J Antimicrob Chemother 2012; **67**: 1138–1144
doi:10.1093/jac/dks005 Advance Access publication 6 February 2012

Journal of Antimicrobial Chemotherapy

Novel inhibitory activity of the *Staphylococcus aureus* NorA efflux pump by a kaempferol rhamnoside isolated from *Persea lingue* Nees

Jes Gitz Holler[1]*, S. Brøgger Christensen[1], Hans-Christian Slotved[2], Hasse B. Rasmussen[1], Alfonso Gúzman[1], Carl-Erik Olsen[3], Bent Petersen[4] and Per Mølgaard[1]

[1]*Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, 2100, Denmark;* [2]*Department of Microbiological Surveillance and Research, Statens Serum Institut, Ørestads Boulevard 5, 2300, Denmark;* [3]*Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871, Denmark;* [4]*Carlsberg Research Center, Gamle Carlsberg Vej 10, 2500, Denmark*

*Corresponding author. Tel: +45-35-33-62-93; Fax: +45-35-33-60-41; E-mail: jgh@farma.ku.dk

Received 30 November 2011; returned 28 December 2011; revised 3 January 2012; accepted 4 January 2012

Objectives: To isolate a plant-derived compound with efflux inhibitory activity towards the NorA transporter of *Staphylococcus aureus*.

Methods: Bioassay-guided isolation was used, with inhibition of ethidium bromide efflux via NorA as a guide. Characterization of activity was carried out using MIC determination and potentiation studies of a fluoroquinolone antibiotic in combination with the isolated compound. Everted membrane vesicles of *Escherichia coli* cells enriched with NorA were prepared to study efflux inhibitory activity in an isolated manner.

Results: The ethanolic extract of *Persea lingue* was subjected to bioassay-guided fractionation and led to the isolation of the known compound kaempferol-3-*O*-α-L-(2,4-bis-*E*-*p*-coumaroyl)rhamnoside (compound 1). Evaluation of the dose–response relationship of compound 1 showed that ethidium bromide efflux was inhibited, with an IC_{50} value of 2 μM. The positive control, reserpine, was found to have an IC_{50} value of 9 μM. Compound 1 also inhibited NorA in enriched everted membrane vesicles of *E. coli*. Potentiation studies revealed that compound 1 at 1.56 mg/L synergistically increased the antimicrobial activity of ciprofloxacin 8-fold against a NorA overexpresser, and the synergistic activity was exerted at a fourth of the concentration necessary for reserpine. Compound 1 was not found to exert a synergistic effect on ciprofloxacin against a *norA* deletion mutant. The 2,3-coumaroyl isomer of compound 1 has been shown previously not to cause acute toxicity in mice at 20 mg/kg/day.

Conclusions: Our results show that compound 1 acts through inhibition of the NorA efflux pump. Combination of compound 1 with subinhibitory concentrations of ciprofloxacin renders a wild-type more susceptible and a NorA overexpresser *S. aureus* susceptible.

Keywords: MRSA, antibiotic resistance, antibiotics, natural compounds

Introduction

Active efflux makes bacteria resistant towards antibiotics by reducing the intracellular concentration to an ineffective level. The multidrug efflux transporter NorA of *Staphylococcus aureus* confers multidrug resistance (MDR) to a broad spectrum of compounds, including fluoroquinolones, quaternary ammonium compounds, reserpine, verapamil and the dyes ethidium bromide, rhodamine and acridines.[1,2] The *S. aureus* chromosome codes for two homologues of NorA, namely NorB and NorC. In half of bloodstream isolates of *S. aureus*, efflux pumps contributed strongly to resistance. All three efflux pumps were overexpressed in more than half of these.[3] Emerging resistant pathogenic bacteria and the contribution to this resistance by MDR pumps have increased interest in clinical methods for inhibiting these pumps. Combination of an antibiotic with an efflux pump inhibitor (EPI) would be expected to re-establish susceptibility of the bacteria to antibiotics that at present cannot be used any longer. Combination therapy might even synergistically increase the susceptibility of the bacteria.[4] At present no EPI/antibiotic combination is used clinically.

Several plant-derived compounds, including the alkaloid reserpine from *Rauvolfia vomitoria*, the terpene carnosic acid from *Rosmarinus officinalis* and the diterpene totarol from

Chamaecyparis nootkatensis, inhibit NorA-induced ethidium bromide efflux from a NorA overexpresser.[5,6] The flavonolignan 5-methoxyhydnocarpin inhibits NorA activity and synergistically increases the activity of the antimicrobial alkaloid berberine present in the same plant.[7]

Encouraged by the presence of pump inhibitors in plants and based on screening of plants used by the Chilean Huilliche people in their traditional medicine, a bioassay-guided fractionation of the constituents of *Persea lingue* Nees was initiated.[8] This led to isolation of the previously known kaempferol 3-*O*-α-L-(2,4-bis-*E*-*p*-coumaroyl)rhamnoside (compound 1).[9,10] Compound 1 and related isomers have previously been found to have anti-methicillin-resistant *S. aureus* (MRSA) activity.[11,12] The 2,3-coumaroyl isomer of compound 1 has been shown not to cause acute toxicity in mice at 20 mg/kg/day.[13] The NorA inhibitory activity of compound 1 reported in this publication has not been reported previously.

Materials and methods

General experimental procedures

NMR spectra were obtained at 800 MHz on a Bruker Avance spectrometer of the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules.

Accurate mass measurements were made with a micrOTOF-Q instrument from Bruker Daltonics, Bremen. They were done in electrospray ionization (ESI)-positive mode.

Plant material

P. lingue Nees (Lauraceae) leaves were collected in March 2009 in Osorno, Region de Los Lagos, Chile. The leaves were dried in a heating cabinet for 72 h at 40°C. The dried plant material was stored in paper bags until use. Voucher specimens are stored in the herbarium at the Botanical Garden and Museum, University of Copenhagen (Herbarium C, voucher number JGH020).

Extraction and isolation

Dried ground leaves (700 g) were ultrasonicated three times with 96% ethanol 1:10 (mass to volume) for 30 min and the mixture was filtered. The combined extracts were concentrated *in vacuo* to give 61.8 g of residue. The residue was extracted in 1000 mL of dichloromethane (DCM)/methanol (1:1) and the extract was concentrated to ∼500 mL *in vacuo*. Methanol (300 mL) and water (200 mL) were added to the solution. The mixture was washed four times with 300 mL of heptane. The heptane phase was extracted with methanol/water (80:20) to ensure maximum yield. The combined aqueous methanolic phases were concentrated *in vacuo* to remove the methanol. The remaining aqueous phase was diluted to a total volume of 1000 mL with H_2O and extracted four times with 300 mL of ethyl acetate. The combined ethyl acetate phases were concentrated *in vacuo* to give 17.5 g of a residue. The residue was stored at −20°C until use.

To isolate compound 1, the residue (17.5 g) was chromatographed over 250 g of silica gel 60 (0.040–0.063 mm, Merck, Germany) and eluted with 200 mL aliquots of heptane/[DCM/methanol (9:1)] (100:0), (70:30), (50:50), (40:60), (30:70) and (0:100) as eluents using vacuum liquid chromatography. A total of 31 fractions of 40 mL were obtained. Fractions with similar thin layer chromatography (TLC) profiles were pooled and concentrated. Fractions revealing activity in the MIC assay and ethidium bromide efflux inhibition activities were combined to give 11.5 g of residue. The active residue was chromatographed over 250 g of silica gel 60 eluted with 200 mL aliquots of toluene/acetone (80:20), (70:30), (60:40), (50:50), (40:60), (25:75) and (0:100) followed by 200 mL of acetone/methanol (80:20) and 200 mL of methanol as eluents. Fractions of 60 mL were pooled according to similar TLC profiles. The fractions revealing activity in the MIC and ethidium bromide efflux inhibition assay were combined and concentrated to give 430 mg of a residue. This residue was further fractionated using a semi-preparative HPLC system: Dionex ASI-100 Automated Sample Injector, P580 pump, PDA-100 Photodiode Array Detector and Chromeleon Client v.6.30 software (Dionex, Sunnyvale, CA, USA) equipped with a Supelco® Discovery HS-F5 pentafluorophenyl column (25 cm×10 mm, 5 μm) using Milli-Q water plus 0.1% trifluoroacetic acid (TFA)/methanol for HPLC with 0.1% TFA (3:1) as an eluent, flow rate 3.6 mL/min. The eluate containing the peaks t_R=15.5 min and 16.4 min was collected and concentrated to yield 22 mg of compound 1 and 19 mg of a mixture containing small amounts of compound 1 and an isomer. Unless otherwise stated, solvents were CHROMASOLV® for HPLC grade obtained from Sigma-Aldrich.

Bacterial strains and growth conditions

Staphylococcus aureus ATCC 29213, SA1199B and K1758 were used. SA1199B is a fluoroquinolone-resistant NorA overexpresser derived from a methicillin-susceptible *S. aureus* bloodstream isolate from a patient with endocarditis.[14] *S. aureus* K1758 is a *norA* knockout strain.[5] Both strains were kindly provided by Dr G.W. Kaatz (Wayne State University, USA). Strains were cultivated in Mueller–Hinton growth medium II (MHB II; Oxoid, Hampshire, UK) with appropriate antibiotics where applicable.

E. coli DH10B background with pTrcHis2C-*norA* was kindly provided by David C. Hooper (Massachusetts General Hospital, Boston, USA).[15] This strain was grown on LB plates containing 25 mg/L ampicillin and 0.128 mg/L norfloxacin. Cells were stored at −80°C for further use. Control strain DH10B containing pTrc99A was constructed by transformation using $CaCl_2$ as described by Sambrook and Russell.[16]

Ethidium bromide efflux inhibition assay

The assay was performed according to Kaatz *et al.*[17] Test solutions were prepared by dissolving plant extracts and compound in DMSO (>99%, Merck, Germany) and then diluted in MHB II to a final concentration of 100 mg/L for plant extracts (final [DMSO] ≤1%). Chemicals used were carbonyl cyanide 3-chlorophenylhydrazone [≥98.0% (HPLC), Fluka, Switzerland] and ethidium bromide solution (BioReagent, for molecular biology, 10 mg/mL in H_2O, Sigma-Aldrich). The positive control was reserpine [≥99.0% (HPLC), Fluka] at 20 μg/mL and the negative control was 1% DMSO in MHB II. Measurements were carried out at least in duplicate on a spectrofluorometer (LS-50B luminescence spectrometer, Perkin Elmer, UK) and mean results were expressed as percentage reduction of efflux compared with control.

Preparation of everted membrane vesicles and measurement of the proton gradient using Acridine Orange and inhibition of Hoechst 33342 efflux

Preparation of everted membrane vesicles was carried out according to Yu *et al.*[15] The method was modified by adding phenylmethanesulphonyl fluoride (≥98.5%, Sigma, USA) (final concentration 0.1 mM) before disruption through a French Press followed by addition of pancreatic DNase (Sigma, USA) (0.1 mg/mL final) to the cell lysate before centrifugation. Vesicles were adjusted to 20 mg/mL using a BCA™ Protein Assay Kit (Thermo Scientific, USA) and stored in aliquots of 0.2 mL at −70°C until use. To ensure viable function, measurement of the proton gradient across the membrane was performed. Vesicles were diluted to a concentration of 80 μg of protein/mL with 2 mL of 50 mM potassium HEPES (HEPES Enzyme Grade—99% minimum, Fisher Scientific, USA)/8.5 mM

sodium chloride (Certified ACS Crystalline, Fisher Scientific)/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 (≥95%, bacterial, Sigma, USA) to a final concentration of 50 μM generated a proton motive force. The ionophores valinomycin (valinomycin, ≥98%, Sigma, USA) and nigericin (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 for excitation and 530 nm for emission, respectively. Measurement of inhibition of Hoechst 33342 efflux was carried out by diluting vesicles to a concentration of 80 μg protein/mL with 2 mL of 50 mM potassium HEPES/8.5 mM sodium chloride/2 mM magnesium (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 energized by the addition of Mg^{2+}-ATP (50 μM final) to generate a proton motive force. At 150–160 s of assay time, the compound of interest was added to inhibit NorA activity. Fluorescence was determined using wavelengths of 355 nm for excitation and 457 nm for emission over a 300 s time course.

Susceptibility and potentiation studies

MIC determination was by the microdilution method. Positive controls were a bacterial suspension in MHB II and a bacterial suspension in MHB II with DMSO in amounts corresponding to the highest quantity present (≈1%). Negative controls were wells with only MHB II and MHB II and plant extract. MIC determination controls for the solvent DMSO and ciprofloxacin were also performed. All MIC determinations were repeated at least in duplicate and in accordance with CLSI/EUCAST guidelines. Potentiation studies were carried out as a chequerboard assay according to Lomovskaya *et al.*,[18] determining ciprofloxacin MIC in the presence of increasing amounts of EPI against *S. aureus* SA1199B, ATCC 29213 and the *norA* knockout strain K1758. Two-fold serial dilutions in MHB II were carried out, yielding end concentrations of 0.05–12.5 and 0.2–25 mg/L for ciprofloxacin and EPI, respectively. Ciprofloxacin–EPI interactions were classified using the fractional inhibitory concentration (FIC) index.

Results

Bioassay-guided fractionation of an extract of *P. lingue* using inhibition of ethidium bromide efflux as a guide led to the isolation of the known compound 1 (Figure 1). The structure was confirmed by HR-MS *m/z* 724.1786 [M—H], (expected mass $C_{36}H_{32}O_{14}$: 724.1792) and comparison of the ^{1}H and ^{13}C NMR data with those previously published for compound 1.[9,10]

Ethidium bromide efflux inhibition

Taking advantage of ethidium bromide being a substrate for many MDR efflux pumps, the ability of an extract or compound to inhibit efflux of ethidium bromide was followed fluorimetrically by measuring the changes in the levels of ethidium bromide bound to intracellular nucleic acids. The inhibitory effect of increasing concentrations of compound 1 and the known inhibitor reserpine can be seen in Figure 2. A concentration-dependent effect was observed for both inhibitors. The IC_{50} value of compound 1 was determined to be 2 μM, more than 4-fold more potent than reserpine (IC_{50}=9 μM). The MIC value for compound 1 was 25 mg/L (~35 μM). This is within the concentration range assayed in the efflux inhibition evaluation, but approximately 17 times higher than the IC_{50} value.

Inhibition of Hoechst 33342 efflux from NorA-enriched everted membrane vesicles

The enriched vesicles and control vesicles were able to generate a proton motive force (PMF) upon addition of Mg-ATP, demonstrated by using the optical probe Acridine Orange and measuring fluorescence changes caused by the activation of F_0F_1 H^+-ATPase (Figure 3). NorA-enriched vesicles and control vesicles showed a 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. Control vesicles did not respond as dramatically but did show a slight change in fluorescence upon addition. This slight change originated in the presence of a residue of

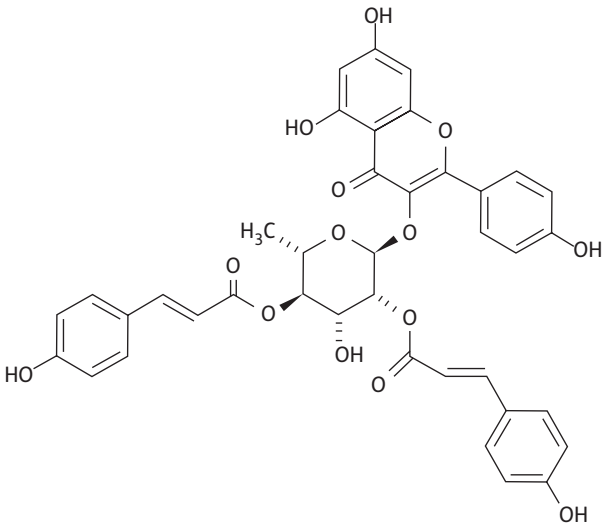

Figure 1. Chemical structure (absolute configuration) of compound 1.

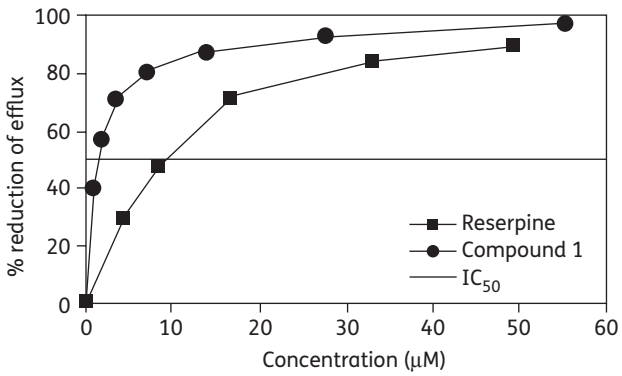

Figure 2. Dose–response relationships of compound 1 (filled circles) and positive control reserpine (filled squares) for inhibition of ethidium bromide efflux in NorA overexpresser SA1199B.

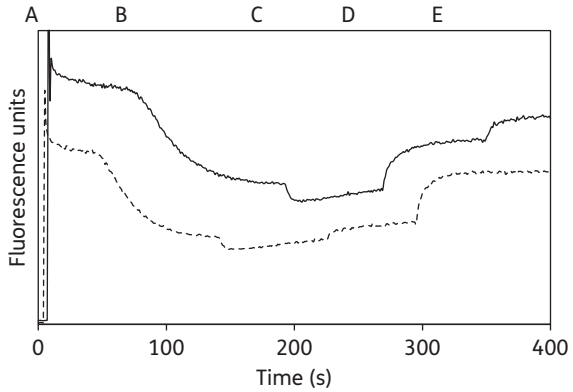

Figure 3. Measurement of the proton gradient across everted membrane vesicles enriched with NorA (continuous line) (pTrcHis2C-*norA*) and control vesicles (broken line) (pTrc99A) using Acridine Orange. A, 0.5 μM Acridine Orange; B, 50 μM Mg^{2+}-ATP; C, 2 μM valinomycin; D, 10 μM norfloxacin; E, 5 μM nigericin.

acetic acid, the solvent used to initially dissolve norfloxacin, as shown by running a control sample corresponding to the dilution of acetic acid without norfloxacin.

When adding compound 1 or the positive control verapamil, a time-dependent increase in fluorescence could be observed, consistent with activity through inhibition of NorA (Figure 4). Compound 1 was added at approximately half the concentration of verapamil and exhibited a much larger inhibition response.

Adding compound 1 and verapamil to vesicles without previously adding ATP produced an instant and small decrease in fluorescence caused by quenching of the Hoechst dye by the added inhibitors (Figure 4). Compound 1 also caused quenching of Hoechst 33342 in the control vesicles. The lack of NorA in these proves that the pump is not involved in the observed changes. We also investigated the effect on control vesicles (pTrc99A) using Acridine Orange instead of Hoechst 33342 to observe changes to the pH and electrical gradient without NorA present. A substantial decrease in fluorescence when adding compound 1 (Figure 5) was observed, similar to the experiment with Hoechst 33342 (Figure 4). When adding verapamil to the control vesicles a fluorescence increase was demonstrated (Figure 5). The solvent DMSO did not interfere with fluorescence.

Susceptibility and synergy studies

The ability of compound 1 and positive controls to enhance the antibacterial activity of ciprofloxacin was evaluated. Combination of compound 1 with ciprofloxacin significantly reduced the MIC values (Table 1). At 1.56 mg/L of compound 1 the MIC of ciprofloxacin for the NorA overexpresser SA1199B was reduced 8-fold from 6.25 to 0.78 mg/L. Reserpine showed the same degree of synergy, but only at a 4-fold higher concentration (6.25 mg/L). Even at 0.2 mg/L, compound 1 was able to halve the MIC of ciprofloxacin. Against ATCC 29213 at least a 4-fold decrease in the MIC of ciprofloxacin was evident at 0.78 mg/L of compound 1. The FIC indices showed that interactions were synergistic when tested against strains SA1199B and ATCC 29213. Verapamil did not show synergy within assay limits for these strains. No potentiation was observed against the K1758 knockout, confirming that compound 1 inhibits NorA.

Discussion

We obtained compound 1 by utilizing ethidium bromide efflux inhibition-guided isolation of an extract of *P. lingue*. The NorA-inhibitory effect of this compound has not previously been recorded. The ability of compound 1 and a positive control, reserpine, to inhibit NorA-mediated ethidium bromide efflux was potent and concentration dependent. The activity of reserpine corresponded to previous findings[19] in which the IC_{50} of reserpine was found to be 10 μM. Several EPIs have been isolated from natural sources. Many of these do not find uses in the clinic due to toxicity, as is the case with reserpine.[6] Previous findings have shown that methoxylated flavones and isoflavones of similar structure to the kaempferol moiety of compound 1 potentiate the activities of the antimicrobial alkaloid berberine and the fluoroquinolone norfloxacin in *S. aureus*.[20,21] However, their potentiation activity is considerably less at 25 and 6.25 mg/L than for compound 1 at 1.56 mg/L, indicating that the structural differences between the kaempferol moiety of compound 1 as a whole and the flavonols is important for activity.

The rather potent inhibitory activity of compound 1 towards NorA ethidium bromide efflux is among the highest found in this MDR system and is similar in magnitude to the various inhibitors found by Markham *et al.*[22] An MIC determination was carried out and compound 1 exhibited antibacterial activity at 25 mg/L (~35 μM). This is within the concentration range assayed in the efflux inhibition evaluation, but approximately 17 times higher than the IC_{50} value of ethidium bromide efflux inhibition. Many EPIs from natural sources have been found to possess intrinsic antibacterial activity and therefore care must be taken in interpreting the results.[6] The significantly lower concentration needed to inhibit ethidium bromide efflux makes it unlikely that the antibacterial activity of the EPI contributes to the efflux-inhibitory activity.

To further address presumed NorA inhibitory activity, everted membrane vesicles enriched with NorA were constructed for studying test compounds in a system without intracellular interfering pathways. Investigation of the ability of compound 1 and the control verapamil to inhibit the efflux of Hoechst 33342 produced a time-dependent increase in fluorescence, consistent with inhibition of Hoechst 33342 efflux. Compound 1 at 2 mg/L (2.76 μM) caused 40% inhibition of the efflux of Hoechst 33342. This inhibition response was several fold larger than the inhibition by verapamil tested at approximately double the concentration. Strong interference of fluorescence from the more potent control reserpine prevented its use in this assay (data not shown).

We observed quenching of Hoechst 33342 when adding compound 1 or verapamil to unenergized vesicles as well as the energized control vesicles. This quenching was in both cases increased with higher concentrations of EPI and was not due to the solvent DMSO. The change was instant and not time dependent.

In the vesicles containing NorA, disruption of PMF is unlikely as Mg^{2+}-ATP was not added and PMF was therefore not generated. The difference in the magnitude of this interference may be explained by the differences between the two types of vesicle. One is enriched with NorA and contains up to 5–10% of this protein in the membrane and the other lacks NorA; the two

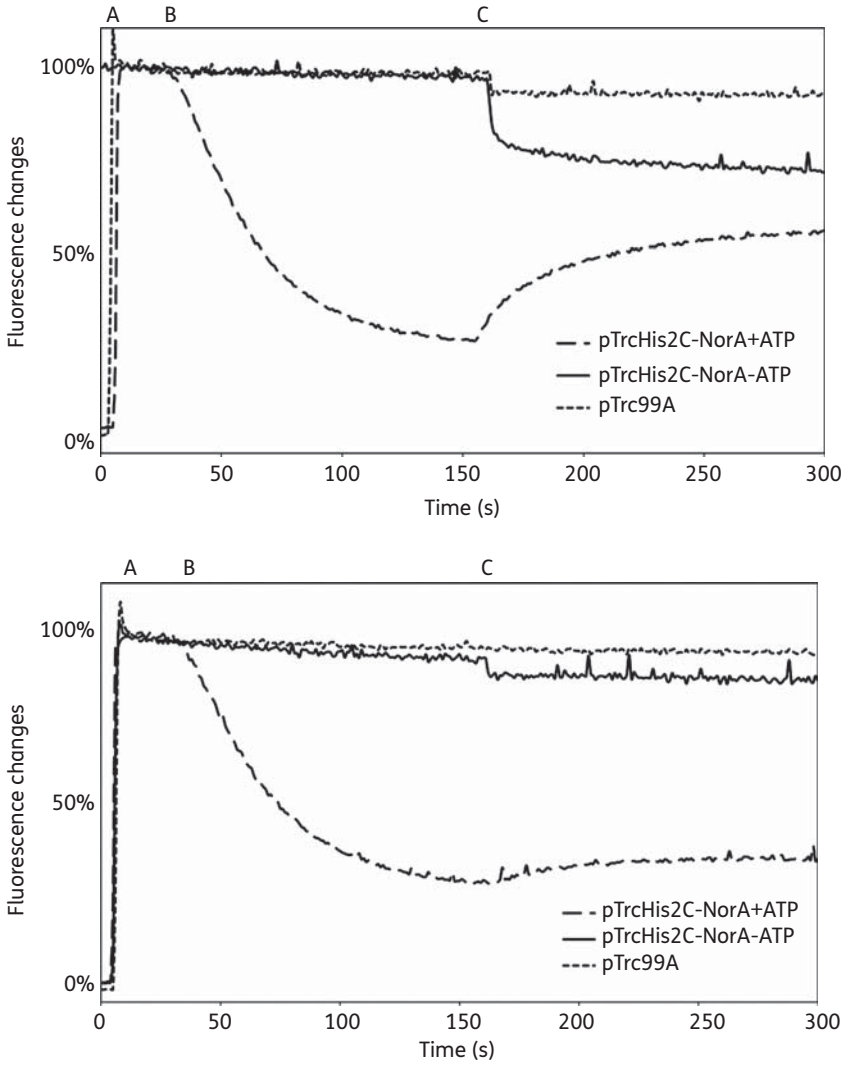

Figure 4. Hoechst 33342 efflux inhibition in pTrcHis2C-NorA vesicles with (broken line with long dashes) and without (continuous line) addition of ATP and pTrc99A control vesicles (broken line with short dashes). Top panel: Hoechst 33342 efflux inhibition with compound 1. A, 100 nM Hoechst 33342; B, 50 μM Mg^{2+}-ATP; C, 2 μg/mL compound 1 (2.76 μM). Bottom panel: Hoechst 33342 efflux inhibition with verapamil. A, 100 nM Hoechst 33342; B, 50 μM Mg^{2+}-ATP; C, 5 μM verapamil.

types of vesicle thus constitute different matrices for Hoechst accumulation and equilibrium in the membrane.[15]

We investigated the effect of inhibitors on the control vesicles (pTrc99A) using Acridine Orange instead of Hoechst 33342 to observe whether changes to the pH and electrical gradient occurred without NorA present. We found a rapid, time-independent quenching of Acridine Orange fluorescence when adding compound 1, as we did when adding compound 1 in the Hoechst 33342 experiment with NorA present.

Since Acridine Orange is a substrate for many MDR pumps, it is likely that the decrease in fluorescence may arise from competitive inhibition of such a pump with the EPIs and, as previously reported by Palmgren,[23] a rapid decrease in Acridine Orange fluorescence can be the result of dimerization of free dye accumulated in the membrane when the concentration of the dye is increased. Therefore, it is questionable whether quenching of Acridine Orange is quantitatively correlated with the proton flux, and it is therefore unlikely that compound 1 has an effect on the PMF.

Addition of verapamil to control vesicles after generation of a pH gradient afforded an increase in fluorescence. This is consistent with inhibition of the H^+-ATPase-generated transmembrane proton electrochemical gradient by verapamil.[24] The solvent DMSO was not responsible for the interference with fluorescence.

According to the two efflux inhibitor assays, compound 1 inhibited NorA and consequently a lower MIC value for antibiotics that are substrates for the pump was expected. We investigated the NorA overexpresser SA1199B as well as an ATCC strain (29213) and K1758, a *norA* knockout strain. Potentiation studies revealed that compound 1 at 1.56 mg/L synergistically increased the antimicrobial activity of ciprofloxacin 8-fold against the NorA overexpresser SA1199B and the synergistic activity was exerted at a fourth the concentration necessary

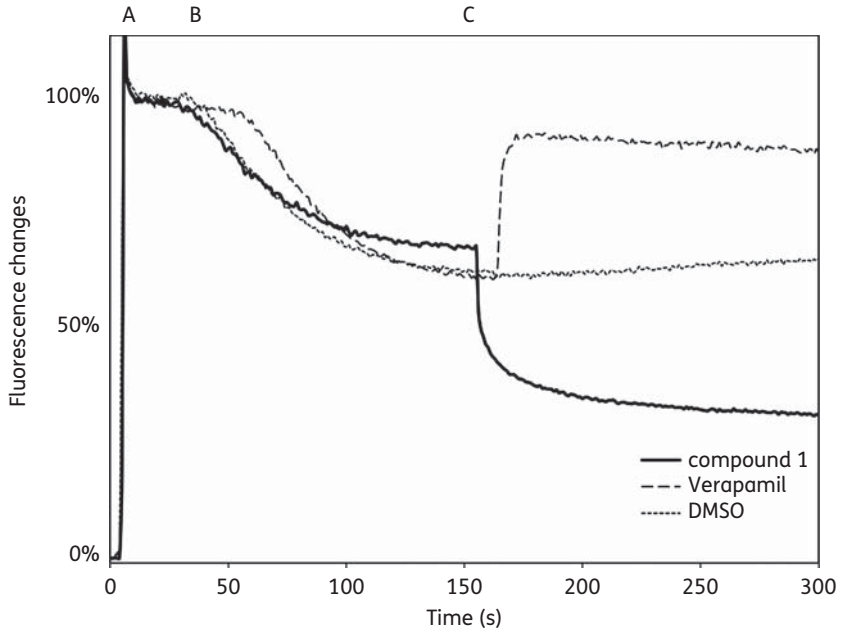

Figure 5. Measurement of fluorescence changes of Acridine Orange in pTrc99A control vesicles upon addition of compound 1 (continuous line), verapamil (broken line with long dashes) and DMSO (broken line with short dashes). A, 500 nM Acridine Orange; B, 50 μM Mg^{2+}-ATP; C, 5 μM verapamil or 2 μg/mL compound 1 or 0.5% DMSO final concentration.

Table 1. Results of potentiation studies of compound 1 with ciprofloxacin against *S. aureus* strains with different expression levels of *norA*; reserpine and verapamil were included as positive controls

	K1758 *norA*−		ATCC 29213		SA1199B		FIC index	FIC index
Compound(s)	MIC	EPI	MIC	EPI	MIC	EPI	(ATCC 29213)	(SA1199B)
ciprofloxacin	0.1	0	0.39	0	6.25	0	—	—
+compound 1	0.1	1.56	0.10	0.78	0.78	1.56	0.28	0.19
+reserpine	0.1	6.25	0.10	6.25	0.78	6.25	0.50	0.37
+verapamil	0.2	25	0.39	25	6.25	25	NAP	NAP

NAP, no antimicrobial potentiation.
For each strain two columns are shown representing the MIC of ciprofloxacin and the corresponding EPI concentration to produce this MIC. MIC and EPI are depicted as mg/L. The results show the combinations that produce a decrease of at least 4-fold in ciprofloxacin MIC. FIC index ≤0.5=synergy.

for reserpine. This finding is consistent with the ratio of IC_{50} values for the two compounds. Compound 1 was not found to exert a synergistic effect on ciprofloxacin in the K1758 *norA* deletion mutant, further confirming the EPI activity of compound 1 towards the NorA efflux pump. FIC indices showed synergic combinations of both inhibitors with ciprofloxacin against the ATCC and SA1199B strains.

Verapamil did not show synergy within assay limits for these strains, due to low concentrations, as this EPI is of lower potency compared with compound 1 and reserpine.[25]

In contrast to earlier findings that compound 1 has MIC values of 1–2 mg/L for a selection of clinically isolated MRSA strains,[11,12] we found that compound 1 had MIC values of 25 mg/L for the *S. aureus* strains used in our study. This might be explained by the use of different strains. In addition, no control of the methanol dilution and a small difference in inoculum size (10^5 cfu/mL) compared with the 5×10^5 cfu/mL of this study could also have an effect; this warrants investigation. Liu *et al.*[26] have demonstrated synergy between compound 1 and several fluoroquinolones towards various *S. aureus* strains, data that indirectly confirm that compound 1 is also an inhibitor of NorA. They hypothesized that compound 1 mainly acts by targeting topoisomerase IV.[26] Our data, based on the lack of cytosolic machinery in the everted membrane vesicles, strongly support that compound 1 acts through NorA inhibition and not at the expression level of the gene.

In summary, we found that compound 1 is a potent inhibitor of NorA-mediated efflux of ethidium bromide in whole cells and Hoechst 33342 efflux in enriched everted membrane vesicles. Moreover, compound 1 synergistically increased the antibiotic effect of ciprofloxacin against a wild-type and a NorA overexpresser without affecting a *norA* knockout strain.

For further development of compound 1 into an antibiotic adjuvant, it should be noted that the flavonoid glycoside

structure of compound 1 is prone to be deglycosylated in the small intestine by β-glucosidases and hence activity might be compromised.[27] Yet it is possible that the kaempferol moiety is indeed the active entity and compound 1 may therefore be utilized as a prodrug. If activity is compromised through oral administration, compound 1 might find uses as a topical remedy in combination with ciprofloxacin. The very high incidence of *S. aureus* infections on the skin, which are observed particularly with community-associated MRSA and may progress to life-threatening diseases,[28] makes this formulation a putative treatment option. In the study by Ibrahim *et al*.[13] the *in vivo* effect of the 2,3 *p*-coumaroyl isomer of compound 1 administered intramuscularly was evaluated and no acute toxicity was evident. This suggests that compound 1 would not exert acute toxicity either, and such toxicity would be even less likely if the compound were used as a topical formulation. Our results provide further evidence that natural products may serve as a source of new and effective EPIs that eventually may be employed to combat MDR microorganisms.

Acknowledgements

We thank Glenn W. Kaatz (John D. Dingell VA Medical Center, Detroit, MI, USA) and David C. Hooper (Division of Infectious Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA) for providing strains. In addition, we especially thank Hiroshi Nikaido (Department of Molecular and Cell Biology, University of California, Berkeley, CA, USA).

Funding

The project was funded by Danish Government Funds.

Transparency declarations

None to declare.

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