Intracellular activity of trovafloxacin against *Staphylococcus aureus*

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The effect of trovafloxacin on *Staphylococcus aureus* ingested by human granulocytes or monocytes was compared with that on *S. aureus* in cell-free medium. Maximum growth inhibition ($E_{t_{\text{max}}}$) by the antibiotic was 0.530 log $10^{10}$/h for *S. aureus* within granulocytes, 0.912 log $10^{10}$/h for *S. aureus* within monocytes, and 1.830–1.916 log $10^{10}$/h for *S. aureus* in medium. EC$_{50}$, the concentration at which 50% of the maximum growth inhibition is achieved, did not differ significantly under the conditions investigated. After inhibition of intracellular killing by granulocytes with sodium fluoride, the intracellular antibacterial activity of trovafloxacin was still less than that in medium. A 3.4 times higher concentration was needed to achieve the same effect on phagocytosed *S. aureus* as in cell-free medium. Trovafloxacin binds more strongly to granulocytes than to monocytes, the respective cellular concentrations being 10 and four times higher than that in medium. In conclusion, the activity of trovafloxacin against *S. aureus* ingested by human granulocytes or monocytes is less than that against *S. aureus* in cell-free medium and is not related to the cell-associated concentration. Intracellular conditions are not favourable for the antibacterial activity of trovafloxacin.

Introduction

Quinolones are antibiotics that attach fairly easily to phagocytes, as is reflected by a cellular to extracellular concentration ratio (C/E ratio) of between five and eight for granulocytes and macrophages. Binding of the antibiotics to these cells is rapid at 37°C, the maximum cellular concentration being reached within about 15 min. When the cells are transferred into quinolone-free medium, there is rapid release of antibiotic from the phagocytes. Quinolones are not concentrated at specific sites in the cells, such as lysosomes, but seem to occur free in the cytosol. The strong attachment of quinolones to phagocytes has been used to explain the success of ciprofloxacin treatment of malakoplakia, a disease consisting of tumour-like lesions of macrophages containing coliform bacteria. Indeed, the intracellular antibacterial activity of quinolones has been demonstrated for a variety of bacteria, e.g. *Staphylococcus aureus*, *Mycobacterium* spp., *Legionella pneumophila*, *Enterococcus faecium* and *Escherichia coli*. Trovafloxacin is one of the new quinolones with increased activity against Gram-positive bacteria. Like the other quinolones the drug binds very strongly to cells. The C/E ratio is around 10 for granulocytes, macrophages harvested from peritoneal effluents from patients undergoing continuous ambulatory peritoneal dialysis, and tissue cells. For guinea pig alveolar macrophages a C/E ratio of over 20 was reported. Trovafloxacin exhibits antibacterial activity against *S. aureus* and *E. faecium* phagocytosed by human granulocytes and *L. pneumophila* ingested by guinea pig alveolar macrophages.

We investigated the intracellular antibacterial activity of trovafloxacin in relation to the activity against *S. aureus* in cell-free medium. The assumption was that there is no direct relationship between cellular binding of an antibiotic and antibacterial activity against ingested bacteria. The intracellular conditions are an important determinant of intracellular activity as found, for example, for β-lactam antibiotics, which bind poorly to phagocytes but can exhibit excellent intracellular activity, depending on the state of the ingested bacteria.

Materials and methods

Antibiotic

A stock solution of trovafloxacin mesylate 1000 mg/L (80.7% activity; Pfizer, Capelle a/d IJssel, The Netherlands) in water was prepared and stored at 4°C in the dark.

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for a maximum of 2 weeks. For each experiment the stock solution was diluted with phosphate-buffered saline (PBS) or medium.

Lysostaphin
A stock solution of lysostaphin 10⁵ U/L (Sigma Chemical Co., St Louis, MO, USA) in PBS was prepared and stored at −20°C. For each experiment the activity of lysostaphin was checked as previously described.³⁰

Serum
Blood from healthy blood group AB donors was clotted at room temperature and centrifuged at 1100g for 20 min. The serum was harvested and stored at −70°C.

Medium
Hanks’ balanced salt solution with 0.1% (w/v) gelatin, 0.5% (v/v) tryptic soya broth (TSB; Oxoid Ltd, Basingstoke, UK) and 10 mM HEPES as buffer (pH 7.3) was used as medium unless otherwise indicated.

Granulocytes and monocytes
Recalcified buffy coat of blood from healthy donors was centrifuged on Ficoll–Isopaque gradients. Granulocytes were obtained from the pellet by sedimentation of erythrocytes with a 40% solution of dextran (mol. wt 200,000) in PBS for 10 min at 37°C. After three washes with PBS containing heparin 500 U/L (heparin–PBS) the granulocytes were suspended in medium to a concentration of 5 × 10⁶/mL. The viability of the granulocytes and monocytes exceeded 95% during the experiments, as determined by trypan blue dye exclusion.

Staphylococcus aureus
S. aureus (phage type 42 D) was stored in aliquots of approximately 1 × 10⁸ bacteria/mL at −70°C. For each experiment a sample was diluted 100-fold in TSB and incubated for 60 min at 37°C in a shaking water bath. Next, the culture was centrifuged for 10 min at 1500g and the pellet was resuspended in medium to a concentration of approximately 1 × 10⁷ bacteria/mL. S. aureus were opsonized by incubation of the suspension in the presence of 10% (v/v) serum for 30 min under rotation (4 rpm) at 37°C. The bacteria were then washed twice with medium and resuspended in medium to a concentration of approximately 1 × 10⁷/mL. The MIC of trovafloxacin for S. aureus was 0.016 mg/L, as determined by agar incorporation after inoculation of 5 × 10³ bacteria per spot.

Measurement of the activity of trovafloxacin against S. aureus in suspension
Aliquots of 1 mL of 1 × 10⁷/mL pre-opsonized S. aureus were incubated in the presence of trovafloxacin under rotation (4 rpm) at 37°C. After 0, 60, 120 and 180 min of incubation a 200 µL sample was taken and added to 1 mL ice-cold medium. The samples were then centrifuged for 10 min at 1500g to remove the antibiotic and the pellets were resuspended in 200 µL medium. The number of viable S. aureus was determined by plating 10-fold serial dilutions on diagnostic sensitivity test agar (DST agar; Oxoid Ltd) and counting the colonies after incubation for 18 h at 37°C.

Measurement of the effect of trovafloxacin on S. aureus phagocytosed by granulocytes
Equal volumes of 1 × 10⁷/mL granulocytes and 1 × 10⁷/mL pre-opsonized S. aureus were combined and incubated under rotation (4 rpm) at 37°C. After 3 min the tubes were cooled in crushed ice to stop phagocytosis. Non-ingested bacteria were removed by differential centrifugation at 110g for 4 min and washing the cells twice with medium. The granulocytes with ingested S. aureus were resuspended to a concentration of 5 × 10⁶ cells/mL and re-incubated in the presence of trovafloxacin under rotation (4 rpm) at 37°C. No serum was added during the incubation to minimize intracellular killing by the granulocytes.³¹ At 0, 60, 120 and 180 min a 100 µL sample was taken, added to 900 µL ice-cold medium and centrifuged at 110g for 4 min at 4°C. The supernatant was removed, the pellet resuspended in 1 mL ice-cold water containing 0.01% bovine serum albumin (water–BSA) and the cells were lysed by vortexing the tube for 1 min. The number of viable cell-associated S. aureus was determined as described above.

Measurement of the effect of trovafloxacin on S. aureus phagocytosed by monocytes
Monocytes with ingested S. aureus, prepared as described for granulocytes, were divided among various tubes, with one tube for each time-point, and incubated in the presence of trovafloxacin under rotation (4 rpm) at 37°C. Killing of S. aureus by the monocytes was prevented by not adding serum during incubation.³² At 0, 60, 120 and 180 min one of the tubes was centrifuged at 110g for 4 min at 4°C, the supernatant removed, and the pellet resuspended in 1 mL ice-cold water–BSA. The cells were lysed by vortexing the tube for 1 min, and the number of viable cell-associated bacteria was determined as described above.
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Calculations

For the experiments in medium and with cells the effect on numbers of S. aureus (\(E_N\)) at time \(t\) was expressed as the logarithm of the ratio of the number of viable \(S. \text{aureus}\) in the control with no antibiotic \((N_c)\) to the number present during incubation with the antibiotic \((N_a)\), hence

\[
E_N = \log N_c - \log N_a
\]  

(1)

The effect on growth rate \((E_R)\) was calculated by linear regression analysis as the slope of the relationship between \(E_N\) and time according to the equation

\[
E_N = E_R \cdot t + b
\]  

(2)

in which \(b\) is the intercept. The concentration–effect relationship for \(E_R\) was calculated by non-linear regression analysis (Systat, Systat Inc., Evanston, IL, USA) according to a Hill equation:

\[
E_R = E_{R,\text{max}} \times \left[\frac{C^s}{(EC_{50}^s + C^s)}\right]
\]  

(3)

in which \(E_{R,\text{max}}\) is the maximum value of \(E_R\) estimated from the experimental results, \(C\) is the concentration of the antibiotic, \(s\) is a variable reflecting the steepness of the concentration–effect curve and \(EC_{50}\) is the concentration of the antibiotic that achieved 50% maximum growth inhibition.

Demonstration of the intracellular localization of cell-associated S. aureus

Samples obtained during the assay to measure the effect of trovafloxacin on phagocytosed \(S. \text{aureus}\) were centrifuged at 1100 g for 4 min. One sample was incubated with lysofatin 1000 U/L for 5 min at 4°C and a second sample was incubated with medium. Cytosin preparations were then made, stained with Giemsa, and the number of bacteria and cells counted in 25 fields of view under a light microscope at 1000× magnification. The results were expressed as the number of bacteria per 100 cells. The lystophin-treated samples reflect the intracellular bacteria and the difference between the number of bacteria in the non-treated and lystophin-treated samples represents the number of extracellular bacteria.

Determination of the cell-associated concentration of trovafloxacin

Monocytes were further purified from the interphase layer of the Ficoll–Isopaque gradient by countercurrent centrifugation in a Beckman J2-21 centrifuge (Beckman Instruments, Palo Alto, CA, USA). The cellular suspension obtained by this procedure contained 85% monocytes with a viability of over 90%, as determined by trypan blue dye exclusion. Granulocytes or monocytes at a concentration of \(4.5 \times 10^7/\text{mL}\) were incubated in medium in the presence of trovafloxacin 2 or 4 mg/L under rotation (4 rpm) at 37°C.

At 0, 30 and 60 min samples were removed and the cells separated from the medium by centrifugation over a silicone oil layer for 45 s at 10,000 g. The cells were lysed in distilled water and the concentration of trovafloxacin was determined by reverse-phase HPLC with a MOS Hypersil 5 \(\mu\)m column (Chrompack Nederland b.v., Bergen op Zoom, The Netherlands), a mobile phase (pH 2.5) containing 85% (w/v) 0.05 M \(\text{KH}_2\text{PO}_4\), 10% (v/v) acetonitrile and 5% (v/v) isopropanol at a flow rate of 1 mL/min, and a UV light detector at a wavelength of 270 nm. The cellular concentration \((C)\) of trovafloxacin was calculated according to the equation

\[
C = \frac{(V_1 \times C_1)}{(G \times V_c)}
\]  

(4)

in which \(V_1\) is the volume of the cell lysate, \(C_1\) the antibiotic concentration in the cell lysate, \(G\) the total number of cells present during incubation, and \(V_c\) the volume of a single cell. The volume of a human granulocyte is 334 fL, that of a monocyte 421 fL.

Results

The effect of trovafloxacin on S. aureus phagocytosed by granulocytes

Trovaflaxacin had a concentration-dependent effect on \(S. \text{aureus}\) ingested by granulocytes. Maximum growth inhibition \((E_{R,\text{max}});\) equation (3) was 0.531 log \(10\)/h and \(EC_{50}\) was 0.03 mg/L (Figure 1). The effect was much less than that on \(S. \text{aureus}\) in medium, in which \(E_{R,\text{max}}\) was 1.830 log \(10\)/h and \(EC_{50}\) was 1.299 (95% confidence interval \(-1.803\) to \(-0.795\)). The \(EC_{50}\) in medium was 0.1 mg/L (95% confidence interval 0.03–0.17); but the difference with respect to the value for intracellular \(S. \text{aureus}\)
was not significant (difference $-0.07$, 95% confidence interval $-0.15$ to $0.01$).

To investigate the influence of intracellular killing by granulocytes on the antibacterial activity of trovafloxacin, granulocytes were incubated with 20 mM sodium fluoride (NaF) for 30 min at 37°C before phagocytosis of *S. aureus* and measurement of the effect of the antibiotic on the phagocytosed bacteria. Analysis of variance of the growth rate of *S. aureus* in medium, in NaF-treated and in non-treated granulocytes in the absence of trovafloxacin showed a significant difference between non-treated granulocytes and NaF-treated granulocytes and medium ($P < 0.001$; Figure 2), and a small but significant difference between NaF-treated granulocytes and medium ($P < 0.02$; Figure 2). Trovafloxacin exhibited a concentration-dependent effect in medium and NaF-treated granulocytes ($P < 0.05$; Figure 2). For the same effect on the growth rate of *S. aureus* a 3.4 times (95% confidence interval 1.1–13.3) higher concentration of trovafloxacin was needed in the presence of NaF-treated granulocytes than in medium. Comparison of the effect of trovafloxacin in medium and non-treated granulocytes also showed concentration dependence ($P < 0.01$; Figure 2), but there was no difference between the two conditions (potency ratio 1.5, 95% confidence interval 0.5–6.0).

**The effect of trovafloxacin on *S. aureus* phagocyted by monocytes**

The antibacterial activity of trovafloxacin against *S. aureus* ingested by monocytes was less than that against *S. aureus* in medium. $E_{R,\text{max}}$ for ingested *S. aureus* was 0.912 log$_{10}$/h and 1.916 log$_{10}$/h for *S. aureus* in medium (Figure 3), the difference being $-1.004$ (95% confidence interval $-1.544$ to $-0.455$). The EC$_{50}$ for the two conditions was not significantly different; 0.04 mg/L for intracellular bacteria and 0.1 mg/L (95% confidence interval 0.02–0.2) for bacteria in medium (difference $-0.074$, 95% confidence interval $-0.17$ to 0.02). Maximum growth inhibition by trovafloxacin for *S. aureus* ingested by monocytes was higher than that for *S. aureus* ingested by granulocytes. In a direct comparison the difference was estimated to be 0.433 log$_{10}$/h (95% confidence interval 0.172–0.693).

**Intracellular localization of cell-associated *S. aureus***

In experiments using lysostaphin to lyse extracellular bacteria the intracellular localization of *S. aureus* in granulocytes and monocytes was confirmed (Figure 4). At the start of incubation with trovafloxacin over 90% of bacteria were localized intracellularly in granulocytes and monocytes. In the absence of trovafloxacin the ratio for granulocytes between intracellular and extracellular bacteria remained within this range for the next 3 h of incubation. For monocytes the proportion of extracellular *S. aureus* increased to 20%. In the presence of trovafloxacin extracellular bacteria were often not seen at all. During incubation with trovafloxacin no significant decrease in the number of intracellular *S. aureus* was observed as indicated by the lysostaphin-treated samples. *S. aureus* exposed to trovafloxacin differed in size, form and colour from non-exposed *S. aureus* but remained visible under the microscope.

**Cell-associated concentration of trovafloxacin**

Incubation of granulocytes and monocytes in the presence of trovafloxacin led to a cell-associated concentration of
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the drug that was about 10 and four times higher than the concentration in medium, respectively (Table). Association of the drug occurred very quickly as indicated by the fact that maximal binding was observed in the sample at 0 min of incubation. Neither time nor concentration contributed significantly to the variation in the C/E ratio (analysis of variance, \( P > 0.1 \)).

Discussion

The results of the present investigations confirm that trovafloxacin does affect \( S. aureus \) ingested by granulocytes or monocytes. The intracellular activity is limited compared with that against \( S. aureus \) in cell-free medium. Inside monocytes, which kill phagocytosed \( S. aureus \) less effectively than granulocytes under the given conditions, trovafloxacin exhibited greater antibacterial activity than within granulocytes. The effect of trovafloxacin on \( S. aureus \) ingested by granulocytes also remained less than that in medium when intracellular killing by the granulocytes was eliminated by pre-incubation of the cells in sodium fluoride, an inhibitor of glycolysis. Our observations indicate that the effect on intracellular bacteria is not determined as much by the binding of antibiotic to cells, which is high in the case of trovafloxacin, as it is by the intracellular environment, which is unfavourable for the antibacterial activity of the antibiotic.

A comparison of the antibacterial activity of a quinolone against ingested bacteria and bacteria in cell-free medium has been reported for \( Enterococcus faecium \), and for two of the six investigated isolates sparfloxacin exhibited diminished intracellular activity.\(^{14}\) Trovafloxacin was effective against vancomycin-sensitive \( E. faecium \) isolates phagocytosed by granulocytes but not against vancomycin-resistant isolates. The intracellular effect was less than expected from the activity against \( E. faecium \) in medium.\(^{15}\) Our results extend these findings on the limited intracellular activity of quinolones.

It has been suggested that oxygen-dependent killing promotes the intracellular activity of quinolones. Diffloxacin, ciprofloxacin, pefloxacin and fleroxacin were less effective against \( S. aureus \) phagocytosed by granulocytes from patients with chronic granulomatous disease (CGD) than against \( S. aureus \) phagocytosed by normal granulocytes, suggesting that absence of the oxygen-dependent killing system is detrimental to the intracellular activity of quinolones.\(^{16}\) Péman et al.\(^{21}\) concluded from their experiments with ciprofloxacin and phenylbutazon-treated granulocytes that oxygen-dependent killing interacts ‘favourably’ with quinolones. Easmon et al.\(^{15}\) investigated the influence of sodium cyanide (an inhibitor of mitochondrial respiration), potassium fluoride and iodoacetamide on the effect of ciprofloxacin against \( S. aureus \) ingested by granulocytes. Only sodium cyanide diminished the intracellular effect slightly. These observations seem to contradict our findings. However, when normal non-treated granulocytes are used the decline in intracellular \( S. aureus \) is due to the
combined effect of intracellular killing by the cells and antibacterial activity of the quinolone. In granulocytes from patients with CGD and with phenylbutazon-treated granulocytes the decrease is caused only by the antibiotic. Although the data allow the conclusion that the combined effect of intracellular killing and quinolone is greater than that of a quinolone on its own, this does not imply synergy as the combined effect may be greater than the individual effects even when it is much less than the sum of the separate effects.

In conclusion, trovafloxacin shows activity against \textit{S. aureus} ingested by human granulocytes or monocytes. The intracellular activity is less than that against \textit{S. aureus} in cell-free medium, is not related to the cell-associated concentration of the drug, and is dependent on intracellular conditions. Insofar as supported by available literature, trovafloxacin does not seem to differ from the other quinolones in this respect.

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References


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