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Novel structural analogues of piperine as inhibitors of the NorA efflux pump of *Staphylococcus aureus*

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***Objectives*: Evaluation of novel synthetic analogues of piperine as inhibitors of multidrug efflux pump NorA of *Staphylococcus aureus*.**

***Methods*: A library of piperine-derived compounds was evaluated for their potential to inhibit ethidium bromide efflux in NorA-overexpressing *S. aureus* SA 1199B. The active compounds were then individually combined with ciprofloxacin to study the potentiation of ciprofloxacin's activity.**

***Results*: Based on the efflux inhibition assay, a library of 200 compounds was screened. Three piperine analogues, namely SK-20, SK-56 and SK-29, were found to be the most potent inhibitors of the NorA efflux pump. These inhibitors acted in a synergistic manner with ciprofloxacin, by substantially increasing its activity against both NorA-overexpressing and wild-type *S. aureus* isolates. These analogues were 2- to 4-fold more potent than piperine at a significantly lower minimal effective concentration. Furthermore, these inhibitors also significantly suppressed the *in vitro* emergence of ciprofloxacin-resistant *S. aureus*.**

***Conclusions*: A newly identified class of compounds derived from a natural amide, piperine, is more potent than the parent molecule in potentiating the activity of ciprofloxacin through the inhibition of the NorA efflux pump. These molecules may prove useful in augmenting the antibacterial activities of fluoroquinolones in a clinical setting.**

Keywords: ciprofloxacin, microbial resistance, efflux pump inhibitors

Introduction

Bacterial multidrug efflux pumps are the major contributors of microbial resistance to several classes of antibiotics.[1,2] Efflux of an antibiotic confers an environment of greater selection of resistant mutants having mutations in drug targets.[3–5] By interfering with the development of resistance, clinical use of some antibiotics can be enhanced. The problem of antibiotic efflux can be overcome by addressing any of the following four strategies: (i) inhibiting drug binding to the cytoplasmic membrane pumps; (ii) inhibiting interaction of different components of a multicomponent pump; (iii) targeting energy sources of a pump; and (iv) targeting the regulatory network that controls the expression of efflux pumps.[6] As the inhibition of an efflux pump can potentially improve the clinical efficacy of an antibiotic and simultaneously decrease the selection of resistant mutants, pharmaceutical companies and research institutes are therefore focusing on identifying novel efflux pump inhibitors (EPIs), which may be clinically useful.[5,7] *Staphylococcus aureus* is a common cause of nosocomial infections and is less susceptible to hydrophilic quinolones due to their active expulsion from the cells by the NorA multidrug resistant (MDR) efflux pump.[8] There has been a continuous search for EPIs that can restore the activity of hydrophilic quinolones by inhibiting the NorA MDR efflux pump.[9–16] We have previously described the role of piperine, a major constituent of *Piper nigrum*, as a putative bacterial EPI.[12] Based on this study, a library of piperine analogues (the SK series) was synthesized and evaluated for the inhibition of the NorA efflux pump of *S. aureus*.[17] The present communication is an in-depth comparative study of piperine with some of the selected potent compounds of this series.

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Materials and methods

Chemicals

A structurally diverse library of ~200 compounds was synthesized.[17] Piperine, ethidium bromide and ciprofloxacin were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Bacterial strains and media

S. aureus ATCC 29213 was obtained from the American Type Culture Collection (Manassas, VA, USA). NorA-overproducing *S. aureus* strain SA 1199B[8,18,19] and *S. aureus* SA 1199 (wild-type) were generously provided as a gift by Dr G. W. Kaatz (Wayne State University). Mueller–Hinton broth (MHB; Becton–Dickinson, Cockeysville, MD, USA) supplemented with calcium (25 mg/L) and magnesium (12.5 mg/L) was used for screening, MIC determination and kill kinetic experiments. Mueller–Hinton agar (MHA; Becton–Dickinson) was used for mutation studies. Trypticase soy agar (TSA; Becton–Dickinson) was used for culturing of bacteria and cfu counts.

Screening for EPIs

Since the objective of this study was to evaluate the efficacy of piperine-like molecules as EPIs for NorA inhibition, *S. aureus* SA 1199B, a NorA-overexpressing mutant, was employed. The efflux of ethidium bromide, a well-known substrate for NorA, was used as a marker. Assays were performed in 96-well microtitre plates (Nunc, Denmark). The stock solutions of compounds were prepared in 100% dimethyl sulphoxide (DMSO); the highest final concentration of DMSO used in assays (<1%, v/v) caused no inhibition of bacterial growth (data not shown).

Test compounds (EPIs) were added to a final concentration of 50 mg/L. Ethidium bromide was added to a final concentration of 12.5 mg/L (1/4 MIC for *S. aureus* SA 1199B). Bacteria at logarithmic growth phase were suspended in normal saline (0.85%) to an optical density of ~0.1 at 625 nm corresponding to 1.5×10^8 cfu/mL. This inoculum was further diluted and a final inoculum of 1×10^5 cfu/mL in cation-adjusted MHB was added to the plate. Plates were incubated for 18 h at 37°C and observed visually for growth. Compounds that inhibited the growth were subsequently tested at a 2-fold serially diluted concentration range of 50–0.8 mg/L to obtain the optimal concentration of the compound that altered the MIC.

Determination of minimum effective concentrations (MECs) of the EPIs

The MIC of ciprofloxacin was determined for *S. aureus* SA 1199 and *S. aureus* SA 1199B in MHB in the presence of increasing amounts of EPIs by a broth chequerboard synergy method in microtitre plates using 2-fold serial dilutions.[5] Each candidate EPI was tested at seven different concentrations (50 to 0.8 mg/L) and ciprofloxacin was tested at 10 different concentrations (16 to 0.03 mg/L). The plates were incubated for 18 h at 37°C and the wells were assessed visually for growth. The MEC was determined to be the minimal concentration of EPI that produced the maximal reduction in MIC of ciprofloxacin. No further decrease in MIC was observed at EPI concentrations greater than the MEC.[13]

Time–kill studies

Kill kinetics of ciprofloxacin in the presence of EPIs was evaluated in MHB by a time–kill curve method.[20] *S. aureus* SA 1199B was used as the test bacterium in this assay. The initial bacterial inoculum (1×10^6 cfu/mL) at its logarithmic growth phase was used. Ciprofloxacin was tested at 2 mg/L (1/4 MIC) alone and in combination with EPIs at MEC concentrations as determined earlier (SK-20 and SK-56 at 6.25 mg/L, SK-29 at 12.5 mg/L and piperine at 50 mg/L). Ciprofloxacin was also tested alone at an MIC of 8 mg/L. Cfu/mL was determined by a serial dilution method in triplicate on TSA at 0 (inoculum control), 2, 4, 8 and 24 h of incubation at 37°C. Because of the initial 1:10 dilution of all samples, no antibiotic carryover was observed.

Selection of resistant mutants in vitro

The frequency of ciprofloxacin-resistant mutants was determined as described previously.[21] A bacterial suspension containing 10^9 cfu (100 μL) of *S. aureus* ATCC 29213 was plated on MHA containing ciprofloxacin at concentrations equal to 4, 8 and 16 times the MIC. The same concentrations of ciprofloxacin were also tested in combination with EPIs (SK-20 and SK-56 at 6.25 mg/L, SK-29 at 12.5 mg/L and piperine at 50 mg/L). Mutation frequency was calculated by counting the total number of colonies appearing after 48 h of incubation at 37°C on the drug-containing plate and by dividing the number by the total number of cfu plated.

Efflux studies

Ethidium bromide efflux was determined as previously reported.[22] *S. aureus* SA 1199B was grown overnight on TSA. Bacterial suspensions (optical density of 0.2 at 550 nm) were prepared in uptake buffer (110 mM NaCl, 7 mM KCl, 50 mM NH_4Cl, 0.4 mM Na_2HPO_4, 52 mM Tris base and 0.2% glucose, adjusted to pH 7.5 with HCl). The suspensions were exposed to 2 mg/L ethidium bromide in the presence of EPIs (SK-20 and SK-56 at 6.25 mg/L, SK-29 at 12.5 mg/L and piperine at 50 mg/L) for 30 min at 37°C. The cells were pelleted down by centrifugation and resuspended in fresh buffer. The loss of fluorescence was recorded for 30 min at 5 min intervals at an excitation wavelength of 530 nm and an emission wavelength of 600 nm in a spectrophotometer (Perkin-Elmer model LS50).

Results

Screening of EPIs in S. aureus SA 1199B

A chemical library consisting of ~200 structurally diverse compounds (molecular weights of ~250–500 Da) was screened for inhibition of the NorA efflux pump. Ethidium bromide was chosen as a standard molecule against *S. aureus* SA 1199B, because active efflux represents the only known mechanism of bacterial resistance to ethidium bromide.

In a preliminary experiment, the MICs of the entire library were determined in order to subsequently use these compounds at concentrations devoid of bacterial toxicity and hence to evaluate their efficiency only as EPIs. All the EPIs were found to lack any intrinsic antibacterial activity (MIC > 100 mg/L).

Compounds from the library were tested at a maximum concentration of 50 mg/L, and those displaying at least 4-fold reversal of resistance to ethidium bromide while being non-toxic to the bacteria were selected. Approximately 50 compounds were found to be positive in the preliminary screening. One-fifth of these compounds were at least as potent as piperine, being active at 50 mg/L. Out of these active compounds, five were

found to be more potent inhibitors than piperine. Three analogues, SK-20, SK-29 and SK-56 (Figure 1), that showed better activity than piperine were selected for further studies. The key modifications in the structure of piperine that led to a significantly higher efflux pump inhibition leading to the potentiation of ciprofloxacin's activity may be summarized briefly as: (i) the introduction of an alkyl group (C1–C10) at the C-4 position is the major contributor to potentiation, with ethyl and *n*-propyl contributing maximally; (ii) replacement of a piperidinyl moiety by an aromatic amine such as anisidine or toluidine (with ethyl or *n*-propyl at C-4) showed maximum potentiation, whereas other basic substituents such as aniline, amino esters, pyrollidine, azepine and alkylamines (except isobutyl amine) were less effective; (iii) unsaturation is an important contributor to potentiation, as di- and tetra-hydro derivatives, as well as tetrahydropiperine, showed much less activity than corresponding unsaturated analogues; (iv) amide carbonyl is important for potentiation, as the reduction of this group led to less potentiation—an effect also observed with reduced piperine; and (v) retention of 3,4-methylenedioxyphenyl or 4-methoxyphenyl analogues (with C-4 bearing ethyl or *n*-propyl group) also contributed towards the enhanced potentiation.

Comparative evaluation of piperine analogues as EPIs for the potentiation of ciprofloxacin's activity

The MIC of ciprofloxacin was reduced in the presence of piperine and its analogues. This reduction in MIC was more prominent for *S. aureus* SA 1199B (NorA-overexpressing, ciprofloxacin-resistant) when compared with *S. aureus* SA 1199 (wild-type, ciprofloxacin-susceptible). The most potent EPIs (SK-20 and SK-56) at an MEC of 6.25 mg/L reduced the MIC of ciprofloxacin by 8-fold. Compared with the MEC of piperine, these EPIs were effective at 8-fold lower concentrations. With respect to the fold reduction of the MIC of ciprofloxacin, both SK-20 and SK-56 were equipotent and four times more potent than piperine (Table 1). Interestingly, these compounds were two to four times more potent than standard NorA inhibitors such as reserpine and verapamil, respectively, at 2- to 8-fold lower MEC. Another piperine analogue SK-29 was also found to be two times more potent than piperine but less effective than the other two compounds (Table 1). Further evaluation of these EPIs was performed at the MEC concentration obtained for *S. aureus* SA 1199B.

Effect of EPIs on the kill kinetics of ciprofloxacin

The objective of the time–kill curve assay of *S. aureus* SA 1199B was to assess the bactericidal effect of the combination of ciprofloxacin with EPIs. Ciprofloxacin was used at its subinhibitory concentration of 2 mg/L (1/4 MIC) alone as well as in combination with EPIs. As expected, ciprofloxacin at 2 mg/L did not show any inhibitory activity while the bactericidal activity (99.9% kill) was achieved at 8 mg/L in 6 h. However, the subinhibitory concentration of ciprofloxacin (2 mg/L) resulted in bactericidal activity when tested in combination with selected EPIs at their MEC concentrations. The bactericidal activity of the combination was equivalent to the bactericidal activity of ciprofloxacin alone at 8 mg/L as is evident from Figure 2. Regrowth of bacteria was observed in all the groups after 24 h of incubation. However, even after regrowth, the combination groups of ciprofloxacin with EPIs could retain the bacteriostatic activity and maintained the log cfu less than the initial log cfu at 0 h (Figure 2). Further, the surviving cells from each experiment recovered after 24 h were again tested for their ciprofloxacin and/or EPI susceptibilities. These cells exhibited the same ciprofloxacin MIC (8 mg/L) and similarly exhibited the reduction of ciprofloxacin MIC in the presence of EPIs by chequerboard, taking *S. aureus* SA 1199B as the control.

Frequency of emergence of ciprofloxacin resistance in the presence of EPIs

A mutant selection study was performed on *S. aureus* ATCC 29213, which is a wild-type strain with no reported mutation in the regulatory domain of NorA and drug target domain (DNA gyrase and topoisomerase IV). Ciprofloxacin at 4 mg/L (16 times the MIC), at which no mutant was selected, has been defined as the mutation prevention concentration (MPC). Ciprofloxacin in combination with EPIs resulted in a significantly lower mutation frequency (Table 2), and there was no mutant detected even at a concentration of 2 mg/L. The MPC of the combination was much lower than the C_{max} of ciprofloxacin (3–4 mg/L), indicating the clinical relevance of these combinations in restricting the selection of resistant mutants.

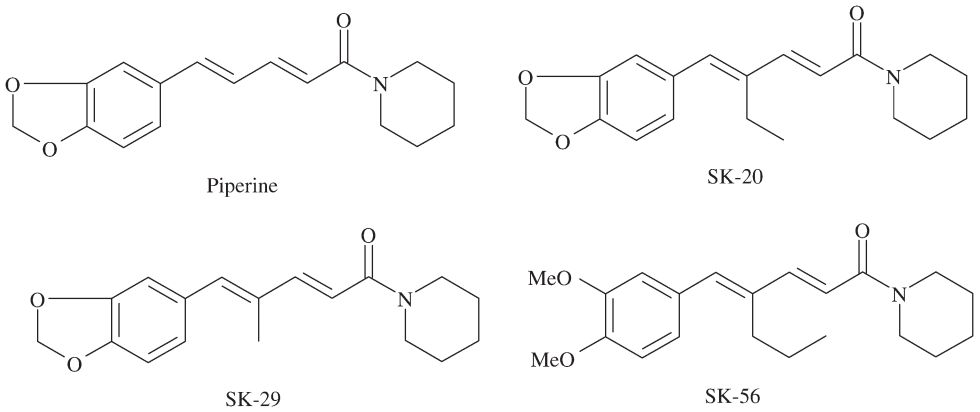

Figure 1. Chemical structures of piperine and its analogues as NorA inhibitors.

Table 1. Effect of EPIs on the activity of ciprofloxacin against *S. aureus* SA 1199 and *S. aureus* SA 1199B

Organism	EPI	MIC of EPI (mg/L)	MEC of EPI (mg/L)	MIC of ciprofloxacin (mg/L)		Reduction (*n*-fold) in MIC of ciprofloxacin
				without EPI	with EPI	
S. aureus SA 1199	piperine	>100	50	0.25	0.12	2
	SK-20	>100	6.25	0.25	0.06	4
	SK-56	>100	12.5	0.25	0.06	4
	SK-29	>100	25	0.25	0.12	2
	reserpine	>100	25	0.25	0.12	2
	verapamil	>100	50	0.25	0.12	2
S. aureus SA1199B	piperine	>100	50	8	4	2
	SK-20	>100	6.25	8	1	8
	SK-56	>100	6.25	8	1	8
	SK-29	>100	12.5	8	2	4
	reserpine	>100	25	8	2	4
	verapamil	>100	50	8	4	2

Effect of EPIs on ethidium bromide efflux

The ability of EPIs to directly inhibit the efflux of ethidium bromide from *S. aureus* SA 1199B was evaluated by using a fluorescence assay.[22] The cells were loaded with ethidium bromide, with and without EPIs, and placed in a fluorimeter cuvette containing fresh medium. The ethidium bromide fluoresces only when it is bound to nucleic acids inside cells. There was a rapid decrease in fluorescence due to NorA-mediated ethidium bromide efflux. Results presented in Figure 3 are the mean values from triplicate samples. As shown in Figure 3, only the control cells without EPIs extruded ethidium bromide, resulting in a significant decrease in fluorescence over the assay period. In the presence of each EPI, loss of fluorescence was significantly reduced, reflecting a strong interference of ethidium bromide efflux by EPIs.

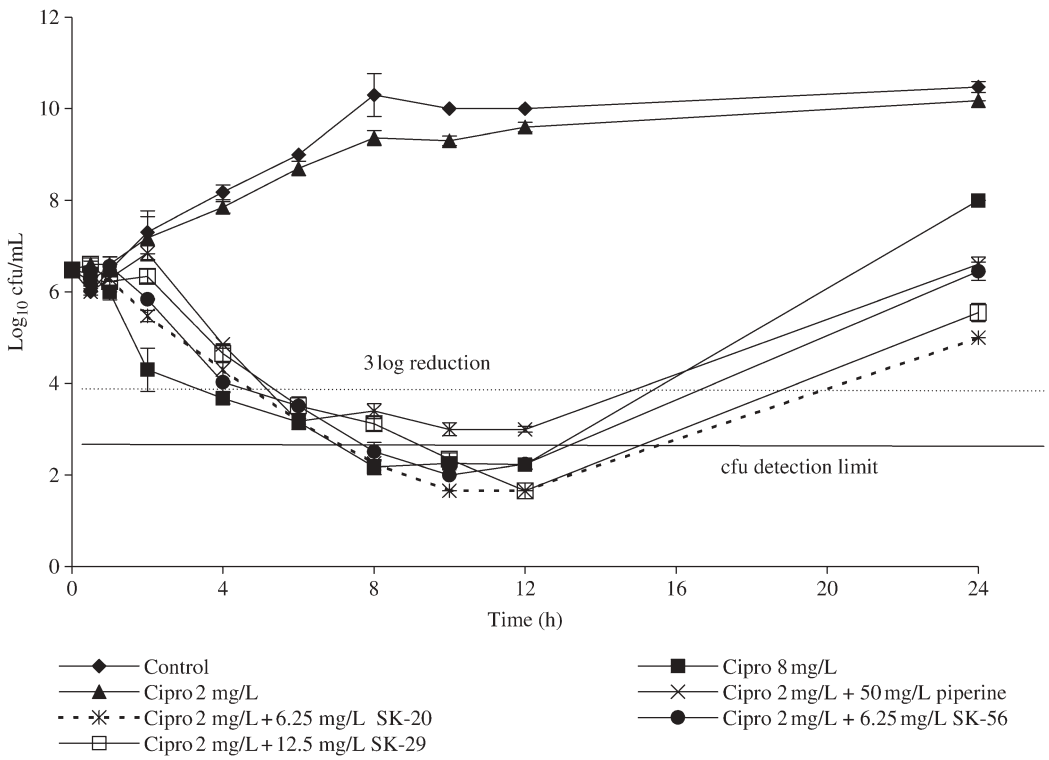

Figure 2. Time–kill curves of *S. aureus* SA 1199B for ciprofloxacin with and without EPIs. Each time point represents the mean $\log_{10} \pm$ SD of three readings. Ciprofloxacin was used at 1/4 MIC (2 mg/L) in combination with EPIs.

Table 2. Frequency of mutation in *S. aureus* ATCC 29213

EPI (mg/L)	Mutation frequency with ciprofloxacin at 2× MIC (0.5 mg/L)	4× MIC (1 mg/L)	8× MIC (2 mg/L)
Without EPI	1.3×10^{-6}	2×10^{-8}	1×10^{-9}
Piperine (50)	8.5×10^{-8}	$<10^{-9}$	$<10^{-9}$
SK-20 (6.25)	1×10^{-9}	$<10^{-9}$	$<10^{-9}$
SK-56 (6.25)	4.5×10^{-9}	$<10^{-9}$	$<10^{-9}$
SK-29 (12.5)	2×10^{-8}	1×10^{-9}	$<10^{-9}$

Discussion

Efflux pumps are primary tools in prokaryotic and eukaryotic cells to remove toxins from the interior of the cell. This protective function enables bacterial cells to survive in hostile environments, including in the presence of antibiotics during the treatment of infections. The up-regulation of efflux systems through physiological induction and spontaneous mutation can significantly lower the intracellular concentration of many antibiotics, causing an impact on clinical efficacy of the antibiotics.[13]

Although there is a limited structural homology between bacterial and mammalian efflux pumps, there is a significant substrate overlap observed.[23] Because of this overlap, it is not surprising that many mammalian MDR inhibitors, such as reserpine, verapamil, GG918 and piperine, also affect bacterial efflux pumps.[23–25] A well-tolerated dually active bacterial and mammalian EPI may have some favourable pharmacological effects, such as: (i) promoting gastrointestinal absorption of antibiotic (altering the pharmacokinetic profile); (ii) improving permeation through the blood–brain barrier for the CNS; (iii) increasing mammalian intracellular antibiotic concentrations for the eradication of invasive pathogens; and (iv) enabling the use of lower concentrations of antibiotics to minimize their undesirable side effects. Such effects could significantly improve antibiotic efficacy by raising physiological levels of an antibiotic and act synergistically by reducing bacterial efflux.[13] In our earlier study, we reported that piperine, a mammalian P-glycoprotein inhibitor, also inhibits bacterial efflux pumps.[12] In the present study, a new series of structurally related analogues of piperine were synthesized in order to obtain new compounds endowed with better activity as bacterial EPIs. These derivatives were then evaluated in *S. aureus* SA 1199B overexpressing the targeted NorA efflux pump. NorA is the initial contributor to the wild-type fluoroquinolone resistance resulting in the emergence of first-step mutants. These mutants decrease the intracellular drug concentrations and bacterial cells subsequently accumulate additional target mutations under treatment, leading to commonly encountered high-level fluoroquinolone-resistant clinical strains.[26] Moreover, NorA is the prototype of other major facilitator superfamily (MFS) pumps with 12 transmembrane segments, such as PmrA in *Streptococcus pneumoniae*,[27] and has generally served as the model for studying EPIs of MDR pumps in Gram-positive organisms.[28]

The newly identified piperine analogues were more potent than the parent molecule in potentiating the activity of ciprofloxacin. The two most active EPIs, namely SK-20 and SK-56, were more potent than known EPIs such as reserpine and verapamil. These newly identified EPIs not only increased the intrinsic susceptibility of *S. aureus* to ciprofloxacin but also significantly reduced the emergence of ciprofloxacin-resistant mutants of *S. aureus*. The recently reported inhibitors of multidrug pump NorA of *S. aureus* include a series of 11 pyrrolo[1,2-*a*]quinoxaline derivatives,[16] *n*-caffeoylphenalkylamide derivatives[29] and 4-[2-(alkylamino)ethylthio]pyrrolo[1,2-*a*]quinoxaline derivatives.[30] We found that the EPIs reported in the present study are more potent inhibitors showing activities at lower concentrations compared with the reported activities of the above-mentioned known inhibitors.

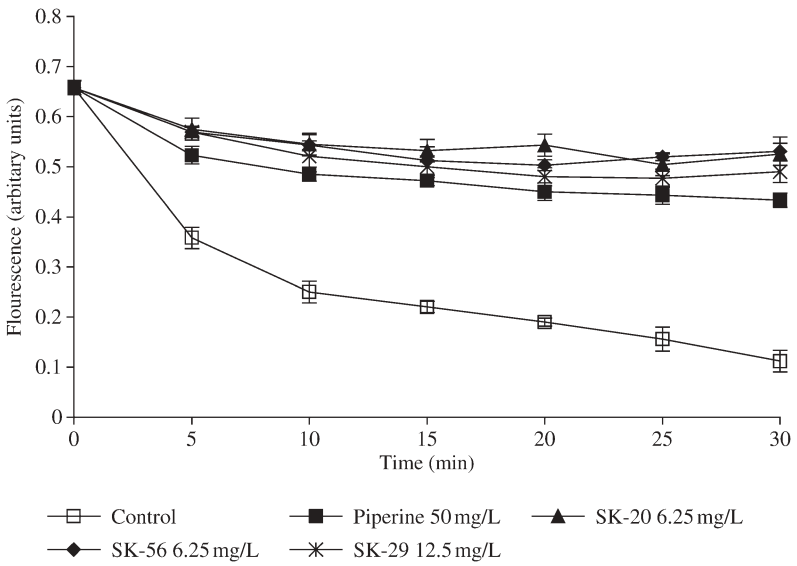

Figure 3. Effect of EPIs on the ethidium bromide efflux from *S. aureus* SA 1199B cells. The cells were loaded with ethidium bromide and the efflux was allowed to occur in the absence of EPI (control) or in the presence of EPIs. Each time point represents the mean $\log_{10} \pm$ SD of three readings.

Currently, there are no EPI/antimicrobial drug combinations on the market, although efforts in identifying potential EPIs are ongoing both in academic institutions as well as in the pharmaceutical industry.[16,29–33] However, identifying EPIs from natural sources is still in its infancy and only a small number of research groups are searching for such efflux inhibitors. No natural products have so far been taken up for further development, as much of the data acquired for such putative EPIs are only preliminary, resulting from potentiation assays and accumulation and efflux studies.[34] The natural molecules offer a number of novel and safe pharmacophores for further chemical modifications. The new class of EPIs reported here in this study are the derivatives of piperine, an amide present in black pepper (*P. nigrum*) and long pepper (*Piper longum*). Piperine is pharmacologically safe and also figures in the US FDA list of 'Generally Regarded as Safe' (GRAS) compounds. This limited series of derivatives of piperine has been synthesized in order to initiate a preliminary structure–activity relationship study for further modifications. These EPIs have no intrinsic antibacterial activity, thereby demonstrating the specificity of their pharmacological effect. Out of the three EPIs reported in this study, SK-20 is currently being evaluated in the pre-clinical safety studies in our institute. In acute toxicity studies, this compound was well tolerated in mice ($LD_{50} > 1$ g/kg body weight) when administered orally (data not shown). The potential clinical utility of this new class of bacterial efflux inhibitors as potentiators of antibiotic activity warrants further investigation.

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Transparency declarations

None to declare.

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