J Antimicrob Chemother 2012; **67**: 2401–2408
doi:10.1093/jac/dks232 Advance Access publication 17 July 2012

Journal of Antimicrobial Chemotherapy

Capsaicin, a novel inhibitor of the NorA efflux pump, reduces the intracellular invasion of *Staphylococcus aureus*

Nitin Pal Kalia[1], Priya Mahajan[2], Rukmankesh Mehra[2], Amit Nargotra[2], Jai Parkash Sharma[3], Surrinder Koul[3] and Inshad Ali Khan[1]*

[1]*Clinical Microbiology Division, Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu 180001, India;* [2]*Discovery Informatics Division, Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu 180001, India;* [3]*Bioorganic Chemistry Division, Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu 180001, India*

*Corresponding author. Tel: +91-191-2569001; Fax: +91-191-2569333; E-mail: iakhan@iiim.res.in

Received 7 March 2012; returned 26 March 2012; revised 27 April 2012; accepted 15 May 2012

Objectives: To delineate the role of capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) as an inhibitor of the NorA efflux pump and its impact on invasion of macrophages by *Staphylococcus aureus*.

Methods: Capsaicin in combination with ciprofloxacin was tested for activity against *S. aureus* SA-1199B (NorA overproducing), SA-1199 (wild-type) and SA-K1758 (*norA* knockout). The role of NorA in the intracellular invasion of *S. aureus* and the ability of capsaicin to inhibit this invasion was established in J774 macrophage cell lines. The three-dimensional structure of NorA was predicted using an *in silico* approach and docking studies of capsaicin were performed.

Results: Capsaicin significantly reduced the MIC of ciprofloxacin for *S. aureus* SA-1199 and SA-1199B. Furthermore, capsaicin also extended the post-antibiotic effect of ciprofloxacin by 1.1 h at MIC concentration. There was a decrease in mutation prevention concentration of ciprofloxacin when combined with capsaicin. Inhibition of ethidium bromide efflux by NorA-overproducing *S. aureus* SA-1199B confirmed the role of capsaicin as a NorA efflux pump inhibitor (EPI). The most significant finding of this study was the ability of capsaicin to reduce the intracellular invasion of *S. aureus* SA-1199B (NorA overproducing) in J774 macrophage cell lines by 2 $\log_{10}$.

Conclusions: This study, for the first time, has shown that capsaicin, a novel EPI, not only inhibits the NorA efflux pump of *S. aureus* but also reduces the invasiveness of *S. aureus*, thereby reducing its virulence.

Keywords: ciprofloxacin, major facilitator superfamily, kill kinetics, post-antibiotic effect, macrophages

Introduction

Bacterial multidrug efflux pumps are the major contributors of microbial resistance to several classes of antibiotics.[1,2] Efflux pumps may be specific for one substrate or may transport various compounds with different structures.[3] The physiological role of efflux pumps appears to be far more complex than merely that of an antibiotic export system, and data are emerging to suggest their importance in the pathogenicity of the organism and/or survival in its ecological niche. Recent reports even document the role of efflux pumps in bacterial survival within professional phagocytes, such as human monocyte-derived macrophages, and some studies have examined *Staphylococcus aureus* survival in neutrophils.[4] Efflux of an antibiotic confers a selective environment for greater selection of resistant mutants having mutations in drug targets.[5–7] 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 the interaction of different components of a multicomponent pump; (iii) targeting the energy sources of a pump; and (iv) targeting the regulatory network that controls the expression of efflux pumps.[8] 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 focusing on identifying novel efflux pump inhibitors (EPIs), which may be clinically useful.[7,9] To date, >10 efflux pumps have been described for *S. aureus*.[10] Most of these pumps belong to the major facilitator superfamily (MFS), namely the chromosomally encoded NorA, NorB, NorC, MdeA and SdrM as well as the plasmid-encoded QacA/B pumps.[11–16] *S. aureus* is less susceptible to hydrophilic quinolones due to their active

expulsion from the cells by the NorA multidrug-resistant (MDR) efflux pump.[17] Apart from NorA, the other recently discovered efflux pumps, NorB and NorC, also contribute to quinolone resistance, particularly resistance to hydrophobic (moxifloxacin and gatifloxacin) quinolones in *S. aureus*. Both of these pumps are negatively regulated by MgrA, a global regulator that positively regulates the NorA pump.[13] There has been a continuous search for EPIs that can restore the activity of hydrophilic quinolones by inhibiting the NorA MDR efflux pump.[18–25] We have previously described the role of piperine, a major constituent of *Piper nigrum*, as a putative bacterial EPI.[21] Further, in our pursuit to identify natural molecules as EPIs we identified capsaicin as a novel EPI of the NorA efflux pump of *S. aureus*.

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the major constituent of hot chilli, a member of the genus *Capsicum*. Because of its interesting pharmacological and toxicological profiles, capsaicin has generated much research interest in the past decade.[26,27] Capsaicin showed an inhibitory effect on the function of P-glycoprotein, which suggests that it can potentially give rise to P-glycoprotein-mediated drug interactions.[28] It also has a wide range of other biological activities in humans, affecting the nervous, cardiovascular and digestive systems as well as finding use as an analgesic.[29] Although possibly detrimental to the human gastric mucosa, capsaicin is also bactericidal to *Helicobacter pylori*.[30] In this report, we describe for the first time the potentiating effect of capsaicin with ciprofloxacin in *in vitro* combination studies against *S. aureus* and its putative role as an EPI. We further examined the influence of capsaicin on the invasiveness of the NorA-overproducing *S. aureus* strain SA-1199B and the *norA* knockout *S. aureus* SA-K1758.

Materials and methods

Chemicals

Capsaicin, ciprofloxacin, ethidium bromide and reserpine were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Bacterial strains, cell lines and growth conditions

S. aureus ATCC 29213 was obtained from the ATCC (Manassas, VA, USA). NorA-overproducing *S. aureus* strain SA-1199B, *S. aureus* SA-1199 (wild-type) and *norA* knockout *S. aureus* SA-K1758 were obtained as a kind gift by Dr G. W. Kaatz (Wayne State University).[17,31–33] Macrophage cell line J774 was gifted by Dr J. N. Agrewala (Institute of Microbial Technology, Chandigarh, India). Cation-adjusted Mueller–Hinton broth (MHB; Becton–Dickinson, Cockeysville, MD, USA) was used for combination studies and kill kinetic experiments. For mutation studies, Mueller–Hinton agar (MHA; Becton–Dickinson) was used. Trypticase soy agar (TSA; Becton–Dickinson) was used for culturing the bacteria and colony counts.

In vitro combination studies for determination of minimum effective concentration (MEC) of capsaicin

The broth chequerboard microdilution method is the most frequently used technique for *in vitro* combination studies.[34] The ciprofloxacin and capsaicin combination was tested in MHB (pH 7.0) against *S. aureus* SA-1199B (NorA overproducing), *S. aureus* SA-1199 (wild-type) and *norA* knockout *S. aureus* SA-K1758. The experiment was performed in 96-well U-bottomed plates (Tarson, India). Ten 2-fold serial dilutions of ciprofloxacin, ranging from 0.03 to 64 mg/L, were prepared in the presence of increasing concentrations of capsaicin (0.8–50 mg/L), in such a way that row B of the microtitre plate received the lowest concentration of capsaicin and row H received the highest concentration. Bacteria grown overnight on TSA plates were suspended in normal saline (0.85%) and the turbidity was adjusted so that it was equivalent to that of a 0.5 McFarland standard, corresponding to 1.5×10^8 cfu/mL. Further dilution of the inoculum in MHB was done in such a manner that each well contained 5×10^5 cfu/mL as a final bacterial inoculum and the plates were then incubated at 37°C for 18 h. Reserpine (a known efflux pump blocker) was used as the control in this study. The MEC of capsaicin that produced the maximal reduction in the MIC of ciprofloxacin was determined.

Time–kill studies

Time–kill studies of ciprofloxacin in the presence of capsaicin were conducted in MHB and evaluated using a time–kill curve method, as described previously by Eliopoulus and Moellering.[35] *S. aureus* SA-1199B was used as the test bacterium in this assay. Bacterial suspension in its logarithmic phase (1×10^6 cfu/mL) was used as the inoculum. Ciprofloxacin at 2 mg/L (0.25× MIC) was tested alone and in combination with capsaicin at the MEC concentration (25 mg/L), as determined above. Ciprofloxacin was also tested alone at an MIC of 8 mg/L. The cfu/mL was determined by a serial dilution method in triplicate on TSA at 0 (untreated 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.

Post-antibiotic effect (PAE)

The PAE was determined by the method described by Craig and Gudmundsson.[36] A final inoculum of 1.5×10^6 cfu/mL of *S. aureus* SA-1199B was achieved in each tube by adding 0.05 mL of an inoculum with a turbidity equivalent to that of a 0.5 McFarland standard ($\sim1.5\times10^8$ cfu/mL) into 5 mL of fresh broth containing ciprofloxacin at concentrations equivalent to the MIC (8 mg/L), 0.5× MIC (4 mg/L) and 0.25× MIC (2 mg/L) alone and in combination with capsaicin at 25 mg/L. After 2 h of incubation at 37°C, with shaking, samples (0.005 mL) from each tube were diluted 1:1000 with fresh broth (5 mL) to effectively remove the drug and capsaicin. Viability counts were determined on TSA from each tube before exposure and immediately after dilution (0 h), and then every 1 h until visual turbidity was observed in the control tube. The PAE calculated by the viable count method was the difference in the time for growth in the exposed culture (T) and the corresponding unexposed control (C) to increase by 1 $\log_{10}$ cfu/mL immediately after drug removal, and is represented by the formula $PAE = T - C$.

Selection of resistant mutants in vitro

The mutation prevention concentration (MPC) of ciprofloxacin against *S. aureus* ATCC 29213 was determined as described previously.[37] A bacterial suspension of 10^9 cfu (0.1 mL) was plated onto MHA containing ciprofloxacin concentrations equal to 2×, 4×, 8× and 16× MIC. The same concentrations of ciprofloxacin were also tested in the presence of capsaicin at 25 and 12.5 mg/L, respectively. The 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 then dividing the number by the total number of cfu plated.

Ethidium bromide efflux studies

The fluorometric determination of ethidium bromide efflux from *S. aureus* SA-1199B loaded with ethidium bromide was performed as described previously.[38] Bacterial suspensions (optical density of 0.2 at 550 nm) of overnight-grown *S. aureus* SA-1199B 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 for 30 min at 37°C in the presence of capsaicin at its MEC, i.e. 25 mg/L, and reserpine, a known efflux inhibitor, at 25 mg/L served as an EPI control. 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).

Macrophage invasion assay

The macrophage invasion assay was performed in macrophage cell line J774. The cell line was maintained using RPMI medium supplemented with 10% (v/v) fetal calf serum. The *S. aureus* strains SA-1199, SA-1199B and SA-K1758 were used to infect the cell monolayers. The macrophage cell monolayers ($\sim 10^5$ cells/well) were prepared in 24-well plates and kept at 37°C with 5% CO_2 for 24 h. Cell monolayers were infected with bacteria by adding $\sim 10^6$ cfu/well (multiplicity of infection 10) of freshly grown bacteria in the absence or presence of capsaicin at its MEC (25 mg/L) and keeping at 37°C with 5% CO_2 for 1 h. The infected monolayers were washed with PBS (pH 7.4) and the remaining extracellular bacteria were killed by using 50 mg/L gentamicin for 30 min. Intracellular bacteria, which had been protected from this treatment, were quantified by host-cell lysis in mild detergent, such as 0.25% (w/v) SDS, and plated on TSA for viable colony counting.[39]

Structure prediction of NorA and docking studies with capsaicin

All the computational studies were carried out in the Schrodinger suite 2010 molecular modelling software. The two-dimensional structure of the ligands (capsaicin and reserpine) was built using Maestro.[40] The structures were then converted to their respective three-dimensional structures, with various conformers, tautomers and ionization states determined using the LigPrep and ConfGen modules.[41–44] The three-dimensional structure of NorA predicted by us using glycerol-3-phosphate transporter from *Escherichia coli* (PDBID: 1PW4) as template was used as the receptor for the docking studies.[45] The receptor was prepared for docking using the Protein Preparation wizard. The probable binding sites of the modelled structure were identified using the SiteMap module of the Schrodinger software and a binding site grid was generated.[46] The various conformers thus generated were docked onto the receptor through this grid using the Glide module and flexible docking was carried out for all the conformers in order to determine the binding mode of the ligand. The Extra Precision scoring function of Glide was used for these studies.[47–49] The amino acid sequence of NorA was searched for conserved domains using the Conserved Domain Database of National Center for Biotechnology Information.[50]

Statistical analysis

All experiments were repeated two or three times with three replicates for each condition tested and similar results were obtained on all occasions. The data are expressed as the means ± SD. Differences between two mean values were calculated by Student's *t*-test. A one-way analysis of variance was performed for comparison of multiple means. The chosen level of significance for all statistical tests was $P < 0.05$.

Results

In vitro combination studies

The MIC of ciprofloxacin was determined alone as well as in the presence of capsaicin (Table 1). The MIC of ciprofloxacin was reduced by 2- to 4-fold in the presence of capsaicin. This reduction in the MIC was more prominent for *S. aureus* SA-1199B (NorA overproducing) as compared with *S. aureus* SA-1199 (wild-type) up to 25 mg/L capsaicin. However, no concentration-dependent effect was observed with capsaicin beyond 25 mg/L, as evident in Table 1. *S. aureus* SA-K1758 (*norA* knockout), on the other hand, did not show any reduction in the MIC of ciprofloxacin.

Effect of capsaicin on kill kinetics of ciprofloxacin

The time–kill curve assay of *S. aureus* SA-1199B was performed to assess the bactericidal effect of the combination of ciprofloxacin with capsaicin. Ciprofloxacin was used at its subinhibitory concentration of 2 mg/L (0.25× MIC) alone as well as in combination with capsaicin. As expected, ciprofloxacin at 2 mg/L did not show any inhibitory activity, while 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 capsaicin at its MEC (25 mg/L). The bactericidal activity of the combination was equivalent to the bactericidal activity of ciprofloxacin alone at 8 mg/L, as is evident in Figure 1. Regrowth of bacteria was observed in all the groups after 24 h of incubation. However, even after regrowth, the combination of ciprofloxacin with capsaicin retained bacteriostatic activity and maintained the $\log_{10}$ cfu below the initial log cfu at 0 h (Figure 1). Further, the surviving cells from each experiment recovered after 24 h exhibited the same ciprofloxacin MIC (8 mg/L) and similarly exhibited a reduced ciprofloxacin MIC in the presence of capsaicin (data not shown).

PAE

The PAE is the phenomenon of continued suppression of bacterial growth after a short exposure of bacteria to antimicrobial agents. The PAE of ciprofloxacin alone and in combination with capsaicin was determined against *S. aureus* SA-1199B. Ciprofloxacin alone exhibited a PAE of 0.3, 1.0 and 1.3 h at 2 mg/L

Table 1. *In vitro* ciprofloxacin/capsaicin combination studies

Capsaicin (mg/L)	MIC of capsaicin	MIC (mg/L) of ciprofloxacin for respective strain with/without test molecule (fold reduction)		
		SA-1199	SA-1199B	SA-K1758
Capsaicin (50)	>100	0.12/0.25 (2)	2/8 (4)	0.125/0.125 (0)
Capsaicin (25)	>100	0.12/0.25 (2)	2/8 (4)	0.125/0.125 (0)
Capsaicin (12.5)	>100	0.25/0.25 (0)	4/8 (2)	0.125/0.125 (0)
Reserpine (25)	>100	0.12/0.25 (2)	2/8 (4)	0.125/0.125 (0)

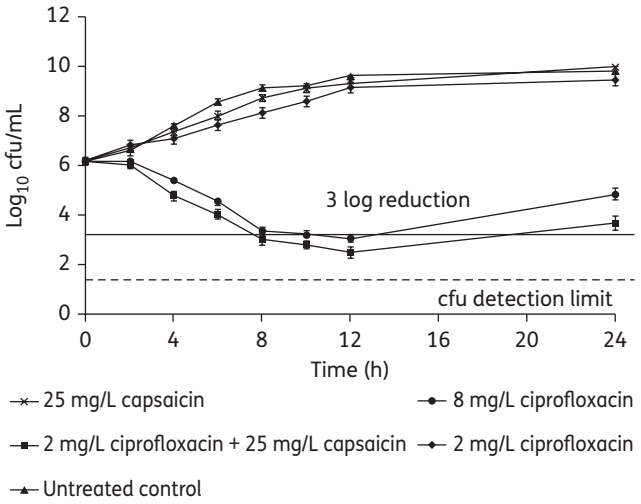

Figure 1. Time–kill curves of *S. aureus* SA-1199B showing the bactericidal effect of ciprofloxacin at 0.25× MIC (2 mg/L) in combination with capsaicin at 25 mg/L. Each timepoint represents the mean $\log_{10} \pm$ SD of three readings.

Table 2. PAE of ciprofloxacin alone and in combination with capsaicin against *S. aureus* SA-1199B after exposure of 2 h

	Mean PAE (h) ± SD		
Regimen	0.25× MIC (2 mg/L)	0.5× MIC (4 mg/L)	MIC (8 mg/L)
Ciprofloxacin	0.3 ± 0.1	1.0 ± 0.1	1.3 ± 0.17
Ciprofloxacin + capsaicin (25 mg/L)	1.0 ± 0.2	1.5 ± 0.17	2.4 ± 0.2

(0.25× MIC), 4 mg/L (0.5× MIC) and 8 mg/L (MIC), respectively. The same concentrations of ciprofloxacin in combination with capsaicin (25 mg/L) resulted in significantly lengthened PAEs of 1.0, 1.5 and 2.4 h, respectively (Table 2).

Frequency of emergence of ciprofloxacin resistance in the presence of capsaicin

The minimum concentration of a drug at which no mutant is selected is defined as its MPC. 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 the drug target domain (DNA gyrase and topoisomerase IV). Ciprofloxacin at 4 mg/L (16× MIC), at which no mutant was selected, has been defined as the MPC. When tested in combination with capsaicin at 12.5 and 25 mg/L, the MPC of ciprofloxacin was reduced to 2 and 1 mg/L, respectively (Table 3). The MPC of the combination was found to be 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.

Table 3. Mutation frequency of *S. aureus* ATCC 29213

	Mutation frequency with ciprofloxacin			
Capsaicin (mg/L)	2× MIC (0.5 mg/L)	4× MIC (1 mg/L)	8× MIC (2 mg/L)	16× MIC (4 mg/L)
0	1.47×10^{-9}	7.7×10^{-9}	4.3×10^{-9}	$<10^{-9}$
12.5	13.5×10^{-9}	3.9×10^{-9}	$<10^{-9}$	$<10^{-9}$
25	3.4×10^{-9}	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$

Effect of capsaicin on ethidium bromide efflux

The ability of capsaicin to directly inhibit the efflux of ethidium bromide from *S. aureus* SA-1199B was evaluated by using a fluorescence assay. The cells were loaded with ethidium bromide, with and without capsaicin, and placed in a fluorimeter cuvette containing fresh medium. Ethidium bromide fluoresces only when it is bound to nucleic acids inside cells. There was a rapid decrease in the fluorescence due to NorA-mediated ethidium bromide efflux. As shown in Figure 2, only the control cells without capsaicin extruded ethidium bromide, resulting in a significant decrease in fluorescence over the assay period. In the presence of capsaicin, the loss of fluorescence was significantly reduced, reflecting a strong interference with ethidium bromide efflux by capsaicin.

Macrophage invasion assay

The invasiveness of *S. aureus* was examined in the presence of capsaicin at its MEC (25 mg/L). The intracellular invasion of *S. aureus* was nearly 2 $\log_{10}$ higher in NorA-overproducing *S. aureus* SA-1199B compared with the wild-type SA-1199 (Figure 3). *S. aureus* SA-K1758, with the *norA* gene deleted, failed to penetrate macrophage cells. In the presence of

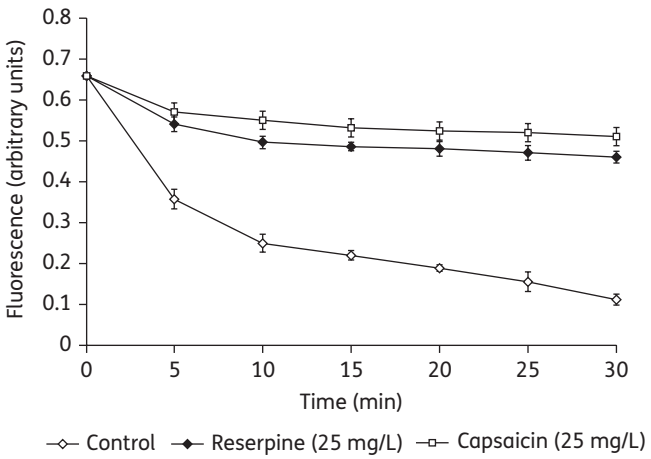

Figure 2. Fluorescence-based determination of ethidium bromide efflux by *S. aureus* SA-1199B cells in the presence and absence (control) of capsaicin at 25 mg/L. Reserpine at 25 mg/L was used as a known EPI control. Each timepoint represents the mean $\log_{10} \pm$ SD of three readings.

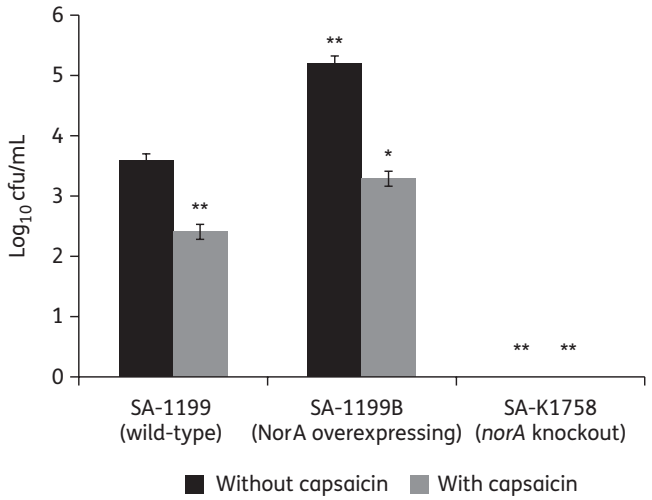

Figure 3. Influence of capsaicin (25 mg/L) on the invasive capacities of *S. aureus* wild-type (SA-1199), NorA-overproducing *S. aureus* SA-1199B and the *norA* knockout *S. aureus* SA-K1758. The invasiveness of SA-1199 and SA-1199B was significantly reduced in the presence of 25 mg/L capsaicin. The assay was performed in triplicate and results are expressed as the means ± SD. $^{*}P<0.05$ and $^{**}P<0.01$ when compared in the absence of capsaicin.

capsaicin the invasiveness of *S. aureus* was reduced. The invasiveness of NorA-overproducing *S. aureus* SA-1199B was reduced to a greater extent (2 $\log_{10}$) compared with the wild-type *S. aureus* SA-1199.

Docking of capsaicin to the active site of NorA

From the docking studies of reserpine (known inhibitor) and capsaicin in the binding site of NorA, it was observed that Arg98 and Ile23 are involved in key interactions and hence play an important role in ligand binding (Figure 4a and b).[23] Also, a hydrophobic cleft formed by Leu26, Val44 and Phe47 at one end, and by Pro24, Phe140, Ile244, Gly248 and Phe303 at the other (particularly for capsaicin) provides some extra stability to the complex. An H-bond formation between reserpine and Gln51 is further supposed to provide strength to the reserpine/NorA complex. A strong H-bond interaction at a distance of 1.89 Å with Ile23 of the protein NorA is observed in case of capsaicin. The orientation of capsaicin within the binding site (as shown in Figure 4b) allows its aliphatic chain to extend into the hydrophobic cleft involving residues Pro24, Phe140, Ile244, Gly248 and Phe303, which enables strong hydrophobic interactions due to the close proximity of the ligand with these residues (distance range: 1.7–3.2 Å). A weak H-bond between Arg98 and the hydroxyl group of the aryl moiety of capsaicin may be attributing extra stability to the capsaicin/NorA complex. The location of key interactive residues in the case of capsaicin with respect to the orientation of the helices is shown in Figure 4c. Multiple sequence alignment of NorA with protein sequences belonging to the MFS transporters revealed that Val44, Phe47, Gln51, Phe140, Ile244, Gly248 and Phe303 form the residues of the conserved domain of the MFS transporter family (see Figure S1 and Table S1, available as Supplementary data at *JAC* Online).

Discussion

Bacteria have evolved sophisticated mechanisms of resistance, including efficient drug efflux pumps that accommodate a wide range of substrates, both antibacterial and non-antibacterial.[51] The wide specificities of multicomponent, multidrug efflux systems suggest that their runaway overexpression might result in the efflux of intracellular concentrations of many such agents, causing an impact on their clinical efficacy. This therapeutic failure stimulates the search for the development of EPIs as adjuvant therapies.[7] Efflux mechanisms should now be taken fully into account in the evaluation of new antibiotics as well as for the future of chemotherapy in the long term.

Although there is a limited structural homology between bacterial and mammalian efflux pumps, there is a significant substrate overlap observed.[52] 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.[52–54] A well-tolerated dually active bacterial and mammalian EPI may have some favourable pharmacological effects, such as: (i) promoting the gastrointestinal absorption of an 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 the physiological levels of an antibiotic and act synergistically by reducing bacterial efflux.[22] In our earlier study, we reported that piperine, a mammalian P-glycoprotein inhibitor, also inhibits bacterial efflux pumps.[21] In the present study, capsaicin, yet another mammalian P-glycoprotein inhibitor, is evaluated as an inhibitor of the NorA efflux pump of *S. aureus*.[28] Capsaicin was evaluated in *S. aureus* SA-1199B overproducing the target NorA efflux pump, which 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.[55] Moreover, NorA is the prototype of other MFS pumps with 12 transmembrane segments, such as PmrA in *Streptococcus pneumoniae*, and has generally served as the model for studying EPIs of MDR pumps in Gram-positive organisms.[56,57]

Capsaicin not only increased the intrinsic susceptibility of *S. aureus* to ciprofloxacin, but also significantly reduced the emergence of ciprofloxacin-resistant mutants of *S. aureus*. Combination of ciprofloxacin (2 mg/L) and capsaicin (25 mg/L) exhibited a bactericidal effect and a >3 $\log_{10}$ reduction in cfu was observed in 8 h, whereas ciprofloxacin alone exhibited the same effect at the higher concentration of 8 mg/L. Furthermore, capsaicin enhanced the PAE of ciprofloxacin in a concentration-dependent manner. In terms of the percentage increase in PAE of ciprofloxacin, a 233% increase in PAE was observed at 2 mg/L (0.25× MIC) ciprofloxacin in combination with capsaicin. However, the most extended PAE of 2.4 h was observed with ciprofloxacin at 8 mg/L when tested in combination with capsaicin at 25 mg/L (Table 2).

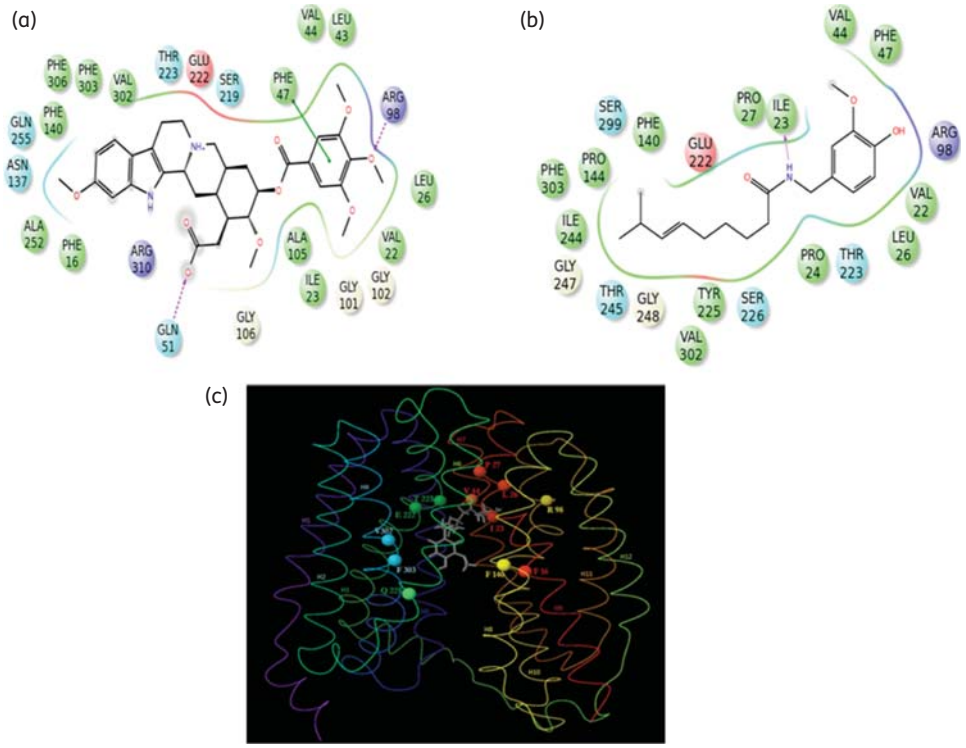

Figure 4. (a) Interaction of reserpine in the binding site of NorA. H-bond formation between reserpine and Gln51 provides stability to the protein/ligand complex. (b) The orientation of capsaicin within the binding site allows its aliphatic chain to extend into the hydrophobic cleft involving residues Pro24, Phe140, Ile244, Gly248 and Phe303, which enables strong hydrophobic interactions due to the close proximity of the ligand with these residues. (c) The relative orientation of the binding site residues with respect to the transmembrane helices of the predicted three-dimensional structure of NorA. All 12 transmembrane helices of the protein are coloured differently and labelled. Capsaicin is also shown bound to the central cavity of the protein. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

The accumulation and efflux of ethidium bromide are good indicators of the involvement of efflux pumps in the resistance mechanism, particularly in Gram-positive bacteria such as *S. aureus*.[21] The fluorescence-based efflux studies of ethidium bromide-preloaded NorA-overproducing *S. aureus* cells in the presence and absence of capsaicin showed reduced efflux in the presence of capsaicin, thus indicating inhibition of the efflux mechanism and confirming capsaicin as another P-glycoprotein inhibitor that inhibits ciprofloxacin efflux from bacterial cells.

The docking results of capsaicin and the *in silico*-predicted three-dimensional structure of NorA revealed the orientation of capsaicin within the binding site (as shown in Figure 4b), which allows its aliphatic chain to extend into the hydrophobic cleft. Also, a weak H-bond between Arg98 and the hydroxyl group of the aryl moiety of capsaicin contribute extra stability to the capsaicin/NorA complex. Apart from the other key interactive residues, it was found that Val44, Phe47, Gln51, Phe140, Ile244, Gly248 and Phe303 form the residues of the conserved domain of the MFS transporter family (see Figure S1 and Table S1, available as Supplementary data at *JAC* Online). Since MFS transporters facilitate the transport of many different substrates, the residues involved in substrate binding may not be strictly conserved among superfamily members.

Though *S. aureus* is not classically considered an intracellular pathogen, gaining an intracellular niche, even briefly, might afford it a window of opportunity to survive and promote disease. In this regard our studies confirmed the role of NorA overproduction in the intracellular invasion of *S. aureus* in macrophage J774 cell lines and, further, the involvement of capsaicin as an EPI in reducing the invasion of NorA-overproducing *S. aureus*. Similar observations have been made by Hirakata *et al.*[58] with diamine compounds in MexAB-OprM-overproducing *Pseudomonas aeruginosa*. They suggested that probably *P. aeruginosa* exports invasion determinants using the MexAB-OprM system and diamine compounds reduce the invasion of MexAB-OprM-overproducing *P. aeruginosa* through the inhibition of this pump.[58]

In conclusion, we have, for the first time, shown that capsaicin, a novel EPI, not only inhibits the NorA efflux pump of *S. aureus* but also reduces the invasiveness of *S. aureus*, thereby reducing its virulence.

Acknowledgements

We are grateful to Dr G. W. Kaatz (Wayne State University School of Medicine, Detroit, MI, USA) for providing *S. aureus* SA-1199, *S. aureus* SA-1199B and *S. aureus* SA-K1758. We are also thankful to Dr J. N. Agrewala (Institute of Microbial Technology, Chandigarh, India) for providing the J774 macrophage cell line.

Funding

This work was funded by the Council of Scientific and Industrial Research, New Delhi, India (grant no. MLP 6003). N. P. K. received a Senior Research Fellowship from the Indian Council of Medical Research, New Delhi, India (80/665/2010-ECD-1).

Transparency declarations

None to declare.

Supplementary data

Figure S1 and Table S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Hooper DC. Efflux pumps and nosocomial antibiotic resistance: a primer for hospital epidemiologists. *Clin Infect Dis* 2005; **40**: 1811–7.

2 Webber MA, Piddock LJV. The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother* 2003; **51**: 9–11.

3 Piddock LJ. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev* 2006; **19**: 382–402.

4 Garzoni C, Kelley WL. *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol* 2009; **17**: 59–65.

5 Van Bambeke F, Balzi E, Tulkens PM. Antibiotic efflux pumps. *Biochem Pharmacol* 2000; **60**: 457–70.

6 Hsieh PC, Siegel SA, Rogers B *et al*. Bacteria lacking a multidrug pump: a sensitive tool for drug discovery. *Proc Natl Acad Sci USA* 1998; **95**: 6602–6.

7 Lomovskaya O, Lee A, Hoshino K *et al*. Use of genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1999; **43**: 1340–6.

8 Kumar A, Schweizer HP. Bacterial resistance to antibiotics: active efflux and reduced uptake. *Adv Drug Deliv Rev* 2005; **57**: 1486–513.

9 Lawrence LE, Barrett JF. Inhibition of bacterial efflux: needs, opportunities, and strategies. *Curr Opin Antiinfect Investig Drugs* 2000; **2**: 145–53.

10 Poole K. Efflux pumps as antimicrobial resistance mechanisms. *Ann Med* 2007; **39**: 162–76.

11 Yoshida H, Bogaki M, Nakamura S *et al*. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. *J Bacteriol* 1990; **172**: 6942–9.

12 Truong-Bolduc QC, Dunman PM, Strahilevitz J *et al*. MgrA is a multiple regulator of two new efflux pumps in *Staphylococcus aureus*. *J Bacteriol* 2005; **187**: 2395–405.

13 Truong-Bolduc QC, Strahilevitz J, Hooper DC. NorC a new efflux pump regulated by MgrA of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2006; **50**: 1104–7.

14 Huang J, O'Toole P, Shen W *et al*. Novel chromosomally encoded multidrug efflux transporter MdeA in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2004; **48**: 909–17.

15 Yamada Y, Hideka K, Shiota S *et al*. Gene cloning and characterization of SdrM, a chromosomally-encoded multidrug efflux pump, from *Staphylococcus aureus*. *Biol Pharm Bull* 2006; **29**: 554–6.

16 Tennent JM, Lyon BR, Gillespie MT *et al*. Cloning and expression of *Staphylococcus aureus* plasmid-mediated quaternary ammonium resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 1985; **27**: 79–83.

17 Kaatz GW, Seo SM, Ruble CA. Efflux mediated fluoroquinolone resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1993; **37**: 1086–94.

18 Gibbons S, Moser E, Kaatz GW. Catechin gallates inhibit multidrug resistance (MDR) in *Staphylococcus aureus*. *Planta Med* 2004; **70**: 1240–2.

19 Kaatz GW, Moudgal VV, Seo SM *et al*. Phenothiazines and thioxanthenes inhibit multidrug efflux pump activity in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2003; **47**: 719–26.

20 Kaatz GW, Moudgal VV, Seo SM *et al*. Phenylpiperidine selective serotonin reuptake inhibitors interfere with multidrug efflux pump activity in *Staphylococcus aureus*. *Int J Antimicrob Agents* 2003; **22**: 254–61.

21 Khan IA, Mirza ZM, Kumar A *et al*. Piperine, a phytochemical potentiator of ciprofloxacin against *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2006; **50**: 810–2.

22 Mullin S, Mani N, Grossman TH. Inhibition of antibiotic efflux in bacteria by the novel multidrug resistance inhibitor biricodar (VX-710) and timcodar (VX-853). *Antimicrob Agents Chemother* 2004; **48**: 4171–6.

23 Schmitz F, Fluit A, Luckefahr M *et al*. The effect of reserpine, an inhibitor of multidrug efflux pumps, on the *in vitro* activities of ciprofloxacin, sparfloxacin and moxifloxacin against clinical isolates of *Staphylococcus aureus*. *J Antimicrob Chemother* 1998; **42**: 807–10.

24 Stermitz FR, Lorenz P, Tawara JN *et al*. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5-methoxyhydnocarpin, a multidrug pump inhibitor. *Proc Natl Acad Sci USA* 2000; **97**: 1433–7.

25 Vidaillac C, Guillon J, Arpin C *et al*. Synthesis of omeprazole analogues and evaluation of these as potential inhibitors of the multidrug efflux pump NorA of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2007; **51**: 831–8.

26 Kang JY, Teng CH, Chen C *et al*. Effect of capsaicin and chilli on ethanol induced gastric mucosal injury in the rat. *Gut* 1995; **6**: 664–9.

27 Park JS, Choi MA, Kim BS *et al*. Capsaicin protects against ethanol-induced oxidative injury in the gastric mucosa of rats. *Life Sci* 2000; **67**: 3087–93.

28 Han Y, Tan TMC, Lim LY. Effects of capsaicin on P-gp function and expression in Caco-2 cells. *Biochem Pharmacol* 2006; **71**: 1727–34.

29 Virus RM, Gebhart GF. Pharmacologic actions of capsaicin: apparent involvement of substance P and serotonin. *Life Sci* 1979; **25**: 1273–84.

30 Jones NL, Shabib S, Sherman PM. Capsaicin as an inhibitor of the growth of the gastric pathogen *Helicobacter pylori*. *FEMS Microbiol Lett* 1997; **146**: 223–7.

31 Kaatz GW, Seo SM, Foster TJ. Introduction of a *norA* promoter region mutation into the chromosome of a fluoroquinolone-susceptible strain of *Staphylococcus aureus* using plasmid integration. *Antimicrob Agents Chemother* 1999; **43**: 2222–4.

32 Kaatz GW, Seo SM. Inducible NorA-mediated multidrug resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1995; **39**: 2650–5.

33 Price CTD, Kaatz GW, Gustafson JE. The multidrug efflux pump NorA is not required for salicylate-induced reduction in drug accumulation by *Staphylococcus aureus*. *Int J Antimicrob Agents* 2002; **20**: 206–13.

34 Eliopoulos GM, Wennersten CB. Antimicrobial activity of quinupristin–dalfopristin combined with other antibiotics against vancomycin resistant enterococci. *Antimicrob Agents Chemother* 2002; **46**: 1319–24.

35 Eliopoulus GM, Moellering RCJ. Antimicrobial combinations. In: Lorian V, ed. *Antibiotics in Laboratory Medicine*, 4th edn. Baltimore: Williams & Wilkins, 1996; 330–6.

36 Craig WA, Gudmundsson S. The postantibiotic effect. In: Lorian V, ed. *Antibiotics in Laboratory Medicine*, 3rd edn. Baltimore: Williams & Wilkins, 1991; 403–31.

37 Drugeon HB, Juvin ME, Bryskier A. Relative potential for selection of fluoroquinolone-resistant *Streptococcus pneumoniae* strains by levofloxacin: comparison with ciprofloxacin, sparfloxacin and ofloxacin. *J Antimicrob Chemother* 1999; **43** Suppl C: 55–9.

38 Brenwald NP, Gill MJ, Wise R. Prevalence of putative efflux mechanism among fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1998; **42**: 2032–5.

39 Cheung AL, Bayles KW. Tissue culture assays used to analyze invasion by *Staphylococcus aureus*. *Curr Protoc Microbiol* 2007; Chapter 9: Unit 9C.4 (pp. 4.1–4.7).

40 *Maestro, Version 9.2*. New York: Schrödinger, 2011. http://www.schrodinger.com/products/14/12/. (27 April 2012, date last accessed).

41 *ConfGen, Version 2.3*. New York: Schrödinger, 2011. http://www.schrodinger.com/products/14/26/. (27 April 2012, date last accessed).

42 *LigPrep, Version 2.5*. New York: Schrödinger, 2011. http://www.schrodinger.com/products/14/10/. (27 April 2012, date last accessed).

43 Watts KS, Dalal P, Murphy RB *et al*. ConfGen: a conformational search method for efficient generation of bioactive conformers. *J Chem Inf Model* 2010; **50**: 534–46.

44 Chen I, Foloppe N. Drug-like bioactive structures and conformational coverage with the LigPrep/ConfGen Suite: comparison to programs MOE and Catalyst. *J Chem Inf Model* 2010; **50**: 822–39.

45 Nargotra A, Sharma S, Datt M *et al*. Structure characterization of NorA, a multidrug resistant efflux pump of *Staphylococcus aureus* using *in silico* approaches. In: *Abstracts of the Sixth International Conference on Bioinformatics, Hong Kong, 2007*. Abstract 33, p. 71.

46 Halgren T. New method for fast and accurate binding-site identification and analysis. *Chem Biol Drug Des* 2007; **69**: 146–8.

47 *Glide, Version 5.6*. New York: Schrödinger, 2010. http://www.schrodinger.com/products/14/5/. (27 April 2012, date last accessed).

48 Friesner RA, Banks JL, Murphy RB *et al*. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* 2004; **47**: 1739–49.

49 Halgren TA, Murphy RB, Friesner RA *et al*. Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. *J Med Chem* 2004; **47**: 1750–9.

50 Marchler-Bauer A, Lu S, Anderson JB *et al*. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res* 2011; **39** (Database issue): D225–9.

51 Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. *Drugs* 2009; **69**: 1555–623.

52 Neyfakh A, Bidnenko VF, Chen LB. Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with the mammalian system. *Proc Natl Acad Sci USA* 1991; **88**: 4781–5.

53 Aeschlimann JR, Dresser LD, Kaatz GW *et al*. Effects of NorA inhibitors on *in vitro* antibacterial activities and post antibiotic effects of levofloxacin, ciprofloxacin, and norfloxacin in genetically related strains of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1999; **43**: 335–40.

54 Gibbons S, Oluwatuyi M, Kaatz GW. A novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*. *J Antimicrob Chemother* 2003; **53**: 13–7.

55 Ba BB, Arpin C, Vidaillac C *et al*. Activity of gatifloxacin in an *in vitro* pharmacokinetic-pharmacodynamic model against *Staphylococcus aureus* strains susceptible to ciprofloxacin or exhibiting various levels and mechanisms of ciprofloxacin resistance. *Antimicrob Agents Chemother* 2006; **50**: 1931–6.

56 Piddock LJ, Johnson MM, Simjee S *et al*. Expression of efflux pump gene *pmrA* in fluoroquinolone-resistant and -susceptible clinical isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 2002; **46**: 808–12.

57 Kaatz GW. Bacterial efflux pump inhibition. *Curr Opin Investig Drugs* 2005; **6**: 191–8.

58 Hirakata Y, Kondo A, Hoshino K *et al*. Efflux pump inhibitors reduce the invasiveness of *Pseudomonas aeruginosa*. *Int J Antimicrob Agents* 2009; **34**: 343–6.