcone analogues on *H. pylori*-infected AGS cells inflammation. Induction of NF-κB activation (A) and IL-8 secretion (B) were inhibited by treatment of chalcones **1**, **7**, and **13**. DMSO or the standard antibiotics amoxicillin (AMX), clarithromycin (CLR), and metronidazole (MTZ) at a concentration of 2.5 μM were used as positive control. The luciferase activity and IL-8 levels in supernatants was determined (see Supplementary data). Results are shown by mean values ± SD from at least three independent experiments. Statistical significance was evaluated using Student's *t*-test with $^{*}P$ <0.05; $^{**}P$ <0.01.

1 and **13** reduced the luciferase activity in the range 39.8–90.6% and 14.7–66.0%, respectively (Fig. 2A).

To identify the mechanism whereby chalcones **1**, **7**, and **13** inhibited NF-κB activation, we further determined the effect of these compounds on IL-8 production from *H. pylori*-infected AGS cells.[17] *H. pylori*-induced IL-8 expression is NF-κB dependent.[19] The production of IL-8 in *H. pylori*-infected AGS cells was measured in the presence or absence of chalcones **1**, **7**, and **13**. In consistent with NF-κB activity assay, IL-8 production in AGS cells infected with *H. pylori* was dramatically reduced when cells were pre-treated with chalcone **7** (Fig. 2B). Even pretreatment of 2.5 μM inhibited IL-8 production comparable with that of DMSO or three positive controls treated cells, and the inhibition reached from 81.9% to 99.7% at concentration of 10.0 μM. On the other hand, chalcones **1** and **13** have relative lower IL-8 inhibitory effect with inhibition of 24.6%, 65.2%, and 87.0% and; 8.0, 23.4, and 59.4%, at concentrations of 2.5, 5.0, and 10.0 μM, respectively (Fig. 2B). The results from our study indicated that reduction of IL-8 by chalcones **1**, **7**, and **13** pretreatment might contribute to attenuate the NF-κB activity by AGS cells in response to *H. pylori* infection.[2] To the best of our knowledge, we demonstrate here for the first time on chalcones **1**, **7**, and **13** inhibited *H. pylori*-associated inflammation in AGS cells.

In conclusion, we have screened 23 3′,4′,5′-trimethoxychalcone analogues against *H. pylori*, and found some of them displayed potent anti-*H. pylori* activity. The chalcones bearing 2,3-di-OMe (**1**), 3-OMe, 4-OH (**7**), and 4-OMe (**13**) in ring-B displayed potent growth inhibition on reference as well as multidrug-resistant strains of *H. pylori*. Additionally, the chalcones **1**, **7**, and **13** inhibited the adhesion and invasion of *H. pylori* to AGS cells. In addition these three chalcones diminish the activation of NF-κB, and secretion of IL-8 in *H. pylori*-infected AGS cells. Therefore, the most potential of chalcone **7** to inhibit *H. pylori* and its associated inflammation, and the chemical nature of this low-molecular-mass compound that translates to lower cost of synthesis compared to cationic antibacterial peptides suggest that it has potential to develop new therapeutic drug for the prevention of *H. pylori*-related diseases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.094.

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