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ORIGINAL ARTICLE

A chalcone with potent inhibiting activity against biofilm formation by nontypeable *Haemophilus influenzae*

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ABSTRACT

Nontypeable *Haemophilus influenzae* (NTHi), an important human respiratory pathogen, frequently causes biofilm infections. Currently, resistance of bacteria within the biofilm to conventional antimicrobials poses a major obstacle to effective medical treatment on a global scale. Novel agents that are effective against NTHi biofilm are therefore urgently required. In this study, a series of natural and synthetic chalcones with various chemical substituents were evaluated *in vitro* for their antibiofilm activities against strong biofilm-forming strains of NTHi. Of the test chalcones, 3-hydroxychalcone (chalcone 8) exhibited the most potent inhibitory activity, its mean minimum biofilm inhibitory concentration ($MBIC_{50}$) being 16 μg/mL (71.35 μM), or approximately sixfold more active than the reference drug, azithromycin ($MBIC_{50}$ 419.68 μM). The inhibitory activity of chalcone 8, which is a chemically modified chalcone, appeared to be superior to those of the natural chalcones tested. Significantly, chalcone 8 inhibited biofilm formation by all studied NTHi strains, indicating that the antibiofilm activities of this compound occur across multiple strong-biofilm forming NTHi isolates of different clinical origins. According to antimicrobial and growth curve assays, chalcone 8 at concentrations that decreased biofilm formation did not affect growth of NTHi, suggesting the biofilm inhibitory effect of chalcone 8 is non-antimicrobial. In terms of structure–activity relationship, the possible substituent on the chalcone backbone required for antibiofilm activity is discussed. These findings indicate that 3-hydroxychalcone (chalcone 8) has powerful antibiofilm activity and suggest the potential application of chalcone 8 as a new therapeutic agent for control of NTHi biofilm-associated infections.

Key words biofilm formation, chalcone, nontypeable Haemophilus influenzae, strong-biofilm producing strain.

Nontypeable *Haemophilus influenzae* is one of the commonest human respiratory pathogens that cause a spectrum of mild (otitis media, sinusitis) to severe (bronchitis, chronic obstructive pulmonary disease, septicemia, meningitis) illnesses. Substantial morbidity, mortality and socioeconomic burden caused by this microorganism are of enormous concern globally (1–3). There is both *in vitro* and *in vivo* evidence that NTHi forms biofilm (4, 5). Biofilm infections are notoriously difficult to eradicate because of their resistance to antibiotics and host immune-mediated clearance (6–8). Communal bacteria in a biofilm are upward of 1000-times more resistant to conventional antibiotic treatment than the same organism growing planktonically (9). Usual clinical dosages of antibiotics may therefore fail to adequately clear infections, allowing bacteria to recover,

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List of Abbreviations: BFI, biofilm forming index; HTM, haemophilus test medium; MBIC, minimum biofilm inhibitory concentration; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NTHi, nontypeable *Haemophilus influenzae*.

persist and spread (10). Increased concentrations or multiple combinations of antibiotics have been suggested for treating biofilm-related infections (11, 12). However, excessive or improper uses of antibiotics may result in development of resistance (13), leading to even greater difficulties in disease treatment. It is therefore important to discover and develop novel, effective antibiofilm agents to improve treatment of NTHi biofilm-mediated infections.

Chalcones are open-chain flavonoids with common skeleton of 1,3-diaryl-2-propen-1-one. Both naturally occurring and synthetic chalcones show an array of biological activities, including anti-bacterial, anti-viral, anti-cancer, anti-oxidant, anti-inflammatory and immunosuppressive activities (14, 15). Because of the simplicity of their chemical structures and their vast variety of actions, compounds with chalcone-based structures are currently receiving a great deal of attention. Their wide range of biological properties is generally attributed to the α,β-unsaturated keto moiety. Chemical substitutions of the two aryl rings is also a subject of interest because it leads to useful structure–activity relationship conclusions and thus facilitates synthesis of pharmacologically active chalcones (14). In recent years, chemically modified chalcone-based compounds have been shown to reduce formation of marine bacterial biofilm (16). Moreover, synthetic chalcones reportedly have strong inhibitory activities against biofilms of bacteria that are pathogenic to humans (17). This raises the possibility of developing chalcone-based compounds as antibiofilm agents.

To our knowledge, there are no reported experimental data regarding the antibiofilm activity of chalcones against NTHi; we therefore investigated these in this study. We quantitatively and qualitatively explored the activities of a series of naturally occurring and synthetic chalcones against biofilm formation by strong-biofilm producing NTHi isolates of clinical origin.

MATERIALS AND METHODS

Chalcones

Chalcones **1** and **2** (Fig. 1) were isolated from the flowers of *Butea monosperma* as described previously (18). Chalcones **3**−**13** (Fig. 1) were synthesized by the Claisen–Schmidt condensation reaction (19) of substituted acetophenone (8.3 mmol) with substituted benzaldehyde (8.3 mmol) in the presence of an aqueous solution of 50% KOH (25 mL) in ethanol (25 mL) at room temperature for 6–12 hr. The reaction mixture was then acidified with dilute HCl and the resulting precipitates filtered, washed with water, dried, and further purified by recrystallization from EtOH−H_2O (1:1 v/v) or by silica gel column chromatography, using *n*-hexane−EtOAc as eluent, to produce a high yield (56–92%) of chalcones (Fig. S1). All structures were confirmed by comparison of spectroscopic data and elemental analysis with those previously reported. Chalcones **4** and **5** have been described previously by Karki *et al.* (20), chalcones **6**–**8** and **13** have been reported by Yadav *et al.* (21), chalcones **9** and **12** have been synthesized by Tatsuzaki *et al.* (22), and chalcones **3**, **10** and **11** have been synthesized by Watanabe and Imazawa (23), Arty *et al.* (24) and Hasan *et al.* (25), respectively.

Bacterial strains and culture conditions

Nontypeable *Haemophilus influenzae* NU7, NU38 and NU48 were used in this study. These bacteria were isolated from different specimen types (sputum, pus and blood) from patients admitted to Buddhachinaraj Hospital (Phitsanulok, Thailand), identified by standard microbiological and biochemical procedures, and PCR-serotyped as described previously (26). All isolates were grown on BHI agar or broth (Oxoid, Basingstoke, UK) supplemented with nicotinamide adenine dinucleotide (Becton Dickinson, MD, USA; 10 μg/mL) and hemin (Becton Dickinson; 10 μg/mL) at 37 °C in an atmosphere containing 5% CO_2.

Microtiter biofilm formation assay

The formation of biofilm by NTHi was evaluated using a method based on that reported previously (4) with some modifications. Overnight cultures of NTHi were washed, diluted 1:200 in fresh HTM and 200 μL was inoculated into the wells of flat-bottomed 96-well polystyrene microtiter plates (Nunc, Roskilde, Denmark). The plates were incubated at 37 °C under 5% CO_2 for 18 hr. Growth was assessed by measuring the OD at 600 nm using a microplate reader (Labsystems iEM Reader MF; Vantaa, Finland) prior to biofilm quantitation. The biofilms were then quantitated by staining the adherent cells with 1% (w/v) aqueous solution of crystal violet for 15 min at room temperature. The dye incorporated by the adherent cells was solubilized in 200 μL of 95% ethanol and the OD measured at 540 nm. The BFI was used to express the amount of biofilm formed by NTHi. It was calculated using the formula (AB − CW)/G, in which AB is the OD of the stained attached microorganisms, CW is the OD of the stained control wells containing microorganisms-free medium only and G is the OD of the cells growth in suspended culture. Biofilm formation was classified semi-quantitatively (strong, moderate, weak or none) based on the BFI readings (27). All NTHi isolates were tested in two independent experiments, with quadruplicate determinations in each.

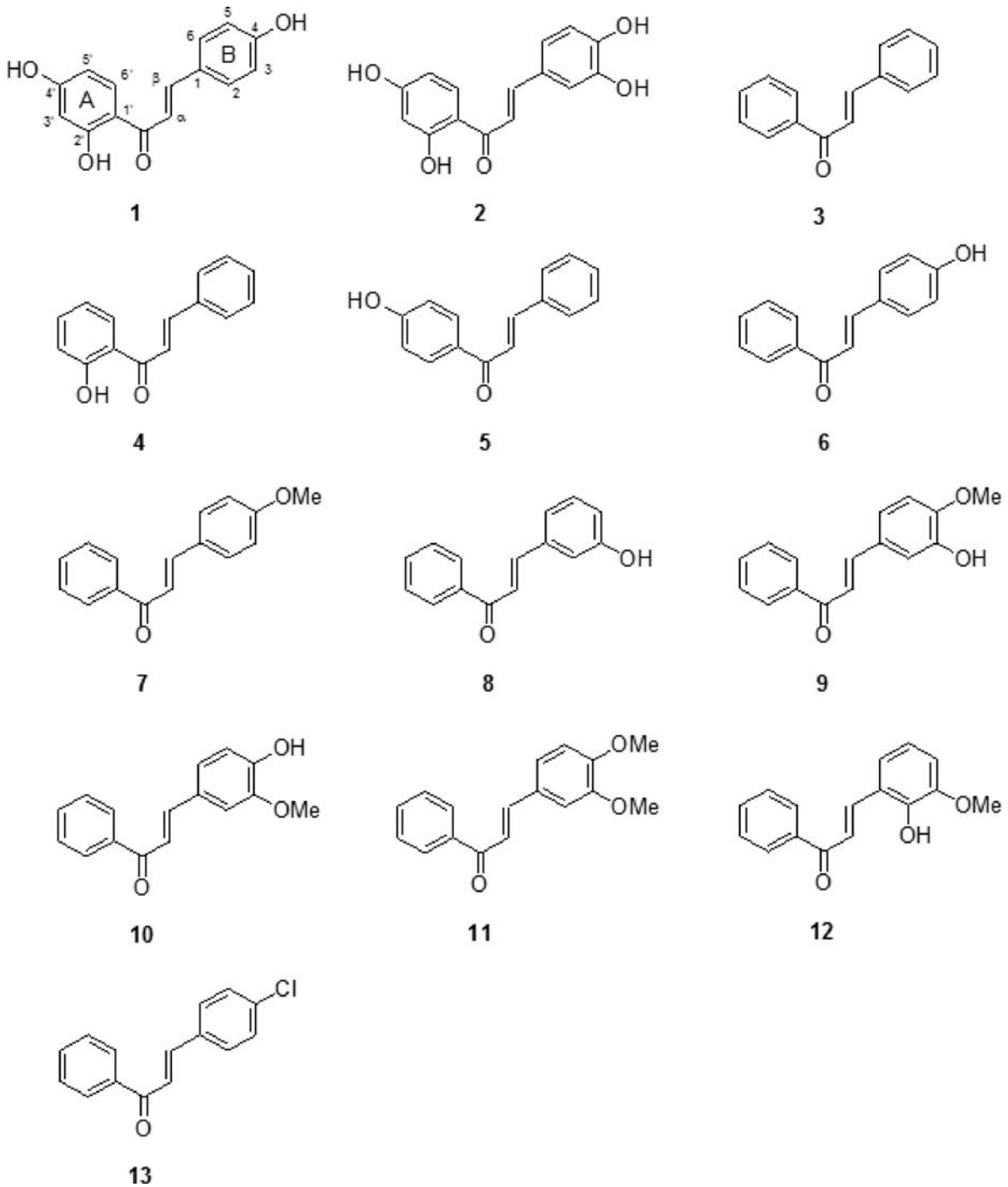

Fig. 1. Chemical structures of the test chalcones.

Biofilm susceptibility assay

The effects of chalcones on NTHi biofilm formation were examined by a microdilution method. Twofold serial dilutions of chalcone were prepared with HTM in the wells of flat-bottomed 96-well microtiter plates (Nunc). Final concentrations of the test chalcones ranged from 0.25 to 512 μg/mL. Azithromycin (Fluka, Buchs SG, Switzerland) was included in the assay because this antibiotic reportedly reduces *H. influenzae* biofilms (28, 29). Aliquots of the bacterial suspension at a final concentration of 5×10^5 CFU/mL were added to

the microtiter wells. Culture without the test compound and medium alone were used as non-treated and blank controls, respectively. Following incubation at 37 °C under 5% CO_2 for 18 hr, the medium containing nonadherent cells was decanted and the wells washed with sterile deionized water. Biofilms were quantitated by a microtiter biofilm formation assay as described above. The percentage of inhibition was calculated using the following equation ([OD_{540} of non-treated control $-$ OD_{540} of the test]/OD_{540} of non-treated control) $\times$ 100. The $MBIC_{50}$ was defined as the lowest concentration of the test compound that resulted in $\geq$50% inhibition of biofilm formation (30).

Time-dependent assay

A time-dependent study was performed using a microdilution method. NTHi strains (5×10^5 CFU/mL) and chalcone **8** (at the concentration equal to $MBIC_{50}$) were incubated in HTM at 37 °C under 5% CO_2. At 0, 1, 2, 4, 8, 12, 18 and 24 hr, biofilm formation was quantitated as described above for the biofilm susceptibility assay.

Antimicrobial assay

The antimicrobial effect of chalcone **8** was assessed by determining the MIC by a broth microdilution method. Twofold serial dilutions of the test chalcone at final concentrations ranging from 0.25 to 512 μg/mL were prepared in HTM in 96-well microtiter plates (Nunc). Adjusted bacterial inoculum (5×10^5 CFU/mL, final concentration) was then seeded. The medium without the test compound served as a non-treated control. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 24 hr and the total bacterial growth determined by OD measurement at 600 nm. The MIC was recorded as the concentration in the first well in which no visible bacterial growth was noted relative to the growth of the non-treated control.

Growth curve assay

The effects of chalcone **8** on planktonic growth of NTHi were examined in 125 mL flasks containing 25 mL of a bacterial culture (5×10^5 CFU/mL, final concentration) and the test chalcone (at the concentration equal to $MBIC_{50}$). The culture without the test compound was used as a bacterial growth control. The cultures were grown at 37 °C under 5% CO_2 and the OD_{600} nm of 1 mL aliquots recorded at 0, 4, 8, 18 and 24 hr.

Scanning electron microscopy

Scanning electron microscopy was used to visually confirm the effect of chalcone **8** on NTHi biofilm formation. Biofilms grown on glass coverslips for 18 hr in the presence or absence of the test chalcone (at the concentration equal to $MBIC_{50}$) were rinsed with PBS (pH 7.2) and fixed with 2.5% glutaraldehyde in PBS (pH 7.2) for 60 min at room temperature. After being carefully washed twice with PBS (pH 7.2), the samples were dehydrated through a graded series of ethanol concentrations (30–100%) and dried with hexamethylsilazine (Sigma−Aldrich, St Louis, MO, USA). Dried samples were sputtered with palladium/gold and then viewed with a Leo 1455VP scanning electron microscope (LEO Electron Microscopy, Cambridge, UK) in high-vacuum mode at 20 kV.

Cytotoxic evaluation

The cytotoxic effect of chalcone **8** against human cells was evaluated by MTT assay as previously described (31). Briefly, peripheral blood mononuclear cells isolated from healthy adults were cultured under 5% CO_2 and 37 °C in RPMI-1640 medium (PAA, Pasching, Austria) supplemented with 10% (v/v) FBS (Gibco, Gaithersburg, MD, USA), 0.01 M HEPES pH 7.4, 2 mM L-glutamine (PAA), 100 U/mL penicillin and 100 μg/mL streptomycin (PAA). Cells were plated in 96-well plates and then exposed for 48 hr to chalcone **8** at concentrations ranging from 0.25 to 32 μg/mL, with untreated cells serving as control. Cell viability was quantified by MTT colorimetric assay. Absorbance was measured at 540 nm and the results expressed as the percentage of cell viability compared with the control.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical significance was assessed using Student's *t*-test. All statistical analyses were performed with SPSS version 11.5 (SPSS, Chicago, IL, USA). Differences were considered to be significant at $P < 0.05$.

RESULTS

Biofilm formation by NTHi

The ability of clinical NTHi isolates NU7, NU38 and NU48 to form biofilm was determined on the surface of polystyrene wells and expressed as BFI. The BFI values for NU7, NU38 and NU48 were 2.07 ± 0.27 (biofilm OD, 0.782; growth OD, 0.377), 2.54 ± 0.15 (biofilm OD, 0.844; growth OD, 0.333) and 2.01 ± 0.78 (biofilm OD, 0.435; growth OD, 0.217), respectively. According to the semi-quantitative classification of biofilm formation, any bacteria with BFI values $\geq$1.10 are defined as strong biofilm producers (27). Thus, the clinical isolates of NTHi in this study were all strong-biofilm producing strains.

Effects of chalcones on NTHi biofilm formation

Initially, the natural chalcones **1** and **2** were tested for their inhibitory activities against biofilm formed by clinical NTHi isolates NU7, NU38 and NU48. We found that these natural compounds inhibit formation of NTHi biofilm (mean $MBIC_{50}$ 166.63 and 254.82 μM, respectively) and are more potent inhibitors than azithromycin (mean $MBIC_{50}$ 419.68 μM), suggesting that chalcones have potential as antibiofilm agents for NTHi (Table 1). Further to these observations, we postulated that chemical modifications of chalcones might strengthen their inhibitory activities. Therefore, compounds with various chemical substituents introduced to the two aryl rings of chalcone backbone were synthesized and their antibiofilm activities assessed. Modification of NTHi biofilm formation to varying degrees was observed in the presence of these synthetic chalcones. Among the synthetic chalcones evaluated, chalcone **8** was the most effective. This compound clearly showed concentration-dependent inhibitory effects on *in vitro* biofilm formation by all NTHi studied, even with the strongest-biofilm producing strain, NU38 (Fig. 2). Consistent with when there is ≥50% inhibition of biofilm formation, chalcone **8** demonstrated the most distinct and potent inhibitory effect, the lowest mean $MBIC_{50}$ value being 16 μg/mL (71.35 μM; Table 1). At this concentration, the biofilm inhibitory activity of chalcone **8** was time-dependent within 24 hr of incubation. Within this time range, obviously reduced biofilm formation by NU38 was seen as early as 4 hr after beginning incubation, whereas reduction of biofilm produced by NTHi strains NU7 and NU48 was observed 8 and 12 hr, respectively, after exposure (results not shown). Inhibitory activities were less pronounced for chalcones **5**, **6** and **9;** little or no inhibition was found in the presence of chalcones **3**, **4**, **7** and **10**–**13** (Table 1). It is interesting to note that chalcone **8** exerted several-fold stronger antibiofilm activity against NTHi than both the tested natural chalcones and azithromycin. Accordingly, chalcone **8** was chosen for further studies.

Effect of chalcone on growth of NTHi

To determine whether biofilm inhibition by chalcone **8** was attributable to a growth-inhibitory effect, growth of NTHi cultures in the presence or not of such a compound was monitored by measuring turbidity. Growth of all NTHi strains in the presence of chalcone **8** at concentrations ranging from 0.25 to 128 μg/mL was comparable to that of non-treated control cultures, although bacterial growth was decreased at 256 and 512 μg/mL (data not shown). Consistent with the growth curve assay, it was also found that all studied NTHi cultures continued to grow after addition of chalcone **8:** the growth curves appeared to be similar to those of the non-treated control cultures after prolonged exposure to the test compound (Fig. 3). Thus, the results of antimicrobial and growth curve assays suggested that NTHi biofilm inhibition by chalcone **8** is not caused by inhibition of growth.

Table 1. Minimum biofilm inhibitory concentrations ($MBIC_{50}$) of chalcones against nontypeable *H. influenzae* NU7, NU38 and NU48

	$MBIC_{50}$[†]			
	Range		Mean	
Compound	μg/mL	μM	μg/mL	μM
1	32–64	124.97–249.93	42.67	166.63
2	16–128	58.81–470.47	69.33	254.82
3	128–512	614.62–2.46×10^3	298.67	1.43×10^3
4	16–512	71.35–2.28×10^3	218.67	975.12
5	16–128	71.35–570.79	69.33	309.16
6	16–256	71.35–1.14×10^3	101.33	451.86
7	64 to >512	268.59 to >2.15×10^3	>512	>2.15×10^3
8	16–16	71.35–71.35	16	71.35
9	64–128	251.69–503.38	85.33	335.58
10	32–512	125.85–2.10×10^3	202.67	797.03
11	256–256	954.12–954.12	256	954.12
12	32–512	125.85–2.01×10^3	202.67	797.04
13	512 to >512	2.11×10^3 to >2.11×10^3	>512	>2.11×10^3
Azithromycin	256–512	341.80–683.60	314.33	419.68

[†]Minimum biofilm concentration of the chalcone tested that showed >50% inhibition of biofilm formation.

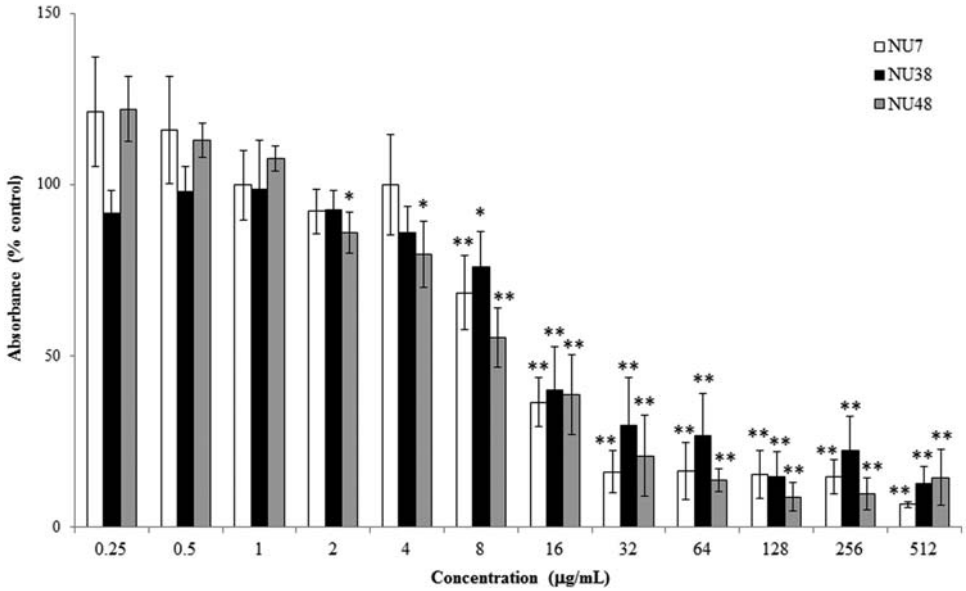

Fig. 2. Effects of chalcone **8** on biofilm formation by nontypeable *H. influenzae*. The bacteria (NU7, NU38 and NU48) were incubated in 96-well microtiter plates with different concentrations of the test compound and without a test compound (non-treated control). After incubation at 37 °C under 5% CO_2, the adherent bacteria were stained with crystal violet and the OD at 540 nm determined to assess the amount of biofilm formed. The results are expressed as percentages of biofilm formed by the non-treated control. Data represent the mean ± SEM of four independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with the control without test compound.

Effect of chalcone 8 on NTHi biofilm morphology

Scanning electron microscopy was performed to more closely examine the effect of chalcone **8** on NTHi biofilm morphology; scanning electron photographs are shown in Figure 4. With the non-treated control, thick and relatively homogenous biofilm was formed on the glass coverslips. Compared to the non-treated control, treatment with chalcone **8** resulted in greatly reduced biofilm formation: only a few scattered bacterial microcolonies were seen. These visual findings confirm the inhibitory activity of chalcone **8** on biofilm formation by NTHi.

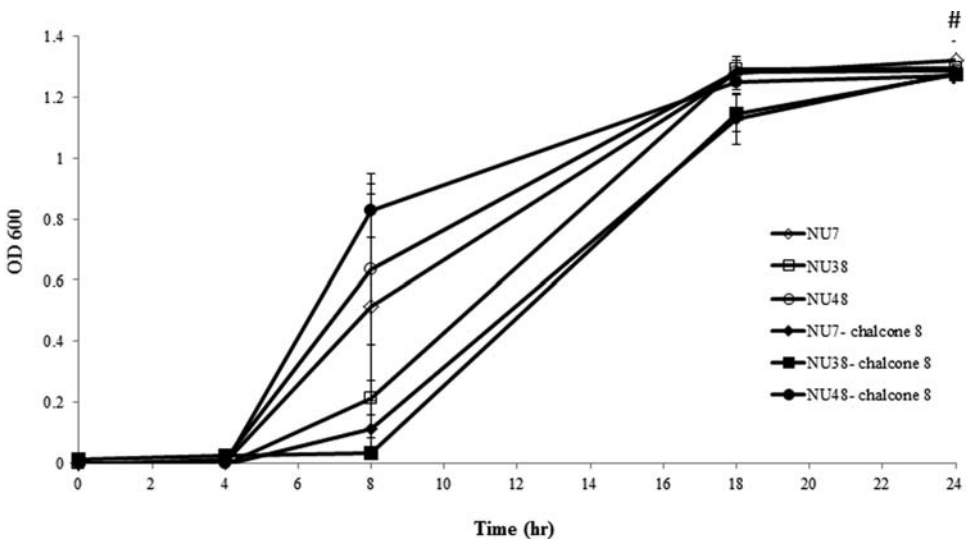

Fig. 3. Effects of chalcone **8** on growth of nontypeable *H. influenzae*. The bacteria (NU7, NU38 and NU48) were grown in HTM with the test chalcone (at the concentration equal to $MBIC_{50}$) at 37 °C, 5% CO_2. A culture without the test compound was used as a bacterial growth control. Bacterial growth was assessed by measuring OD_{600} at the indicated time points. Data represent the mean ± SEM of two independent experiments. #, not statistically different to control ($P > 0.5$).

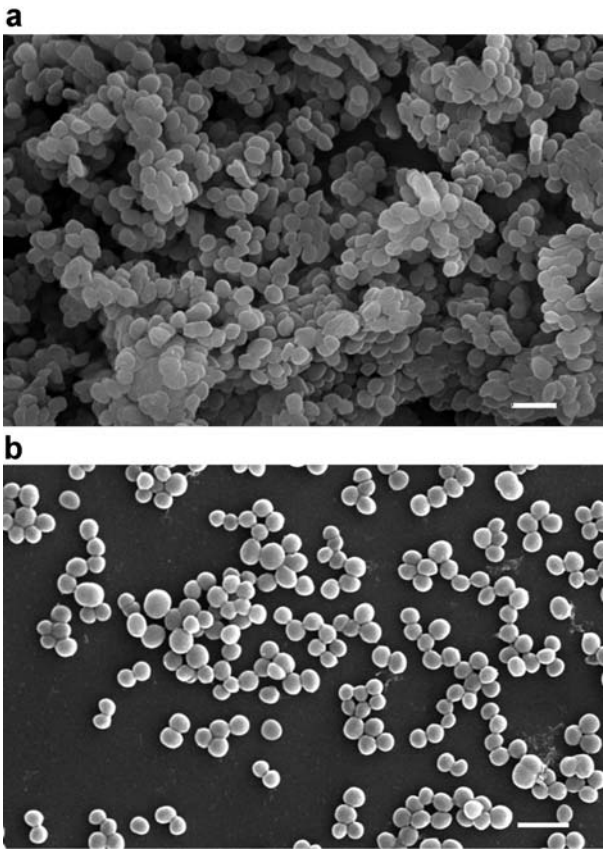

Fig. 4. Effect of chalcone 8 on nontypeable *H. influenzae* biofilm morphology. Biofilm of nontypeable *H. influenzae* was grown on glass coverslips (a) without chalcone or (b) with compound **8** at the concentration equal to $MBIC_{50}$. The images are representative of the results for NU7 (observations for NU38 and NU48 were similar). Magnification, ×3000. Scale bars, 2 μm.

Cytotoxicity of chalcone 8

Regarding future therapeutic applications of chalcone **8**, not only its potent antibiofilm activity, but any toxicity against human cells is very important. Therefore, the cytotoxic effect(s) of chalcone **8** against human peripheral blood mononuclear cells were evaluated. As quantified by MTT assay, chalcone **8** had no effect on the viability of human cells at 48 hr of incubation (Fig. S2). Of particular note, a high percentage (approximately 90%) of cells exposed to chalcone **8** survived at the concentration at which it displayed antibiofilm activity (16 μg/mL), suggesting that chalcone **8** has little cytotoxic effect.

DISCUSSION

The present study investigated the inhibitory activities of chalcone-based compounds on biofilm formation of NTHi. Significant findings of this study are that (i) chalcone **8** is the most active of the chalcones evaluated; (ii) the antibiofilm activity of chalcone **8**, a chemically modified chalcone, is superior to those of the natural chalcones tested; (iii) its excellent inhibitory action was demonstrated both quantitatively and qualitatively in all studied NTHi strains; (iv) chalcone **8** has stronger antibiofilm activity against NTHi than the previously reported azithromycin, with a considerably lower $MBIC_{50}$ value; and (v) at active concentrations, chalcone **8** has little toxic effect on human cells.

In this study, we found that chemical modification of the chalcone backbone markedly increases inhibitory activity against NTHi biofilms. The NTHi used in this study were clinical isolates collected from various specimen types and were all proven to be strong biofilm producers. Thus, we demonstrated that the antibiofilm activity of the chemically modified chalcone, chalcone **8,** occurs across multiple strong biofilm-forming NTHi isolates of different clinical origins. These findings indicate that chalcone **8** has powerful antibiofilm activity and convincingly suggest such a compound has the potential to improve treatment of NTHi biofilm-associated diseases in various clinical settings.

Remarkably, growth of NTHi was unaffected by chalcone **8** at concentrations that decreased biofilm formation, indicating the biofilm inhibitory effect of this compound is non-antimicrobial. In the biofilm susceptibility assay, we added the test compound to polystyrene wells of microtiter plates at the same time as bacterial cells; time-dependent assays showed that biofilm formation decreased as early as 4 hr after incubation. The initial adherence to and accumulation of microorganisms on surfaces is a critical step in both establishment of infection and the subsequent stage of biofilm formation. It is possible that chalcone **8** interferes with the early step of bacterial adhesion and that formation of biofilm is subsequently inhibited. Our findings are of clinical significance because chalcone **8** may serve as an anti-adherent and antibiofilm agent: its presence would undoubtedly prevent establishment of infection and inhibit biofilm formation. With bacteria that can form biofilm, once attached to a surface, they initially continue to grow into a sessile biofilm, later detaching from it and dispersing into the surroundings to colonize new sites. Thus, the presence of chalcone **8** would limit the spread of biofilm diseases by preventing bacterial cells that have detached from a biofilm colony from colonizing new unoccupied tissues. Targeting the virulence determinants involved in bacterial adherence, rather than inhibiting cellular components necessary for growth or viability, thus provides an alternative strategy for limiting or ameliorating infections (32). Though unconventional, this promising new approach has

received attention during the last decade (17, 33). Natural compounds, in particular the flavonoids, have increasingly been documented to interfere with a number of bacterial virulence factors, including quorum-sensing signal receptors, enzymes and toxins (17, 34, 35). In this study, we did not address the mechanisms by which the open chain flavonoid chalcone **8** inhibits NTHi biofilm formation. Whether this compound interferes with virulence factors or other additional mechanisms required for such inhibitory actions requires further investigation. Nevertheless, because its antibiofilm activity does not interfere with bacterial growth, chalcone **8** will probably exert low selection pressure on NTHi and may therefore contribute minimally to development of problematic antibiotic resistance.

Assessment of antibiofilm activities of a series of compounds with different chemical structures would deliver certain correlations between structure and activity, even if such correlations are not specifically being investigated. Both the natural chalcones **1** and **2** possess hydroxyl groups in both the A and B rings. In order to investigate the effects of oxygen functions in the molecule, we synthesized a chalcone with no hydroxyl group (chalcone **3**) and evaluated it for antibiofilm activity: we found that this compound was much less active than the natural chalcones **1** and **2** (Table 1). The low activity of chalcone **3** against NTHi biofilms indicates that oxygen function is essential for antibiofilm potential. To determine the effects of hydroxyl groups on the A ring, we synthesized chalcones **4** and **5**. Although these two chalcones have stronger antibiofilm activity than the unsubstituted chalcone **3**, they have less activity than the natural chalcones **1** and **2** (Table 1). We therefore synthesized chalcone **6**, a chalcone with a hydroxyl group at position 4 of the B ring. The assay results indicate that, though chalcone **6** is twofold more active than chalcone **4**, it is slightly less active than chalcone **5** and azithromycin (Table 1). Methylation of chalcone **6** to chalcone **7** resulted in sharp decrease in activity. However, chalcone **8**, a chalcone with a 3-hydroxyl group on the B ring, exhibited very strong antibiofilm activity (mean $MBIC_{50}$ 71.35 μM); it was approximately sixfold more active than azithromycin. Placement of an extra methoxyl group at the 4-position of the chalcone **8** to yield chalcone **9** caused decrease in activity. This implies that the hydroxyl group should be free and that the presence of a methoxyl group results in decrease in activity. This was further evident from the low activity of chalcones **10**−**12** (Table 1). The 4-chloro analogue **13** is much less active than its hydroxyl analogue **6**, which indicates the importance of a free hydroxyl group. Taken together, these observations indicate that a free hydroxyl group at position 3 on the B ring is a crucial structural requirement for anti-NTHi biofilm activity.

In conclusion, the present study has clearly demonstrated, for the first time, that 3-hydroxychalcone (chalcone **8**) possesses antibiofilm activity against NTHi. This compound inhibits biofilm formation by clinically strong-biofilm producing isolates of NTHi. Although further studies on the mechanisms of action as well as *in vivo* assessments are still required, our results indicate that chalcone **8** has potential as a novel therapeutic agent that would be of value in treatment of NTHi biofilm-related infections. Future research on eradication of already formed biofilms by chemically modified chalcones is also required. Because flavonoids are often influenced by pH, the effects of pH on stability and antibiofilm activity of the active chalcones should also be further investigated.

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DISCLOSURE

The authors declare they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Synthesis of chalcones **3**−**13**. Chalcones **3**−**13** were synthesized by the Claisen–Schmidt condensation reaction of substituted acetophenone with substituted benzaldehyde in the presence of an aqueous solution of 50% KOH in ethanol at room temperature for 6–12 hr.
Fig. S2. Viability of human peripheral blood mononuclear cells after exposure to chalcone **8**. Cells were cultured in the absence or presence of chalcone **8** at varying concentrations for 48 hr at 37 °C in a humidified CO_2 incubator. Cell viability was determined by MTT colorimetric assay. Results are expressed as percentage viability of the control without test compound. Data represent the mean ± SEM of two independent experiments.