A novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*

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GG918, a synthetic inhibitor of P-glycoprotein-mediated mammalian tumour multidrug resistance, was found to be equipotent to reserpine in enhancing the in vitro activity of norfloxacin and ciprofloxacin against strains of *Staphylococcus aureus* expressing distinct efflux-related multidrug resistance pumps. Four- to eight-fold reductions in MICs of these fluoroquinolones were observed for SA-1199B, a strain that overexpresses NorA (the major *S. aureus* multidrug transporter), and SA-K2068, which possesses a multidrug efflux-related pump distinct from NorA. Neither inhibitor potentiated the activity of newer fluoroquinolones such as levofloxacin or moxifloxacin by more than two-fold, and this effect was observed only in SA-1199B and SA-K2068. GG918 and reserpine exposure resulted in two- to four-fold reductions in norfloxacin and ciprofloxacin MICs in a fluoroquinolone-susceptible control strain and in strains expressing the MsrA and TetK proteins, which mediate efflux-related resistance to macrolides and tetracyclines, respectively, suggesting inhibition of as yet uncharacterized pumps for which norfloxacin and ciprofloxacin are substrates. In the MsrA- and TetK-expressing strains no more than a two-fold augmentation of erythromycin or tetracycline activity was observed with either inhibitor, suggesting minimal, if any, inhibitory activity against these efflux proteins. Using GG918 as a lead compound, a structure–activity evaluation may reveal a more potent and broader spectrum inhibitor of *S. aureus* antibiotic efflux pumps.

Keywords: multidrug efflux, GG918, *Staphylococcus aureus*

Introduction

Multidrug (MDR) efflux is an increasingly reported phenomenon and has been described for many organisms, including bacteria, fungi and protozoa, and as a mechanism of resistance in mammalian tumour cells.1 Bacteria possess a wide array of drug efflux proteins and a number of clinically relevant species, most notably *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, utilize these transporters as part of their resistance strategy.2 Many of these efflux mechanisms export an extensive range of structurally unrelated antibiotics from the cell, resulting in a reduced intracellular concentration and thus reduced susceptibility. Examples of efflux-related resistance mechanisms that have been described for *S. aureus* include those conferred by QacA and NorA, which are MDR transporters, and the more specific MsrA and TetK transport proteins.3–6 These export proteins were originally described to efflux quaternary ammonium salts (antiseptics), fluoroquinolones, macrolides and tetracyclines, respectively, although these efflux proteins, especially QacA and NorA, actively export a broad array of structurally dissimilar drugs from the bacterial cell.

GG918 (Figure 1) is a synthetic compound that was originally discovered as part of a screening programme designed to identify inhibitors of mammalian P-glycoprotein (P-gp). P-gp is an ABC-type transporter that exports numerous anti-
neoplastic agents from cancer cells, making them drug-resistant.\textsuperscript{1,7} It has been shown that co-administration of GG918 with paclitaxel significantly increases the systemic exposure to this anti-neoplastic agent.\textsuperscript{8} Toxicities associated with GG918 were not observed in this study and the mean maximal serum concentration of the compound was 0.43 $\pm$ 0.27 mg/L.

The mean area under the plasma concentration–time curve of paclitaxel after oral administration of 1 g of GG918 was comparable to that achieved with oral paclitaxel in combination with another P-gp inhibitor, cyclosporin A.\textsuperscript{9} Unlike cyclosporin A, GG918 has no known immunosuppressive activity and may be a better candidate for clinical use as a P-gp inhibitor.

Previous work on inhibitors of MDR pumps in \textit{S. aureus} includes the screening of a synthetic library against the NorA MDR transporter.\textsuperscript{10} These inhibitors acted in a synergic manner with ciprofloxacin and dramatically suppressed the emergence of ciprofloxacin-resistant \textit{S. aureus} upon \textit{in vitro} exposure to the drug. Other inhibitors of MDR pumps in \textit{S. aureus} include the anti-hypertensive plant alkaloid reserpine, the porphyrin phophorbide A, \textit{S}-m ethoxyhydnocarpin D (a flavonolignan) and selected flavones.\textsuperscript{11} The antimicrobial activity of berberine, a natural antibiotic found in some plants and also a NorA substrate, was found to be potentiated by low concentrations of \textit{S}-methoxyhydnocarpin D by inhibition of its efflux.\textsuperscript{12} Identification of effective inhibitors of NorA and other \textit{S. aureus} efflux pumps could restore the clinical utility of pump substrates, which prompted the current investigation of the activity of GG918 as an inhibitor of such pumps.

\section*{Materials and methods}

\subsection*{Bacterial strains and media}

\textit{S. aureus} RN420 containing plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein, and strain CD-1281, which possesses the TetK tetracycline efflux protein, were generously provided as gifts from J. Cove (University of Leeds, UK) and C. Dowson (University of Warwick, UK), respectively. SA-1199B, which overexpresses the NorA MDR efflux protein, SA-K2068, which exhibits an MDR efflux phenotype conferred by a pump distinct from \textit{NorA}, and \textit{S. aureus} ATCC 25923 were also used.\textsuperscript{13,14} All strains were cultured on nutrient agar (Oxoid, Basingstoke, UK) before determination of MICs. Cation-adjusted Mueller–Hinton broth (MHB; Oxoid) was used for susceptibility tests.

\subsection*{Antibiotics and chemicals}

Tetracycline, norfloxacin and erythromycin were obtained from Sigma (Poole, UK). Ciprofloxacin, levofloxacin and moxifloxacin were obtained from their respective manufacturers. GG918 was provided by GlaxoSmithKline (Stevenage, UK).

\subsection*{Susceptibility tests}

MICs were determined at least in duplicate by microdilution techniques according to the NCCLS guidelines, using \textit{S. aureus} 25923 as a quality control strain.\textsuperscript{15} The effects of GG918 and reserpine (final concentrations 10 and 20 mg/L, respectively) on MICs were also determined. Both of these compounds were dissolved in DMSO before dilution into MHB for use in MIC determinations. The highest concentration of DMSO remaining after dilution (25%, v/v) caused no inhibition of bacterial growth (data not shown).

\subsection*{Ethidium efflux}

Ethidium bromide (EtBr) is a substrate for many Gram-positive MDR pumps, including NorA. The efficiency of efflux pumps for which EtBr is a substrate can be assessed fluorometrically by the loss of fluorescence over time from cells loaded with EtBr. SA-1199B and SA-K2068 were loaded with EtBr as previously described, and the effect of varying concentrations of reserpine and GG918 on EtBr efflux was determined to generate a dose–response profile for each compound.\textsuperscript{16} Results were expressed as percentage reduction of the total efflux observed for test strains in the absence of inhibitors.

\section*{Results and discussion}

Susceptibility data for test strains in the presence and absence of inhibitors are shown in Table 1. Neither reserpine nor GG918 by themselves had inhibitory activity against any test strain at the concentrations employed (data not shown). The presence of either compound resulted in at least a four-fold reduction in norfloxacin MICs for all strains possessing efflux-related resistance phenotypes, regardless of that phenotype. With respect to ciprofloxacin, a four-fold or greater potentiation of activity was observed only with SA-1199B and SA-K2068; no more than two-fold MIC changes were observed for the other test strains. The inhibitors only minimally augmented the activity of levofloxacin and moxifloxacin, which are more recently developed fluoroquinolones with

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Structure of GG918.}
\end{figure}
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Table 1. MICs of test strains (mg/L)

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>RN4220 (MsrA)</th>
<th>CD-1281 (TetK)</th>
<th>SA-1199B (NorA)</th>
<th>SA-K2068 (MDR)</th>
<th>ATCC 25923</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norfloxacin</td>
<td>1</td>
<td>1</td>
<td>32</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>+Reserpine</td>
<td>0.25 (4)</td>
<td>0.25 (4)</td>
<td>8 (4)</td>
<td>1 (8)</td>
<td>0.125 (4)</td>
</tr>
<tr>
<td>+GG918</td>
<td>0.25 (4)</td>
<td>0.25 (4)</td>
<td>4 (8)</td>
<td>2 (4)</td>
<td>0.25 (2)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.25</td>
<td>0.25</td>
<td>8</td>
<td>4</td>
<td>0.125</td>
</tr>
<tr>
<td>+Reserpine</td>
<td>0.125 (2)</td>
<td>0.125 (2)</td>
<td>1 (8)</td>
<td>0.5 (8)</td>
<td>0.063 (2)</td>
</tr>
<tr>
<td>+GG918</td>
<td>0.25 (NC)</td>
<td>0.125 (2)</td>
<td>1 (8)</td>
<td>1 (4)</td>
<td>0.125 (NC)</td>
</tr>
<tr>
<td>Levofoxacin</td>
<td>0.25</td>
<td>0.25</td>
<td>2</td>
<td>1</td>
<td>0.125</td>
</tr>
<tr>
<td>+Reserpine</td>
<td>0.25 (NC)</td>
<td>0.25 (NC)</td>
<td>1 (2)</td>
<td>0.5 (2)</td>
<td>0.125 (NC)</td>
</tr>
<tr>
<td>+GG918</td>
<td>0.25 (NC)</td>
<td>0.25 (NC)</td>
<td>1 (2)</td>
<td>0.5 (2)</td>
<td>0.125 (NC)</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25</td>
<td>0.25</td>
<td>0.031</td>
</tr>
<tr>
<td>+Reserpine</td>
<td>0.125 (NC)</td>
<td>0.125 (NC)</td>
<td>0.125 (2)</td>
<td>0.125 (2)</td>
<td>0.031 (NC)</td>
</tr>
<tr>
<td>+GG918</td>
<td>0.125 (NC)</td>
<td>0.125 (NC)</td>
<td>0.25 (NC)</td>
<td>0.25 (NC)</td>
<td>0.031 (NC)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>64</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>0.125</td>
</tr>
<tr>
<td>+Reserpine</td>
<td>64 (NC)</td>
<td>0.25 (2)</td>
<td>0.125 (2)</td>
<td>0.5 (NC)</td>
<td>0.125 (NC)</td>
</tr>
<tr>
<td>+GG918</td>
<td>32 (2)</td>
<td>0.25 (2)</td>
<td>0.125 (2)</td>
<td>0.5 (NC)</td>
<td>0.125 (NC)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.25</td>
<td>32</td>
<td>0.125</td>
<td>1.0</td>
<td>0.25</td>
</tr>
<tr>
<td>+Reserpine</td>
<td>0.25 (NC)</td>
<td>16 (2)</td>
<td>0.063 (2)</td>
<td>0.5 (2)</td>
<td>0.25 (NC)</td>
</tr>
<tr>
<td>+GG918</td>
<td>0.125 (2)</td>
<td>16 (2)</td>
<td>0.063 (2)</td>
<td>0.5 (2)</td>
<td>0.25 (NC)</td>
</tr>
</tbody>
</table>

*Antimicrobials were tested alone, or in combination with reserpine (20 mg/L) or GG918 (10 mg/L).*

MDR, non-NorA multidrug efflux phenotype; NC, no change.

Improved potency compared with ciprofloxacin against *S. aureus*.

The presence of GG918 or reserpine resulted in no more than a two-fold reduction in erythromycin and tetracycline MICs for all strains. The equivalence in activity regardless of the efflux-related resistance trait present suggests that the modest MIC reductions observed are not related to specific inhibition of MsrA or TetK.

Overall, the effects of GG918 and reserpine on susceptibility data were equivalent for all test strains. In general, the activities of norfloxacin and ciprofloxacin were the most potentiated. This was especially true for SA-1199B, which overexpresses NorA, and SA-K2068, which possesses a novel non-NorA MDR pump. Both norfloxacin and ciprofloxacin are quite hydrophilic molecules with small substituents at the C7 and C8 positions, characteristics that make them more favourable substrates for NorA, and probably for the SA-K2068 efflux pump. The lack of significant activity of either inhibitor on MICs of levofloxacin and moxifloxacin for strains bearing these pumps may relate to molecular hydrophobicity in the case of levofloxacin and structural features in the case of both compounds, characteristics that may reduce recognition and transport.

The sequence of the *S. aureus* genome has recently been published, and examination of the data reveals the presence of up to 17 open reading frames encoding putative drug transporters. The four-fold potentiation of norfloxacin activity by inhibitors in RN4220 (MsrA), CD-1281 (TetK) and ATCC 25923, strains not possessing known quinolone efflux systems, is likely to be related to the inhibition of one or more of these as yet uncharacterized pumps. These data indicate that GG918 and reserpine may have more affinity for MDR-type pumps than for more limited spectrum pumps such as MsrA and TetK, or at least for pumps for which fluoroquinolones are substrates.

The effect of inhibitors on the EtBr efflux capability of SA-1199B and SA-K2068 compared with the effect observed for reserpine is shown in Figure 2. For SA-1199B, concentrations of ≤10 µM GG918 were more potent than the same concentrations of reserpine. Both inhibitors were very potent versus SA-K2068, with GG918 appearing more effective at concentrations of ≤5 µM. These data indicate that at low concentrations GG918 is more potent than reserpine as an inhibitor of MDR pump-mediated EtBr efflux in *S. aureus*.

The development of efflux pump inhibitors, which could be used in conjunction with existing antibiotics, could extend the useful lifetime of some antibiotics by improving therapeutic efficacy and by suppressing the emergence of resistant variants that might otherwise arise during treatment. The former phenomenon has been evaluated in an animal model of a *P. aeruginosa* infection caused by a strain overexpressing the MexAB–OprM MDR efflux system, with treatment consisting of levofloxacin plus inhibitor or levofloxacin alone. Improved therapeutic efficacy was observed in animals treated with the combination versus those treated with only levofloxacin. The latter phenomenon has been demonstrated.
for both NorA and PmrA, a recently described Streptococcus pneumoniae MDR transporter, where it has been shown that the combination of reserpine and a fluoroquinolone markedly reduced the emergence of resistant variants in vitro compared with what was observed with the fluoroquinolone alone. However, the concentrations of reserpine required for the observed effect were not clinically relevant as the potential for adverse effects, such as neurotoxicity, eliminates the use of reserpine as an efflux pump inhibitor in the clinical setting.

GG918 is a first step toward developing an inhibitor active against S. aureus antibiotic efflux pumps, especially NorA. A further effort to identify an even more potent compound with a good toxicity profile and broader spectrum of activity seems reasonable. The combination of a broad-spectrum MDR pump inhibitor with antibiotics that are known pump substrates could reduce the morbidity and mortality that might result from a delay in the institution of effective therapy for serious S. aureus infections.

Acknowledgements

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