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Chalcone inhibitors of the NorA efflux pump in *Staphylococcus aureus* whole cells and enriched everted membrane vesicles

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ABSTRACT

A library of 117 chalcones was screened for efflux pump inhibitory (EPI) activity against NorA mediated ethidium bromide efflux. Five of the chalcones (**5**–**7**, **9**, and **10**) were active and two chalcones (**9** and **10**) were equipotent to reserpine with IC_{50}-values of 9.0 and 7.7 μM, respectively. Twenty chalcones were subsequently proved to be inhibitors of the NorA efflux pump in everted membrane vesicles. Compounds **5**, **7,** and **9** synergistically increased the effect of ciprofloxacin on *Staphylococcus aureus*. Our results suggest that chalcones might be developed into drugs for overcoming multidrug resistance based on efflux transporters of microorganisms.

1. Introduction

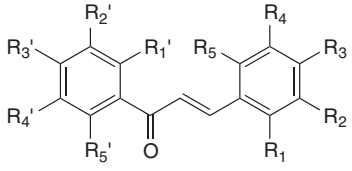
The emergence of multidrug resistance among pathogenic microorganisms makes presently used antibiotics inferior in the combat of infections.[1] Frequently the resistance is mediated by multidrug resistance (MDR) pumps, which are members of the large classes of transporters, known to be involved in uptake and exchange of key nutrients, ions, metabolic vaste, chemotactics, communication and hazardous substances.[2] The *Staphylococcus aureus* native pump NorA of the Major Facilitator Superfamily (MFS) belongs to a class of proton motive force (PMF) dependant pumps that include symporters and antiporters. These transporters are involved in the utilization of sugars, anions, metabolites and drugs.[3,4] Among the drugs identified as substrates for this pump are fluoroquinolones,[5,6] phenothiazines, thioxanthenes,[7] quarternary ammonium compounds and anti-septics,[8,9] verapamil,[10] omeprazole,[11] reserpine, totarol, ferruginol, carnosic acid, and dyes like ethidium bromide, rhodamine, and acridines.[12] Overexpression of the transporters mediates drug resistance.[13] A survey conducted in the USA revealed that about half of bloodstream isolates of *S. aureus* ($n = 232$) possess efflux pumps that contributed strongly to resistance and 22.8% of these were identified as NorA-overexpressors.[13] Recent clinical results indicate that combination of an efflux pump inhibitor (EPI) with an antibiotic is a possibility for overcoming MDR.[14] Recently we found a *p*-coumaroyl ester of rhamnose that act as a potent inhibitor of the NorA pump.[15] Encouraged by this finding we screened a library of 117 chalcones assuming that the cinnamoyl moiety was crucial for the pump inhibitory activity.

The present study reports the NorA inhibitory activities of the chalcones and their ability to potentiate the activity of ciprofloxacin against resistant *S. aureus*.

2. Material and methods

2.1. Preparation of compounds

Compound **1**–**7**, **12**–**16** and **20** were synthesized as described previously.[16–20] Compounds **8**–**11**, **17**–**19** are new compounds. Their preparation is reported below. The nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury 300 MHz instrument and high-resolution mass-spectrometry (HRMS) on a Bruker micrOTOF-Q mass spectrometer.



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1. $R_{1,2,3,4,5}$ = H, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OCH_2–CH_2–$N(CH_3)_2$.
2. $R_{1,2}$ = OCH_3, $R_{3,4,5}$ = H, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OH.
3. $R_{1,4,5}$ = H, $R_{2,3}$ = OCH_3, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OH.
4. $R_{1,3}$ = OCH_3, $R_{2,4,5}$ = H, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OH.
5. R_1 = OCH_3, $R_{2,4}$ = H, R_3 = –OH, R_5 = –CH_3, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OH.
6. $R_{1,4}$ = OCH_3, $R_{2,3,5}$ = H, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OH.
7. $R_{1,2,4,5}$ = H, R_3 = –OCH_2–CO–OH, $R_{1',2',3',4'}$ = H, $R_{5'}$ = –OCH_2–CH–CH_2.
8. R_1 = F, $R_{2,3,4,5}$ = H, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OCH_2–CH_2–$N(CH_3)_2$.
9. $R_{1,2,4,5}$ = H, R_3 = OC_6H_5, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OCH_2–CH_2–$N(CH_3)_2$.
10. $R_{1,2,4,5}$ = H, R_3 = –$N(CH_3)_2$, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OCH_2–CH_2–$N(CH_3)_2$.
11. $R_{1,3,5}$ = H, $R_{2,4}$ = OCH_3, $R_{1',3',4',5'}$ = H, $R_{2'}$ = –O–OC–C_6H_5.
12. $R_{1,3,4,5}$ = H, R_2 = $N(CH_3)_2$, $R_{1',2',3'}$ = OCH_3, $R_{4',5'}$ = H.
13. $R_{1,3,5}$ = H, $R_{2,4}$ = OCH_3, $R_{1',2',3'}$ = OCH_3, $R_{4',5'}$ = H.
14. $R_{1,2}$ = OCH_3, $R_{3,4,5}$ = H, $R_{1',2',4',5'}$ = H, $R_{3'}$ = O–CH_2–CH–CH_2.
15. $R_{1,3}$ = OCH_3, $R_{2,4,5}$ = H, $R_{1'}$ = OCH_3, $R_{2',3',4',5'}$ = H.
16. $R_{1,3,5}$ = OCH_3, $R_{2,4}$ = H, $R_{1',2',4',5'}$ = H, $R_{3'}$ = OH.
17. $R_{1,2,4,5}$ = H, R_3 = –CN, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OCH_2–CH_2–$N(CH_3)_2$.
18. $R_{1,3,4,5}$ = H, R_2 = –F, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OCH_2–CH_2–$N(CH_3)_2$.
19. $R_{1,2,4,5}$ = H, R_3 = –F, $R_{1',2',4',5'}$ = H, $R_{3'}$ = –OCH_2–CH_2–$N(CH_3)_2$.
20. $R_{1,3,4,5}$ = H, R_2 = –OCH_2–CH_2–$N(CH_3)_2$, $R_{1',2',3'}$ = OCH_3, $R_{4',5'}$ = H.

2.1.1. Preparation of 2-fluoro-4′-dimethylaminethoxychalcone (8)

To an aqueous solution of sodium hydroxide (6 M, 0.5 mL) was added a solution of 4′-(2-dimethylaminoethoxy)acetophenone (99 mg, 0.48 mmol) and 2-fluorobenzaldehyde (60 mg, 0.48 mmol) in MeOH (2 mL). The reaction mixture was stirred at room temperature for 2 h and left at 4 °C overnight to give a precipitate. Recrystallization (MeOH–water) afforded the title compound (107 mg, 70%), mp 60–61 °C.

^{1}H NMR (300 MHz, methanol-d_4): δ 2.34 (s, 6H, [$(CH_3)_2$N-CH_2]) 2.79 (t, J = 5.42 Hz, 2H, N-CH_2) 4.19 (t, J = 5.57 Hz, 2H, O-CH_2) 7.07 (d, J = 9.08 Hz, 2H, H3′ and H5′) 7.18 (ddd, J = 11.0, 8.0, 1.0 Hz, 1H, H3) 7.25 (td, J = 7.00, 1.00 Hz, 1H, H5) 7.38–7.50 (m, 1H) 7.76–7.94 (m, 1H, H6) 7.89 (d, J = 16.00 Hz, 1H, COCH=C) 7.81 (d, J = 16.00 Hz, 1H, COC=CH) 8.08 (d, J = 8.79 Hz, 2H, H2 and H6).

^{13}C NMR (75 MHz, $CDCl_3$): δ 45.96 [$(CH_3)_2$N-CH_2], 59.02 (N-CH_2), 67.04 (O-CH_2), 124.33 (C1), 163.06 (d, J = 250 Hz, C2), 117.19 (d, J = 22 Hz, C3), 133.46 (d, J = 9 Hz, C4), 126.05 (C5), 125.09 (d, J = 4.5 Hz, C6), 130.35 (C1′), 132.29 (C2′ and C6′), 115.73 (C3′ and C5′), 164.57 (C4′), 190.30 (CO), 126.01 (CO-C), 137.17 (d, J = 3.7 Hz, CO-C=C). HRMS: Calcd for $[C_{19}H_{21}FNO_2]^+$: 314.1551. Found 314.1540.

2.1.2. Preparation of 4-phenoxy-4′-dimethylaminoethoxychalcone (9)

Compound (**9**) was prepared as described above for **8** using 4′-(2-dimethylaminoethoxy)acetophenone (99 mg, 0.48 mmol) and 4-phenoxybenzaldehyde (72 mg, 0.48 mmol) in MeOH (2 mL) as starting materials, 101–102 °C.

^{1}H NMR (300 MHz, methanol-d_4) δ: 2.35 (s, 6H, $(CH_3)_2$N) 2.79 (t, J = 6.00 Hz, 2H, N-CH_2) 4.19 (t, J = 6.00 Hz, 2H, CH_2-O) 7.07 (d, J = 15.00 Hz, 1H, HC=C) 7.01 (d, J = 15.00 Hz, 1H, HC=C) 6.99 (d, J = 7.50 Hz, 2H, H2″ and H6″) 7.07 (d, J = 7.33 Hz, 2H, H3′ and H5′) 7.18 (t, J = 8.00 Hz, 1H, H4″) 7.40 (t, J = 8.00 Hz, 2H, H3″ and H5″) 7.71 (d, J = 7.00 Hz, 2H, H2 and H6) 7.73 (d, J = 8.50 Hz, 2H, H3 and H5) 8.09 (d, J = 8.50 Hz, 2H, H2′ and H6′).

^{13}C NMR (75 MHz, methanol-d_4) δ: 45.98 [$(CH_3)_2$N], 59.04 (C-N), 67.05 (C-O), 157.59 (C1″), 119.43 (C2″ and C6″), 131.24 (C3″ and C5″), 121.60 (C4″), 132.37 (C1), 131.73 (C2 and C6), 120.94 (C3 and C5), 161.38 (C4), 190.66 (CO), 125.47 (CO-C=), 144.97 (C-C=), 132.20 (C2′ and C6′), 115.66 (C3′ and C5′), 164.42 (C4′). HRMS: Calcd for $[C_{25}H_{26}NO_3]^+$: 388.1907. Found 388.1896.

2.1.3. Preparation of 4-dimethylamino-4′-dimethylaminoethoxychalcone (10)

The title compounds was prepared as described above for (**8**) using 4′-(2-dimethylaminoethoxy)acetophenone (99 mg, 0.48 mmol) and 4-dimethylaminobenzaldehyde (72 mg, 0.48 mmol) in MeOH (2 mL) as starting materials, mp 124–125 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ 2.36 (s, 6H, $(CH_3)_2$N-CH_2) 2.77 (t, J = 5.71 Hz, 2H, CH_2-N) 3.05 (s, 6H, $(CH_3)_2$N-C4) 4.15 (t, J = 5.71 Hz, 2H, CH_2-O) 6.70 (d, J = 8.79 Hz, 2H, H3′ and H5′) 7.00 (d, J = 8.79 Hz, 2H, H2 and H6) 7.36 (d, J = 15.24 Hz, 1H, CO-CH) 7.56 (d, J = 8.79 Hz, 2H, H2 and H6) 7.80 (d, J = 15.24 Hz, 1H, CO-C=CH) 8.03 (d, J = 9.08 Hz, 2H, H2′ and H6′).

^{13}C NMR (75 MHz, $CDCl_3$): δ 40.41 [$(CH_3)_2$N-CH_2], 45.97 [$(CH_3)_2$N-C4], 59.07 (N-CH_2), 67.00 (O-CH_2), 123.99 (C1), 131.85 (C2 and C6), 113.13 (C3 and C5), 153.99 (C4), 132.94 (C1′), 131.92 (C2′ and C6′), 115.55 (C3′ and C5′), 164.12 (C4′), 191.11 (CO), 116.93 (CO-C), 147.27 (CO-C=C). HRMS: Calcd for $[C_{21}H_{27}N_2O_2]^+$: 339.2067. Found 339.2062.

2.1.4. Preparation of 3,5-dimethoxy-3′-benzoyloxychalcone (11)

A solution of sodium hydroxide (6 M, 1 mL) was added to a solution of 3-hydroxyacetophone (113 mg, 0.83 mmol) and 3,5-dimethoxybenzaldehyde (138 mg, 0.83 mmol) in MeOH (1 mL). The reaction mixture was stirred at 50 °C for 18 h and at 4 °C overnight. The precipitate was washed with MeOH and water to give 3,5-dimethoxy-3-hydroxychalcone (100 mg, 43%). To a solution of the crude chalcone (90 mg, 0.3 mmol) in dichloromethane (3 mL) was added pyridine (0.7 mL) and benzoyl chloride (53 mg, 0.38 mmol). The reaction mixture was stirred at room temperature for 4 h.

To the reaction mixture was added a saturated aqueous solution of $NaHCO_3$ (30 mL), and the mixture was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were sequentially washed with an aqueous solution of NaOH (2 M, 10 mL), HCl (2 M, 10 mL), and brine (20 mL), dried over magnesium sulfate, and concentrated in vacuo. Recrystallization of the residue (MeOH and water) afforded the title compound 66 mg (53.6%), mp 110–111 °C.

^{1}H NMR (300 MHz, acetonitrile-d_3): δ 3.81 (s, 6H, [CH_3O]$_2$) 6.57 (t, J = 2.34 Hz, 1H, H4) 6.92 (d, J = 2.34 Hz, 2H, H2 and H6) 7.52 (ddd, J = 8.00, 2.50, 1.00 Hz, 1H, H4′) 7.59 (t, J = 8.00 Hz, 2H, H3″ and H5″) 7.63 (t, J = 8.00 Hz, 1H, H5′) 7.74 (dt, J = 7.50, 1.50 Hz, 1H, H4″) 7.67 (d, J = 15.50 Hz, 1H, COCH=C) 7.73 (d, J = 15.50 Hz, 1H, COC=CH) 7.96 (t, J = 1.90 Hz, 1H, H2′) 8.03 (dt, J = 7.50, 1.50 Hz, 1H, H6′) 8.20 (dt, J = 7.00, 2.00 Hz, 2H, H2″ and H6″).

^{13}C NMR (75 MHz, acetonitrile-d_3): δ 56.24 (CH_3O), 130.33 (C1), 107.47 (C2), 162.14 (C3), 103.77 (C4), 162.14 (C5), 107.47 (C6), 137.79 (C1′), 122.97 (C2′), 152.37 (C3′), 127.07 (C4′), 127.56 (C5′), 130.92 (C6′), 189.74 (CO), 123.30 (CO-C), 145.50 (CO-C=C), 166.05 (OCO), 140.50 (C1″), 130.92 (C2″ and C6″), 129.85 (C3″ and C5″), 134.93 (C4″). HRMS: Calcd for $[C_{24}H_{20}NaO_5]^+$: 411.1203. Found 411.1191.

2.1.5. Preparation of 4-cyano-4′-dimethylaminethoxychalcone (17)

To an aqueous solution of sodium hydroxide (6 M, 0.5 mL) was added a solution of 4′-(2-dimethylaminoethoxy)acetophenone (99 mg, 0.48 mmol) and 4-cyanobenzaldehyde (63 mg, 0.48 mmol) in MeOH (2 mL). The reaction mixture was stirred at room temperature for 2 h and left at 4 °C overnight to give a precipitate. Recrystallization (MeOH–water) afforded the title compound (107 mg, 70%), mp 120–121 °C.

^{1}H NMR (300 MHz, methanol-d_4) δ 2.80 (t, J = 5.42 Hz, 2H, NCH_2) 4.21 (t, J = 5.42 Hz, 2H, OCH_2) 7.06–7.12 (m, 2H, H3′ and H5′) 7.77 (d, J = 7.03 Hz, 2H, H2 and H6) 7.90 (m, 4H, COC═CH, COCH═C, and H3 and H5) 8.07–8.16 (m, 2H, H2′ and H6′). ^{13}C NMR (75 MHz, $CDCl_3$): δ 45.98 [$(CH_3)_2$N-CH_2], 59.03 (N-CH_2), 67.09 (O-CH_2), 141.06 (C1), 130.31 (C2 and C6), 133.91 (C3 and C5), 114.36 (C4), 131.93 (C1′), 132.41 (C2′ and C6′), 115.76 (C3′ and C5′), 164.70 (C4′), 189.91 (CO), 126.26 (CO-C), 142.75 (CO-C═C), 119.56 C≡N. HRMS: Calcd for $[C_{20}H_{21}N_2O_2]^+$: 321.1598. Found 321.1581.

2.1.6. Preparation of 3-fluoro-4′-dimethylaminethoxychalcone (18)

The title compounds was prepared as described above for **8** using 4′-(2-dimethylaminoethoxy)acetophenone (99 mg, 0.48 mmol) and 3-fluorobenzaldehyde (60 mg, 0.48 mmol) in MeOH (2 mL) as starting materials, mp 59–60 °C.

^{1}H NMR (300 MHz, methanol-d_4) δ: 2.28 (s, 6H, [$(CH_3)_2$N-CH_2]) 2.72 (t, J = 5.57 Hz, 2H, N-CH_2) 4.09 (t, J = 5.57 Hz, 2H, O-CH_2) 6.94 (d, J = 8.79 Hz, 2H, H3′ and H5′) 6.98–7.07 (m, 1H, H2) 7.24–7.36 (m, 3H, H4, H5, and H6) 7.48 (d, J = 16.00 Hz, 1H, CO-CH═C) 7.65 (d, J = 16.00 Hz, 1H, CO-C═CH) 7.95 (d, J = 8.79 Hz, 2H, H2′ and H6′).

^{13}C NMR (75 MHz, $CDCl_3$): δ 45.75 [$(CH_3)_2$N-CH_2], 58.01 (N-CH_2), 65.97 (O-CH_2), 137.27 (d, J = 7.5 Hz, C1), 132.00 (d, J = 22 Hz, C2), 163.04 (d, J = 244 Hz, C3), 117.48 (d, J = 22 Hz, C4), 130.62 (d, J = 22 Hz, C5), 124.67 (d, J = 2.2 Hz, C6), 131.62 (C1′), 131.09 (C2′ and C6′), 114.56 (C3′ and C5′), 162.89 (C4′), 189.03 (CO), 123.02 (CO-C), 142.95 (CO-C═C). HRMS: Calcd for $[C_{19}H_{21}FNO_2]^+$: 314.1551. Found 314.1537.

2.1.7. Preparation of 4-fluoro-4′-dimethylaminethoxychalcone (19)

The title compounds was prepared as described above for (**8**) using 4′-(2-dimethylaminoethoxy)acetophenone (99 mg, 0.48 mmol) and 4-fluorobenzaldehyde (60 mg, 0.48 mmol) in MeOH (2 mL) as starting materials, 109–110 °C.

^{1}H NMR (300 MHz, methanol-d_4): δ 2.35 (s, 6H, [$(CH_3)_2$N-CH_2]) 2.80 (t, J = 5.42 Hz, 2H, N-CH_2) 4.20 (t, J = 5.42 Hz, 2H, O-CH_2) 7.08 (d, J = 8.79 Hz, 2H, H3′ and H5′) 7.17 (t, J = 8.79 Hz, 2H, H3 and H5) 7.74 (s, 2H, HC═CH) 7.80 (dd, J = 8.79, 5.27 Hz, 2H, H2 and H6) 8.11 (d, J = 9.08 Hz, 2H, H2′ and H6′).

^{13}C NMR (75 MHz, $CDCl_3$): δ 45.98 [$(CH_3)_2$N-CH_2], 59.05 (N-CH_2), 67.06 (O-CH_2), 133.00 (C1), 132.00 (d, J = 16 Hz, C2 and C6), 117.15 (d, J = 22 Hz, C3 and C5), 164.9 (d, J = 248 Hz, C4), 132.71 (C1′), 132.27 (C2′ and C6′), 115.69 (C3′ and C5′), 164.51 (C4′), 190.54 (CO), 122.78 (CO-C), 144.20 (CO-C═C). HRMS: Calcd for $[C_{19}H_{21}FNO_2]^+$: 314.1551. Found 314.1549.

2.2. Bacterial strains and growth conditions

S. aureus ATCC 29213, SA1199B and K1758 was used for potentiation and MIC determinations. K1758 is a *norA* knock-out and SA1199B is a *norA* overexpressor derived from a methicillin susceptible *S. aureus* bloodstream isolate.[21,22] These were provided by Dr. Glenn W. Kaatz, John D. Dingell VA Medical Center, Detroit MI, USA. Strains were cultivated in Mueller Hinton growth medium II (Oxoid, Hampshire, UK).

Escherichia coli DH10B background with pTrcHis2C-*norA*[23] was kindly provided by Dr. David C. Hooper. Control strain DH10B containing pTrc99A was constructed by transformation using $CaCl_2$ as stated by.[24]

2.3. Ethidium bromide efflux inhibition assay

The assay was performed according to.[25] Test solutions was prepared by dissolving compound in dimethyl sulfoxide (DMSO) (>99%, Merck, Germany) due to lower solubility of chalcones in aqueous media. Then diluted in MHB II (MHB II, Oxoid, Hampshire, UK) to a final concentration of 20 μg/mL (final [DMSO] ⩽ 1%). This preparation allowed clear solutions with no precipitation. Chemicals used were CCCP (carbonyl cyanide 3-chlorophenylhydrazone, ⩾98.0% (HPLC), Fluka) and EtBr (ethidium bromide solution (BioReagent, for molecular biology, 10 mg/mL in H_2O, Sigma–Aldrich). The positive control was reserpine (⩾99.0%, HPLC, Fluka, Switzerland) at 20 μg/ml, and the negative control was 1% DMSO in MHB II. Background was measured on a resuspended pellet in clean MHB II. Measurements were carried out *in duplicate* on a spectrofluorometer (Perkin Elmer, LS-50B luminescence spectrometer, England) using excitation λ_{ex} = 530 nm, emission: λ_{em} = 600 nm. Slit 5 mm excitation and 10 mm emission. Mean results were expressed as percentage reduction of efflux compared to control.

2.4. Preparation of everted membrane vesicles. Measurement of the proton gradient using acridine orange and inhibition of Hoechst 33342 efflux

To investigate NorA inhibitory activity of the inhibitors in absence of cytosolic machinery a subcellular model of everted membrane vesicles with the NorA efflux pump, was constructed. The method is based on the cloning of the *norA* gene into an *E. coli* expression vector and the subsequent transformation of the vector into *E. coli* and expression of NorA. The advantage is that NorA can be expressed in absence of other *S. aureus* native efflux pumps. Preparation of everted membrane vesicles was carried out from an overnight culture of *E. coli* DH10B bearing the plasmid pTrcHis2C-norA or pTrc99A according to Yu et al., 2002.[23] The method was modified by adding protease inhibitor PMSF (phenylmethanesulfonyl fluoride, ⩾98.5%, Sigma, USA) (0.1 mM final) before disruption through French Press and Pancreatic DNAse (Sigma, USA) (0.1 mg/mL final) to the cell lysate before centrifugation. Vesicles were adjusted to 20 mg/mL in 50 mM potassium phosphate buffer (pH 7.2) supplemented with 10% glycerol using BCA™ Protein Assay Kit (Thermo Scientific, USA) and stored in aliquots of 0.2 mL at −70 °C until use. To ensure viable function a measurement of the proton gradient across the membrane was performed. Vesicles were diluted to a concentration of 80 μg protein/mL in 2 mL 50 mM potassium HEPES (HEPES Enzyme Grade, 99% minimum (Fischer Scientific, USA), 8.5 mM sodium chloride (sodium chloride, Certified ACS Crystalline (Fischer Scientific, USA) 2 mM magnesium (magnesium chloride hexahydrate, GR, ACS Crystals (EMD Chemicals Inc., USA) (pH 7.2) buffer. Acridine orange was added to a final concentration of 500 nM. Addition of Mg^{2+}-ATP (adenosine 5′-triphosphate magnesium salt ⩾95%, bacterial. Sigma, USA) to a final concentration of 50 μM generated a proton motive force. The ionophores valinomycin (⩾98% Sigma, USA) and nigericin (sodium salt >98%, Sigma, USA) were added (2 and 5 μM, respectively) to dissipate the electrical and proton gradients, respectively. Norfloxacin (⩾98%, Sigma, USA) as a positive control, was added to a final concentration of 10 μM. Fluorescence was determined using a Shimadzu RF-5301PC spectrofluorometer and wavelengths of 494 nm (excitation) and 530 nm (emission). Measurement of inhibition of Hoechst 33342 efflux was carried out by diluting vesicles to a concentration of 80 μg protein/mL in 2 ml 50 mM potassium HEPES, 8.5 mM sodium chloride, 2 mM magnesium chloride (pH 7.2) buffer. Within 10 s of running time Hoechst 33342 (⩾98%, Sigma, USA) was added to a final concentration of 50 μM. When fluorescence became stable (∼30 s), NorA was activated by addition of Mg^{2+}-ATP (50 μM final concentration) to generate proton motive force. At 150–160 s of assay time the inhibitor was added. Fluorescence was determined using wavelengths of excitation λ_{ex} = 355 nm, emission: λ_{em} = 457 nm over a 300 s time course.

Measurements were carried out in triplicate on independent vesicle batches and expressed as mean of three with standard deviations.

2.5. Susceptibility and potentiation studies

Minimum inhibitory concentration (MIC) determination was by the microdilution method. Final volume in each well was 200 μL with an inoculum size of 5×10^5 cfu/mL. The MIC was the lowest concentration at which no visible growth was observed after 20 h of incubation at 35 °C as inspected on a Micur-Viewer. Positive controls were a bacterial suspension in MHB II and a bacterial suspension in MHB II with DMSO in concentrations corresponding to the highest quantity present (≈1%). Negative controls were wells with only MHB II and MHB II with test compound. A MIC-determination control for solvent DMSO and ciprofloxacin (Ratiopharm ApS, Denmark) were used as positive controls. All MIC-determinations were performed in accordance with CLSI/EUCAST guidelines *in duplicate* or more.

Potentiation studies was carried out as a checkerboard assay determining ciprofloxacin MIC in the presence of increasing amounts EPI against *S. aureus* SA1199B, ATCC 29213 and the *norA* knock-out strain K1758. Assays were carried out in microtitre plates using the checkerboard synergy method as adapted from Lomovskaya et al. 2001 and Smith et al. 2005.[26,27] Twofold serial dilutions in MHB were carried out, yielding end concentrations of 0.05–12.5 μg/mL and 0.2–25 μg/mL for ciprofloxacin and EPI, respectively. Final volume in each well was 200 μL with an inoculum size of 5×10^5 cfu/mL. Plates were incubated at 35 °C for 20 h and read as above. Ciprofloxacin-EPI interactions were classified using Fractional Inhibitory Concentration (FIC) index. The FIC index was calculated as the sum of Fractional Inhibitory Concentrations (FICs) of each of the drug.

FICs = (MIC of drug A or B in combination)/(MIC of drug A or B alone). FIC indices are $\leqslant$0.5 = synergy. 1.0 = Additivity. 2.0 = Indifferent and $\geqslant$4.0 = antagonism.[7]

3. Results

3.1. Origin of chalcones

The majority of the chalcones initially screened were previously prepared in an attempt to find new drugs against malaria.[17] The new chalcones (**8–11**, and **17–19**) have been prepared by a Claisen–Schmidt condensation of the appropriate benzaldehyde with the appropriate acetophenone. Structures of new compounds were substantiated by ^{1}H, ^{13}C NMR, and HRMS.

Table 1
Inhibition of EtBr efflux in SA1199B by the 10 most potent inhibitors

Compound	Concentration of EPI (μg/mL)				IC_{50} (μg)	IC_{50} (μM)
	2.5	5	10	20		
1	21.8	36.1	56.9	83.6	8.4	28.4
2	11.7	38.0	73.3	90.8	6.9	24.3
3	14.0	38.3	64.3	87.9	7.6	26.5
4	17.3	24.1	62.7	89.6	8.3	29.1
5	36.6	63.7	84.3	91.7	3.8	13.4
6	23.3	56.6	82.7	91.0	4.3	15.0
7	14.7	46.2	79.9	87.1	5.7	16.9
8	33.7	48.5	70.3	97.0	5.7	18.1
9	43.4	68.8	97.5	99.1	3.5	9.0
10	48.0	96.5	97.4	98.7	2.6	7.7
Reserpine	30.0	48.1	71.8	85.1	5.1	8.2

Data are presented are the mean of two measurements and expresses percent reduction of ethidium bromide efflux at a given concentration.

3.2. Inhibition of NorA mediated ethidiumbromide efflux

Among the 117 chalcones previously screened for antiplasmodic activity 10 compounds inhibited the NorA transporter just as efficient as or better than the positive control reserpine at a concentration of 20 μg/mL. The IC_{50}-values for the 10 most potent chalcones as inhibitors of ethidium bromide efflux are presented in Table 1. Chalcones **9** and **10** are equipotent to the positive control reserpine with IC_{50}-values of 9.0 and 7.7 μM, respectively. Compounds **5–8** are approximately half as potent. Examples of concentration–response relationships for **5**, **7**, **9,** and reserpine are depicted in Figure 1. The high MIC-value (25 μg/mL) showed that the inhibition of EtBr efflux by **9** was not caused by a killing of the *S. aureus*. The remaining compounds did not exhibit antibacterial activity within the range 0.2–25 μg/mL.

3.3. Inhibition of the NorA efflux pump in enriched membrane vesicles

The functionality of the NorA pump enriched vesicles and control vesicles upon addition of Mg^{2+}-ATP were verified by measuring fluorescence changes caused by the activation of F_0F_1 H^+-ATPase (Data not shown) using the optical probe acridine orange.

NorA-enriched vesicles and control vesicles showed similar magnitude of change in fluorescence. Valinomycin dissipated the electrical gradient and nigericin the proton gradient. When norfloxacin was added at 10 μM, the fluorescence changed dramatically for the NorA-enriched vesicles indicating loss of proton gradient due to the operation of the NorA efflux pump. Test compounds and the positive control verapamil provoked a time-dependent increase in fluorescence as expected for NorA inhibitors.

Analogously the 20 most active chalcones that inhibited the efflux of Hoechst 33342 dye is depicted in Figure 2. No decrease in fluorescence was observed from control vesicles lacking NorA, consistent with Hoechst 33342 being a substrate for this efflux pump.[23] Chalcones **2**, **3**, and **11–15** exerted activity similar to or less than verapamil, whereas the remaining 13 compounds were better at inhibiting Hoechst 33342 efflux.

When adding the chalcones or verapamil to the vesicles a time-dependent increase in fluorescence occurred, consistent with activity through inhibition of NorA (Fig. 3). Some compounds also exert quenching of the Hoechst 33342 dye when testing them without first adding the Mg^{2+}-ATP, for example, **20** (Fig. 3). The quenching appeared instantly, and the magnitude was increased with increasing concentrations of the inhibitor. In the control vesicles pTrc99A, lacking NorA a similar, but smaller quenching was evident. The lack of NorA in these proves that the pump is not involved in the observed changes (Fig. 3).

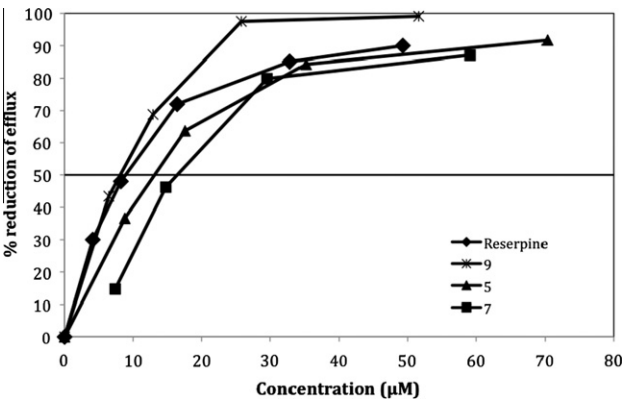

Figure 1. Dose–response relationships of compounds **5**, **7**, **9** and positive control reserpine at inhibiting EtBr-efflux in the NorA-overexpressor SA1199B.

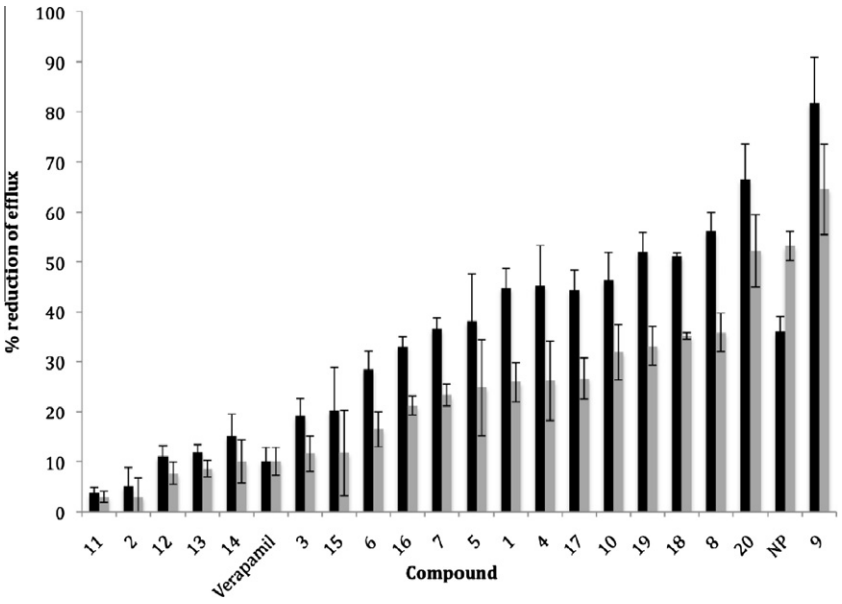

Figure 2. Inhibition of NorA-mediated Hoechst 33342 efflux by the chalcones. NP is the natural product kaempferol-3-*O*-α-L-(2,4-bis-*E*-p-coumaroyl)rhamnoside isolated from *Persea lingue* Nees.[15] Black bars represents EPI activity at 2 μg/mL and grey bars the activity when normalized to verapamil equivalents. EPI levels presented are the mean of three measurements with standard deviation depicted as error bars.

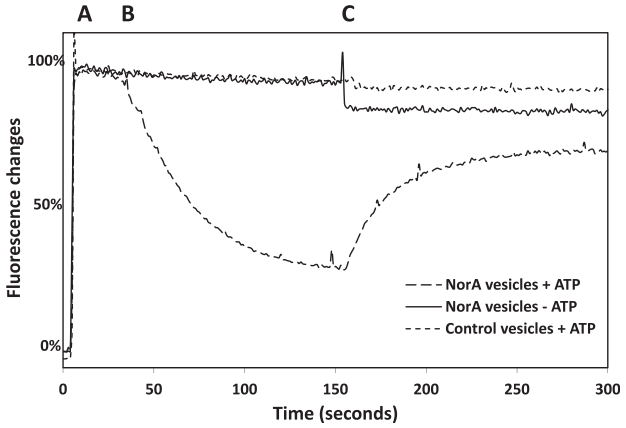

Figure 3. Hoechst 33342 efflux inhibition in vesicles pTrcHis2C-NorA with and without addition of ATP and control vesicles pTrc99A. (A) 100 nM Hoechst 33342, (B) 50 μM Mg^{2+}-ATP; (C) 2 μg/mL chalcone 20.

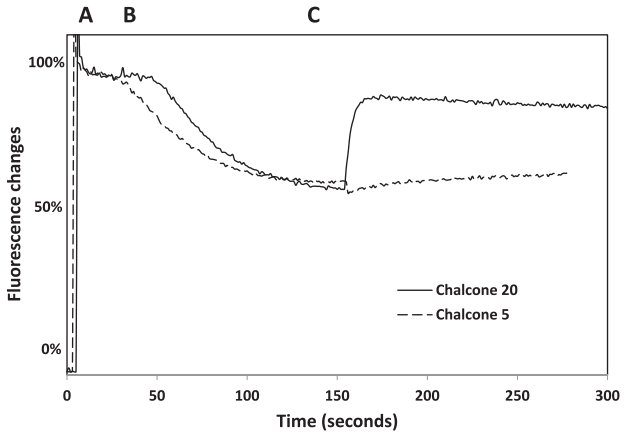

Figure 4. Measurement of fluorescence changes of acridine orange in control vesicles pTrc99A. (A) 500 nM acridine orange, (B) 50 μM Mg^{2+}-ATP; (C) 2 μg/ml chalcone 5 or 20.

In the control vesicles with no NorA present (pTrc99A) and using acridine orange instead of Hoechst 33342, compound **1–10** and **17–20** and verapamil exerted an activity similar to that of nigericin (e.g., **20** in Fig. 4) indicating a disruption of the proton gradient. Compound **2–7** showed no changes of acridine orange fluorescence (e.g., chalcone **5** Fig. 4). Norfloxacin did not exert activity, nor did DMSO.

3.4. Susceptibility testing and potentiation studies

Compounds were studied for antimicrobial activity by means of a MIC determination and potentiating studies were carried out by checkerboard combinations of inhibitors with known NorA substrate ciprofloxacin. The ability of the 10 most potent chalcones to enhance the antibacterial activity of ciprofloxacin was evaluated against three strains of *S. aureus* (SA1199B, ATCC 29213 and K1758) and is comprised in Table 2. A FIC-index $\leqslant$0.5 represents synergic activity.

All compounds, except **1**, **8**, **10** and verapamil exerted fourfold reductions of ciprofloxacin MIC against both strains expressing NorA. Positive control reserpine showed same degree of potentiation at 6.25 μg/mL and compound **5**, **7** were equally potent. Compound **9** showed the highest degree of potentiation (fourfold at 3.13 μg/mL) and was approximately twice as potent as reserpine and chalcones **5** and **7**. FIC-indices calculated for the combinations exerting a minimum of fourfold reduction in $MIC_{ciprofloxacin}$ were <0.5 for all but **1**, **8**, **10** and verapamil against both strains. These combinations are therefore synergic in their activity. The potentiation was concentration dependent and compounds **5** and **7** both showed an 8-fold and 16-fold reduction of ciprofloxacin MIC at 12,5 and 25 μg/mL, respectively. Compound **9** exerted 8-fold and 16-fold reduction of ciprofloxacin MIC at 6.25 and 12.5 μg/mL, respectively. However at 25 μg/mL **9** was antibacterial alone. None of the other compounds showed antibacterial activity at 0.2–25 μg/mL. The concentration of compound **9** needed for exhibiting a synergistic activity was approximately eight times less than the MIC value, making it unlikely that the antibacterial activity is contributing essentially to more than the efflux activity. Chalcone **10** exerted surprisingly low potentiating activity, which is contradictory to the potent EtBr efflux inhibitory activity. None of the

Table 2
Results of potentiation studies of chalcones with ciprofloxacin against *S. aureus* strains with different expression levels of *norA*

Compound(s)	Δ norA K1758		ATCC29213		SA1199B		FIC-index (ATCC 29213)	FIC-index (SA1199B)
	CIP MIC	[EPI]	CIP MIC	[EPI]	CIP MIC	[EPI]		
Ciprofloxacin	0.1	0	0.4	0	6.25	0	NAP	NAP
+1	0.1	25	0.2*	25	3.13	25	>0.5	>0.5
+2	0.1	12.5	0.1	6.25	1.56	12.5	0.25	0.25
+3	0.1	25	0.1	25	1.56	25	0.25	0.25
+4	0.1	12.5	0.1	6.25	1.56	12.5	0.25	0.25
+5	0.1	6.25	0.1	12.5	1.56	6.25	0.25	0.25
+6	0.1	12.5	0.1	12.5	1.56	12.5	0.25	0.25
+7	0.1	6.25	0.1	3.13	1.56	6.25	0.25	0.25
+8	0.1	25	NAP	25	1.56	25	>1	0.25
+9	0.1	3.13	0.1	6.25	1.56	3.13	0.26	0.38
+10	0.2	25	0.2	12.5	NAP	25	0.5	NAP
+Reserpine	0.2	6.25	0.1	6.25	1.56	6.25	0.25	0.25
+Verapamil	0.4	25	0.4	25	6.25	25	>1	>1

Reserpine and verapamil was included as positive controls.
NAP = no antimicrobial potentiation. Presented results are the combinations that produce at least fourfold decrease in ciprofloxacin MIC except marked * producing only twofold decrease. For each strain two columns are shown representing the MIC of ciprofloxacin (CIP MIC) and the corresponding EPI concentration ([EPI]) to produce this MIC. MIC and EPI are μg/mL.

compounds potentiated the activity of ciprofloxacin against the NorA deletion strain, confirming that the activity was directed at the NorA function.

4. Discussion and conclusion

A kaempferol rhamnoside has been shown to be a potent NorA inhibitor.[15] Inspired by the structural similarities of the coumaroyl substituents and chalcones a series of 117 chalcones were tested as EPI inhibitors. At 20 μg/mL 10 of the chalcones inhibited NorA as efficient as did reserpine (Table 1). The IC_{50} values of standard compounds like reserpine, thioridazine and prochlorperazine are reported to be 10 μM and a tryptamine derivative and SSRI paroxetine 18 and 25 μM, respectively.[7,29,30] A synthetic compound of a bisaryl urea EPI attached to ofloxacin (one of the most potent NorA EPI found) have been shown to produce an inhibition response over 84% at 10 μM,[31] and natural products carnosic acid,[12] totarol,[22] orizabin IX, XV[32] has EtBr-efflux inhibitory activity 10–50 μM, respectively. Consequently **1–10** can be concluded to be medium potent EPI inhibitors.

To verify that the effect was caused by NorA inhibitory activity a simpler system consisting of everted membrane vesicles enriched with NorA were constructed. The purpose was to show the activity of the EPIs against NorA in an isolated manner. The system was prepared by overexpression of *norA* in *E. coli* and the construction of everted membrane vesicles. The absence of native *S. aureus* membrane transporter proteins as well as cytosolic machinery in these vesicles constituted a strong tool for the evaluation of NorA inhibitory activity. Showing that the EPIs exerts inhibition of this system provided further confirmation of the activity against NorA and also a separation of the activity from e.g. cellular targets and the possible interference of other efflux pumps of the *S. aureus* membrane.

The chalcones and the control verapamil produced a time-dependent increase in fluorescence, consistent with an inhibition of the Hoechst 33342 efflux. Thirteen of the chalcones (**1**, **4**–**10**, and **16**–**20**) were more potent inhibitors than verapamil. The more potent inhibitor reserpine could not be used as a positive control because of interference with the fluorescence. Also the use of EtBr was found unsuited to study NorA activity in the everted membrane vesicles due to the lack of intracellular components, which afforded only a poor difference in fluorescence of intra- and extra-vesicular EtBr. Similar other dyes including Pyronin Y, Acriflavine, TMA-DPH, Nile Red, NPN, DMP and NBD-X generated poor results (data not shown).

Some compounds instantly exerted quenching of the Hoechst 33342 fluorescence when added to unenergized vesicles as well as the energized pTrc99A control vesicles. This quenching was in both cases increased with higher concentrations of the test compound. A difference in the magnitude of this interference may be explained by the differences of the two types of vesicles, one being enriched with NorA and containing up to 5–10% of this protein in the membrane and the other lacking NorA thereby being different matrices for Hoechst 33342 accumulation and equilibrium in the membrane.[23]

Inoue et al. (1982) has shown that quenching of fluorescence for some chalcones could be the result of interference with PMF. This possibility was precluded by measuring fluorescence changes in the control vesicles using the proton gradient probe acridine orange in presence of the EPIs.[33] Chalcone **1**, **8–10**, **17–20** and verapamil exerted activity similar to nigericin, which interferes with the proton gradient. The remainder did not show changes of acridine orange fluorescence (e.g., chalcone **5,** Fig. 4). Neither norfloxacin nor the solvent DMSO interferes with the fluorescence observed.

Verapamil is known to inhibit NorA induced norfloxacin efflux,[10] but also for being a calcium channel blocker. However, verapamil does also inhibit H^+-ATPase generated trans-membrane proton electrochemical gradient.[34] These properties of verapamil could explain the observed PMF interruption. Interestingly all the more potent chalcones (**1**, **7**–**10**, and **17**–**19**) carry a dimethylaminoethoxy substituent. This structural feature seems to interfere with the PMF or acridine orange in some way similar to the action of verapamil and nigericin. The observed NorA effect of the dimethylaminoethoxy chalcones may therefore be the result of a proton gradient disruption as well as a specific inhibition of NorA.

A mechanism could be inhibition of the H^+-ATPase and thereby indirectly the NorA pump through elimination of the energy source. Of interest is the finding that phenothiazines and thioxanthenes in addition to affect MDR efflux pumps pertubates the membrane energetics of the staphylococcal membrane.[7] These findings are interesting as the phenothiazines and thioxanthenes contain amino-sidechains and especially chlorpromazine has a dimethylamino-propyl sidechain which is similar to the dimethylaminoethoxy side chain of the potent chalcones.

Chalcone inhibitors bearing the dimethylamino-ethoxy sidechain might therefore also act by interfering with membrane energetics and consequently indirectly the NorA function, but this matter warrants further investigation.

Six chalcones (**2–7**) exerted good efflux inhibitory activity, but did not show any interference with the PMF according to the

investigations with acridine orange. These six compounds probably are specific inhibitors of NorA-activity.

Five of these chalcones are all hydroxylated at the 4′-position. This feature is also found in flavones and isoflavones previously studied.[35,36] In addition 4′-position hydroxylated chalcones isolated from *Dalea versicolor* were able to potentiate synergistically the activity of berberine, against *S. aureus* and *Bacillus cereus*.[37]

The present study suggests that in addition to the dimethylaminoethoxy group, a hydroxy at 4′-position of the B-ring and a methoxy group in the 2-position of the A-ring is important for the synergistic activity. The substitution pattern with a methoxy at 2-position in the A-ring and an additional methoxy at either position 3, 4 or 5 does not interfere with activity, while compounds with no substituents or a methoxy group at different positions, potentiate less. A chalcone tested with a hydroxy at 4′-position in the B-ring and a methyl group in 2 and 4-position in the A-ring showed no NorA inhibition determined in the EtBr-efflux assay (data not shown). This is comparable to the findings of Guz et al. (2001) who have investigated structure activity relationships of several flavonolignans and flavones with affinity to NorA and found importance of having a methoxy group instead of hydroxy at 4′-position of flavones.[38]

We investigated the 10 most potent EPIs for intrinsic antimicrobial activity in a MIC determination against assay strains as well a checkerboard combinations with the known NorA substrate ciprofloxacin. The majority of the compounds exhibited fourfold reductions of ciprofloxacin MIC against both strains expressing NorA and no potentiating effect against the NorA-knock out strain. These findings in conjunction with the efflux inhibitory assays are strong evidence that the chalcones inhibit NorA-activity. The degree of potentiating was similar to reserpine for several compounds (**5**, **7** and **9**) at 6.25 μg/ml. Compound **9** showed a fourfold potentiation at 3.13 μg/ml, being twice as potent as reserpine, and compounds **5** and **7**. The activity was concentration dependent in that a concentration of 12.5 μg/ml EPI compounds **5** and **7** showed an eightfold decrease, and at 25 μg/ml a 16-fold reduction was observed without having intrinsic antibacterial activity. Compound **9** exhibited 8-fold and 16-fold reduction of ciprofloxacin MIC at 6.25 and 12.5 μg/ml, but MIC-determination showed that **9** was antibacterial at 25 μg/ml. This is approximately eight times the concentration needed for the fourfold reduction of ciprofloxacin MIC towards SA1199B and an IC_{50} to MIC ratio of 0.14 which makes it unlikely that the antibacterial activity is contributing to the efflux-inhibition. The activity of these EPIs is comparable to e.g. *p*-cyanophenyl amine,[39] totarol[22] and stolineferin I.[40] It is puzzling why compound **10** does not potentiate ciprofloxacin when inhibiting well in both efflux assays. An explanation could be that **10** as a dicationic compound is very much like pentamidine, which is a splendid transporter substrate.[41] Pentamidine has a strong affinity to the NorA pump as well as QacA and MepA MDR-systems and therefore is readily pumped out and not saturating the efflux systems.[42,43]

It has been shown that compounds **2**, **3**, **4,** and **6** inhibit the growth of *Leishmania major* promastigotes, but at higher concentrations they interfere with proliferation of human lymphocytes provoked by PHA. Compounds **2** and **6** almost completely inhibit this response at 10 μg/mL. As potentiating agents a fourfold reduction of ciprofloxacin MIC was exerted at 12.5 μg/mL, and this small difference makes it unlikely that **2** and **6** are safe to use for systemic application. Compound **3** showed slight toxic levels at 10 μg/mL. This chalcone only reveal potentiating effect at 25 μg/mL, making it unlikely that this compound possess a potential for treatment of infections with multi resistant microorganisms. In contrast, compound **4** did not affect the proliferation of human lymphocytes in the concentrations in which it potentiated ciprofloxacin. Compound **4** was further investigated in mice and was found to inhibit a parasitic infection with *Plasmodium yoelii* at a dose of 60 mg/day without killing the host.[44] The very small structural difference in between **2**, **3**, **4** and **6** (substitution pattern of the methoxy group) reveals that it is difficult to predict selectivity of the compounds from their structures. However, the results of the present study reveal that appropriate substitution of the chalcone skeleton might afford drugs for combating infection with multi resistant microorganisms.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.025.

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