Piperine as an inhibitor of Rv1258c, a putative multidrug efflux pump of Mycobacterium tuberculosis

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Objectives: To evaluate the role of piperine as an inhibitor of Rv1258c of Mycobacterium tuberculosis.

Methods: Rifampicin, in combination with piperine, was tested against M. tuberculosis H37Rv and rifampicin-resistant (rifr) M. tuberculosis. A laboratory-generated rifampicin-resistant mutant (rifr) of M. tuberculosis was tested for drug susceptibility and the expression level of the putative efflux protein (Rv1258c) by real-time PCR. The three-dimensional (3D) structure of Rv1258c was also predicted using an in silico approach.

Results: In the present study, rifampicin in combination with piperine, a trans-trans isomer of 1-piperoyl-piperidine, reduced the MIC and mutation prevention concentration (MPC) of rifampicin for M. tuberculosis H37Rv, including multidrug-resistant (MDR) M. tuberculosis and clinical isolates. Moreover, piperine effectively enhanced the bactericidal activity of rifampicin in time–kill studies and also significantly extended its post-antibiotic effect (PAE). In the presence of rifampicin, M. tuberculosis rifr showed a 3.6-fold overexpression of Rv1258c. The 3D structure of Rv1258c, using in silico modelling, was analysed to elucidate the binding of piperine to the active site.

Conclusions: The results of this study are suggestive of piperine’s involvement in the inhibition of clinically overexpressed mycobacterial putative efflux protein (Rv1258c). Piperine may be useful in augmenting the antimycobacterial activity of rifampicin in a clinical setting.

Keywords: rifampicin, PAE, kill kinetics, MPC, efflux pump inhibitors, docking

Introduction

Mycobacterium tuberculosis infection is a major cause of morbidity and mortality in large parts of the world and is considered to be one of the most important global health problems. Despite the use of vaccine and effective antibiotics, currently 8.8 million new cases of tuberculosis (TB) are diagnosed annually and it still remains one of the most common and deadly infectious diseases worldwide. Rifampicin and isoniazid are the two main drugs used in current first-line anti-TB chemotherapy. Rifampicin is reported to be active against M. tuberculosis in the exponential growth phase and also possesses activity against non-replicating persistent bacilli. Rifampicin inhibits bacterial RNA polymerase activity by binding to the β-subunit encoded by the rpoB gene and forming a stable drug–enzyme complex.

The mechanism by which M. tuberculosis develops resistance to rifampicin has been the subject of intensive research, and it may operate in several ways. The first and foremost important mechanism is the mutational alteration(s) of the rpoB gene. Such mutation(s) can result in the continued function of this essential mycobacterial enzyme despite the presence of concentrations of rifampicin lethal to wild-type strains. The second mechanism may involve the efflux of rifampicin from the cell, which generally results in a lower level of resistance than has been seen in rpoB mutants. For example, several clinical rifampicin-resistant M. tuberculosis isolates do not harbour mutations in the rpoB gene. Therefore, it is evident that other less-well-defined mechanisms could play a crucial role in drug resistance. To combat the increasing drug resistance problem successfully, extensive knowledge of the molecular mechanisms underlying mycobacterial antibiotic resistance is required. The analysis of genome sequences has shown that M. tuberculosis has many open reading frames encoding putative efflux pumps. However, the role of these pumps in intrinsic and acquired resistance has not been elucidated as a major cause of the antibiotic resistance of mycobacteria. Rv1258c is one of the efflux proteins, a proteinaceous active transporter localized in the cytoplasmic
membrane of the cells. Its gene encodes a tetracycline/aminoglycoside resistance (TAP-2)-like efflux pump. Studies have shown that the deletion of this gene from the *Mycobacterium* *bovis* BCG chromosome increases the susceptibility of the organism to these two drugs. This gene plays a role in multidrug-resistant (MDR) TB; the association between drug resistance and transcription levels of this tap-like pump prevents cytosolic accumulation of drugs. Overexpression of Rv1258c, under rifampicin pressure, has been reported previously and thus explains the involvement of this clinically important efflux protein in efflux-mediated rifampicin resistance.

Piperine, the trans-trans isomer of 1-piperoyl-piperidine, is isolated from black pepper. It is reported to be a bioavailability enhancer and has potential immunomodulatory activity. We have previously characterized the contribution of the *Staphylococcus aureus* NorA efflux pump to ciprofloxacin resistance and demonstrated piperine as a NorA efflux pump inhibitor. We now report the effect of piperine/rifampicin combination on *M. tuberculosis*. Several lines of evidence suggest that piperine is an efflux pump inhibitor of Rv1258c of *M. tuberculosis*.

### Materials and methods

#### Bacterial strains and growth conditions

*M. tuberculosis* H37Rv (ATCC 27294) was purchased from the ATCC Collection (Manassas, VA) and used throughout the studies. *M. tuberculosis* rifr was the laboratory-generated rifampicin-resistant mutant used. *M. tuberculosis* was the MDR clinical isolate obtained from the Central JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR, Tajganj, Agra, India). The cultures were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, USA) supplemented with 0.5% (v/v) glycerol, 0.25% (v/v) Tween 80 (Himedia, Mumbai, India) and 10% ADC (albumin–dextrose–catalase, Becton Dickinson, Sparks, MD, USA), with shaking at 0.25% (v/v) Tween 80 (Himedia, Mumbai India) and 10% ADC (albumin–dextrose–catalase, Becton Dickinson, Sparks, MD, USA), with shaking at 37°C until mid-log phase.

#### Drugs

Rifampicin, piperine and reserpine (potency >99%) were purchased from Sigma-Aldrich (St Louis, MO, USA). The drugs were dissolved in DMSO and further diluted in broth to obtain the desired concentration. The final concentration of DMSO was <1% and this concentration had no effect on *M. tuberculosis* growth.

#### In vitro rifampicin/piperine combination studies

The rifampicin/piperine combination studies were performed on *M. tuberculosis* H37Rv, *M. tuberculosis* rifr and *M. tuberculosis*. The MIC of rifampicin was determined in the presence of increasing concentrations of piperine by the broth chequerboard method in microtitre plates. The 2-fold serial dilutions of rifampicin, ranging from 0.03 to 128 mg/L, were tested in combination with piperine at five different concentrations (1.56–25 mg/L). The final bacterial inoculum of 5×10^7 cfu/mL was added to each well. The plates were incubated at 37°C in 5% CO₂ for 3–4 weeks. The minimum concentration of piperine that produced the maximal reduction in the MIC of rifampicin was determined.

#### Time–kill studies

The kill kinetics of rifampicin, in the presence or absence of piperine, were evaluated in Middlebrook 7H9 supplemented with 10% ADC medium. The turbidity of the mycobacterial suspension was adjusted to be equivalent to that of a 1 McFarland standard (~1.5×10^7 cfu/mL) in sterile normal saline containing 0.5% (v/v) Tween 80. A 0.1 mL aliquot of this suspension was used to inoculate 5 mL of Middlebrook 7H9 supplemented with 10% ADC, containing increasing concentrations of rifampicin (ranging from 0.25 to 1 mg/L) individually, as well as in combination with piperine (25 mg/L, the most effective concentration as determined above). The suspension was incubated at 37°C with shaking, and the number of cfu was determined on Middlebrook 7H10 agar supplemented with 10% oleic acid–ADC (OADC; Becton Dickinson), using a serial dilution method at a single timepoint on alternate days for 10 days.

#### Selection of resistant mutants in vitro

The first step mutants of *M. tuberculosis* H37Rv were selected as previously described by Dong et al. The bacterial suspension containing 10^6 cfu (0.1 mL) was plated on Middlebrook 7H10 agar supplemented with 10% OADC and containing rifampicin at different concentrations varying from 0.5 to 2 mg/L individually and in combination with piperine at 25 mg/L. The mutation frequency was calculated by counting the total number of colonies appearing after 28 days of incubation at 37°C in 5% CO₂ on the rifampicin-containing plate divided by the total number of cfu plated.

#### Post-antibiotic effect (PAE)

The PAE was determined by the method described by Chiu et al. A final inoculum of 1.5×10^5 cfu/mL of *M. tuberculosis* was achieved in each tube by adding 0.5 mL of 1 McFarland (~1.5×10^6 cfu/mL of *M. tuberculosis*) adjusted inoculum into 5 mL of fresh broth containing rifampicin at concentrations equivalent to the MIC, 2× the MIC and 4× the MIC, alone and in combination with piperine at 25 mg/L. After 2 h of incubation at 37°C, with shaking, the samples were diluted 1:1000 with fresh broth to effectively remove the drug and piperine. Samples were taken from the flask after every 24 h for 15 days and the number of cfu was determined on Middlebrook 7H10 agar supplemented with 10% OADC. The agar plates were incubated for 3–4 weeks and colonies were counted. The PAE calculated by the viable count method was the difference in time for growth in the exposed culture (T) and the corresponding unexposed control (C) to increase by 1 log₃ cfu/mL immediately after drug removal, and is represented by the formula PAE = T–C.

#### Selection and susceptibility of resistant mutants of *M. tuberculosis*

Rifampicin-resistant mutants were selected by plating ~10^8 cfu of *M. tuberculosis* H37Rv on Middlebrook 7H10 agar supplemented with 10% OADC, containing 2 mg/L rifampicin. A total of 20 colonies were picked up and their susceptibilities to rifampicin were determined (in the presence or absence of 25 mg/L piperine) by the broth microdilution method as described above. One of the rifampicin-resistant mutants showed an MIC of 128 mg/L and was designated as *M. tuberculosis* rifr. This mutant and *M. tuberculosis* H37Rv were used to check the restoration of the MIC of rifampicin and ethidium bromide by piperine using the broth microdilution method. Reserpine (a known efflux pump inhibitor) at 25 mg/L was used as the control in this study.

#### qRT–PCR analysis of the putative efflux pump protein of *M. tuberculosis*

In order to analyse the transcriptional profile of the putative efflux pump Rv1258c, *M. tuberculosis* H37Rv and *M. tuberculosis* rifr were grown to mid-log phase in the presence of a subinhibitory concentration of rifampicin (1/4 the MIC). *M. tuberculosis* H37Rv was included as a control. RNA
was extracted using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. In order to remove DNA contamination, RNA samples were treated with RNase–free DNase. The RNA was dissolved in DNase– and RNase–free water. The primers were designed by using PRIM3R 3 software and these were purchased from Sigma.28 The primer sets and sequences of oligonucleotides used in this study were: Rv1258c FP 5′-GGATGGCTGGTGTTGACCTATC-3′ and RP 5′-GACGTCATCAACCC GACTAC-3′ with a product size of 232 bp; and sigA FP 5′-ATCGTGA ACCCAGGAAAA-3′ and RP 5′-GACCTCTTCTGCGGCGTGTA-3′ with a product size of 177 bp. The real-time reactions were performed using a Light Cycler 1.5 (Roche) using one-step RT–PCR in a total reaction volume of 0.02 mL. The RT–PCR mixture consisted of 10 pmol of each primer, 0.004 mL of one-step RT–PCR mixture containing SVBR green I, 3 mM MgCl₂, 0.0004 mL of Light Cycler RT–PCR Enzyme Mix (Roche) and 0.004 L of extracted RNA (600–800 ng). The real-time PCR run protocol consisted of reverse transcription at 55°C for 10 min, followed by PCR activation at 95°C for 30 s and 45 cycles of amplification (10 s at 95°C, 20 s at 58°C and 25 s at 72°C). Each experiment was performed three times independently. The sigA-normalized gene expression was obtained using the ΔΔCt method with the equation:

\[
\text{normalized expression} (\text{reference gene:target gene ratio}) = \frac{E^{(\text{reference gene})}}{E^{(\text{target})}}
\]

where \(E\) stands for the amplification efficiency of the PCR and was determined using the linear regression equation from the standard curve, \(E = 10^{-1/\text{slope}}\).

### Structure prediction of Rv1258c and docking studies with piperine

The amino acid sequence of Rv1258c of M. tuberculosis H37Rv from the NCBI databank was submitted to the PHYRE Protein Fold Recognition Server 29 and, based on the results, a multidrug transporter EmrD from Escherichia coli (PDB ID: 2GFP) was selected as the template for model building, using the methodology described previously for the NorA efflux pump of S. aureus.29–31 The probable binding sites of the modelled structure were identified using the sitemap module of the Schrödinger software.12 The modelled three-dimensional (3D) structure of the protein was prepared for docking by adding the missing hydrogen, correcting the bond orders followed by the energy minimization (RMSD ('root mean square deviation') = 0.03; force field: OPLS ('optimized potential for liquid simulations') 2005) using the protein preparation wizard of the Schrödinger suite. The missing residues in the protein were corrected. The structural validation of the derived protein was carried out using the structural analysis and validation server.31 The prepared receptor was used for Glide XP docking using the Glide module of the Schrödinger suite; the Glide Receptor Grid Generation panel was used to define the receptor active site, based on the selection of entry of probable binding sites, one by one. For docking studies, two inhibitors, namely reserpine (a known efflux pump inhibitor) and piperine, were used.

### Results

#### In vitro combination studies

The MIC of rifampicin was determined alone as well as in the presence of piperine (Table 1). The MIC of rifampicin was reduced by 4- to 8-fold in the presence of piperine. This reduction in the MIC was more prominent for M. tuberculosis rifr as compared with M. tuberculosis H37Rv. However, piperine on its own did not show any antibacterial activity when tested up to 100 mg/L.

#### Effect of piperine on the kill kinetics of rifampicin

Time–kill kinetics were measured against M. tuberculosis H37Rv to determine the bactericidal effect of the combination of rifampicin with piperine. Rifampicin alone was bactericidal at 1 mg/L. However, at 0.25 mg/L (the MIC) and 0.5 mg/L (2× the MIC) the cfu count failed to reach 3 log reduction during the 10 day study period (Figure 1a). Interestingly 0.25 and 0.5 mg/L concentrations of rifampicin in combination with 25 mg/L piperine were sufficient for bactericidal activity (>3 log reduction). Moreover, these combinations reduced the cfu count of M. tuberculosis H37Rv to below the detection limit (<50 cfu/mL) on day 8 (Figure 1b).

#### PAE

The PAE of rifampicin alone and in combination with piperine was determined against M. tuberculosis H37Rv. Rifampicin alone exhibited a PAE of 12, 24 and 48 h at 0.25 mg/L (the MIC), 0.5 mg/L (2× the MIC) and 1.0 mg/L (4× the MIC), respectively. The same concentrations of rifampicin in combination with piperine (25 mg/L) resulted in significantly higher PAEs of 24, 36 and 72 h (Table 2).

#### Frequency of emergence of rifampicin resistance in the presence of piperine

A mutant selection study was performed using M. tuberculosis H37Rv. The minimum concentration of a drug at which no mutant is selected is defined as its mutation prevention

<table>
<thead>
<tr>
<th>Table 1. In vitro rifampicin/piperine combination studies</th>
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<tr>
<td><strong>Organism</strong></td>
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<tr>
<td>-------------</td>
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<tr>
<td>M. tuberculosis H37Rv</td>
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<tr>
<td>M. tuberculosis rifr</td>
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<tr>
<td>M. tuberculosis clinical isolate</td>
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concentration (MPC). It was observed that the MPC of rifampicin could not be determined up to 2.0 mg/L (8 × the MIC), whereas rifampicin in combination with piperine at 25 mg/L exhibited a significantly lower mutation frequency and there was no mutant detected even at a concentration of 2 mg/L (Table 3). The MPC of the combination was much lower than the $C_{\text{max}}$ of rifampicin (9–16 mg/L), indicating the clinical relevance of these combinations in restricting the selection of resistant mutants.

**In vitro combination studies of piperine and ethidium bromide against mycobacteria**

The MICs of rifampicin and ethidium bromide for *M. tuberculosis* H37Rv and *M. tuberculosis* rifr are given in Table 4. In the

![Figure 1](https://academic.oup.com/jac/article-abstract/65/8/1694/740469)

**Table 2.** PAE (h)\(^a\) of rifampicin alone and in combination with piperine against *M. tuberculosis* H37Rv following 2 h of exposure

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>Without Piperine</th>
<th>With Piperine (25 mg/L)</th>
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<tr>
<td></td>
<td>PAE (h)</td>
<td></td>
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<tr>
<td>0.25 mg/L (MIC)</td>
<td>12 ± 2</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>0.5 mg/L (2× MIC)</td>
<td>24 ± 1</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>1 mg/L (4× MIC)</td>
<td>48 ± 3</td>
<td>72 ± 2</td>
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Values are means ± SEM from three independent determinations.

\(^a\) Viable count method.

**Table 3.** Frequency of mutation in *M. tuberculosis* H37Rv with rifampicin in the presence and absence of piperine

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>Mutation Frequency with Rifampicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2× MIC (0.5 mg/L)</td>
</tr>
<tr>
<td>Without Piperine</td>
<td>$2 \times 10^{-9}$</td>
</tr>
<tr>
<td>With Piperine (25 mg/L)</td>
<td>$1.2 \times 10^{-9}$</td>
</tr>
</tbody>
</table>
M. tuberculosis rif mutant there was a 512-fold increase in the MIC of rifampicin and a >2-fold increase in the MIC of ethidium bromide as compared with that of the wild-type. The increases in MICs of rifampicin and ethidium bromide for both the isolates was reversed by piperine and reserpine. Since efflux is the only known mechanism for ethidium bromide resistance, the reversal of its MIC by piperine indicates its role as an efflux pump inhibitor.

Assessment of expression of Rv1258c in M. tuberculosis by qRT–PCR

We used qRT–PCR assay to measure the mRNA level of the Rv1258c gene in M. tuberculosis H37Rv and M. tuberculosis rif mutant in the presence and absence of 1/4 the MIC of rifampicin. The qRT–PCR analysis revealed that the sigA-normalized expression of the Rv1258c gene in M. tuberculosis rif was significantly induced in the presence of rifampicin whereas the expression was not significant in M. tuberculosis H37Rv (Figure 2).

Docking of piperine to the active site of Rv1258c

The sequence identity of Rv1258c and the template sequence [a multidrug transporter EmrD from E. coli (PDB ID: 2GFP)] was found to be 36%. Using the site map module of the Schrödinger suite, the predicted structure of Rv1258c revealed five probable binding pockets. The docking studies of reserpine, a known inhibitor, and piperine on these binding pockets revealed one of the binding sites showing better binding affinity for these inhibitors. Reserpine and piperine had a Glide score of −3.75 kcal/mol and −5.97 kcal/mol, respectively, showing better binding affinity of piperine for the receptor than reserpine. The figure showing the interaction of piperine with Rv1258c revealed that it is involved in H-bond interaction (2.06 Å) with Arg141 to form a protein–ligand complex (Figure 3a). Furthermore, the 1,3-dioxol ring of the piperine molecule is surrounded by Leu55, Leu56, Gln342 and His343 residues at a favourable distance (a ≤ 3 Å) for H-bonding, thereby providing more stability to the ligand in the pocket (Figure 3b). Additionally, piperine and the heterocyclic ring of His343 are seen to be favourably oriented to interact with a π–π interaction between the two.

Discussion

Rifampicin is the backbone of modern anti-TB chemotherapy. Although mutations in the rpoB gene in rifampicin-resistant M. tuberculosis isolates have been described, there is evidence to suggest that efflux-related rifampicin resistance mechanisms may play a role in M. tuberculosis. Furthermore, rifampicin has been shown to up-regulate the expression of a putative efflux pump Rv1258c in clinical isolates of M. tuberculosis. For the first time, we describe the potentiating effect of piperine on the bioefficacy of rifampicin and its role in putative efflux pump Rv1258c inhibition. A combination of rifampicin (0.5 mg/L) and piperine (25 mg/L) exhibited bactericidal activity and showed a >3 log reduction in cfu in 8 days, whereas rifampicin alone exhibited the same effect at the higher concentration of 1 mg/L. Piperine also prevented the emergence of rifampicin-resistant mutants at a clinically achievable concentration of 0.25 mg/L. Previous findings have revealed the MPC of rifampicin against M. tuberculosis to be >80 mg/L. These results are of significant importance because monotherapy with rifampicin in M. tuberculosis leads to emergence of drug resistance.

Rifampicin is reported to exhibit the longest PAE amongst all first-line anti-TB drugs. In our study, interestingly piperine was found to further extend the PAE of rifampicin in a concentration-dependent manner. The most extended PAE of 72 h was observed with rifampicin at 1 mg/L when tested in combination with piperine.

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. However, uptake of ethidium bromide by slow-growing
mycobacteria is very slow and interpretation of the uptake experiments is complicated by the high amount of surface-adsorbed compounds.\textsuperscript{8,35} This problem is even more pronounced for antibiotics which are substrates for drug efflux pumps.\textsuperscript{14} Since efflux is the only known mechanism of resistance for ethidium bromide, reversal of the MIC of ethidium bromide by any substance is an indication that the substance is probably working as an inhibitor of bacterial efflux pumps.\textsuperscript{36,37} It was observed that there was an increase in the MIC of ethidium bromide in \textit{M. tuberculosis} rifr and that piperine reversed the MIC of ethidium bromide, thus indicating the inhibition of the efflux mechanism. However, piperine did not completely restore the MIC of rifampicin in \textit{M. tuberculosis} rifr because of the mutation in the \textit{rpoB} gene, generally seen as responsible for rifampicin resistance (codon 531, TCG->TTG, Ser->Leu) as was revealed in the sequence analysis of the hotspot region of the \textit{rpoB} gene (data not shown).\textsuperscript{38}

The qRT–PCR analysis of Rv1258c in \textit{M. tuberculosis} rifr grown in the presence of rifampicin (1/4 the MIC) showed a 3.6-fold
increase in the transcription level of this gene as compared with bacilli grown without rifampicin. The induced expression of Rv1258c under rifampicin pressure has also been reported previously.\textsuperscript{13,15} This indicates the involvement of this clinically important efflux protein in the efflux-mediated rifampicin resistance, which may contribute to the emergence of drug resistance. The molecular mechanism of piperine-induced potentiation of rifampicin efficacy was also investigated by a molecular modelling approach. Due to non-availability of the crystal structure of Rv1258c, the 3D structure of this protein was predicted on the basis of homology modelling, using Schrödinger software. The docking studies on the derived protein structure revealed one of the binding pockets showing greater affinity for the inhibitors, i.e. reserpine and piperine. In that binding pocket (probable active site), it was observed that piperine is involved in H-bonding with Arg141, among several other interactions with the binding site residues of the protein. We assume that the involvement of Arg141 contributes to the inhibitory activity of piperine and may facilitate the inhibition of the efflux-related rifampicin resistance mechanism in \textit{M. tuberculosis}.

In conclusion, we have, for the first time, shown that piperine plays a significant role in the inhibition of Rv1258c. In our previous work, we demonstrated piperine and its analogues to be inhibitors of the NorA efflux pump of \textit{S. aureus}.\textsuperscript{20,21} Inhibition of the rifampicin efflux pump Rv1258c by an efflux pump inhibitor can be useful in: (i) lowering the dose administered; (ii) reducing the mutation frequency; and (iii) increasing the efficacy of rifampicin against \textit{M. tuberculosis} isolates. However, the effective concentration of piperine described herein may not be clinically achievable, but this study paves the way for the exploration of more potent inhibitors of Rv1258c, which may result in combinations (drug and an efflux pump inhibitor) with high clinical utility.

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\section*{Transparency declarations}

None to declare.

\section*{References}


30 Nargotra A, Sharma S, Datt M et al. Structure characterization of NorA, a multidrug resistant efflux pump of Staphylococcus aureus using in silico approaches. Abstracts of the Sixth International Conferences on Bioinformatics, Hong Kong, 2007. Abstract 33, p. 71. Hong Kong University of Science and Technology, Hong Kong.


