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ORIGINAL ARTICLE

Activity of sitafloxacin against extracellular and intracellular *Staphylococcus aureus in vitro* and *in vivo*: comparison with levofloxacin and moxifloxacin

Gongming Shi, Xiangdong Chen, Hui Wang, Siwen Wang, Xin Guo and Xulei Zhang

Antibiotic activity can differ depending on whether the bacterial target is extracellular or intracellular. To determine extracellular and intracellular activities of sitafloxacin (STX) against *Staphylococcus aureus* in comparison with levofloxacin (LVX) and moxifloxacin (MXF) *in vivo* and *in vitro*, three *S. aureus* strains (ATCC25923, 29213, 43300) were evaluated. MIC, MBC and mutant prevention concentration (MPC) of the test quinolone for *S. aureus* were determined by microdilution in broth, and intracellular activity was determined in RAW264.7 cells after phagocytosis of bacteria. Cellular quinolone accumulation was determined by HPLC. The time- and concentration-kill relationships were examined *in vitro* (in broth and in RAW264.7 cells, respectively) and *in vivo* by use of a mouse peritonitis model. The results showed that the activity of STX in broth cultures, including the MIC, MBC, MPC and the time- and concentration-kill relationships, were greater for STX than those for LVX and MXF. In particular, STX exhibited the strongest activity against intramacrophage *S. aureus*. The intracellular effects could be ranked in the following order as the mean change in the log10 number of cfu ml^{-1} (log10 cfu ml^{-1}) between treated and untreated mice: STX > LVX > MXF. It also showed that the dominant factor of intracellular activity *in vivo* was the frequency of doses. There was a poor correlation between the intracellular accumulation of the three different quinolones and the actual intracellular effect. The results of the intracellular and extracellular time- and concentration-kill relationships indicated that STX has the potential to display useful activity against extracellular and intracellular *S. aureus*.
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INTRODUCTION

Staphylococcus aureus is a common pyogenic bacterium and can cause infections in both the hospital and community environments.[1] In addition to the minor skin and wound infections,[2] *Staphylococcus aureus* may also cause serious diseases, including pneumonia,[3] endocarditis,[4] osteomyelitis[5] and meningitis.[6] Therapy for *S. aureus* may often be ineffective in preventing recurrence and the ensuing mortality.[7,8] This can be due to the following reasons: (1) different subcellular localization of the antibiotic and the bacteria; (2) the increased intracellular MIC due to the impaired antibacterial activity of the antibiotic, resulting from drug metabolism, altered local pH, or protein binding; or (3) the altered bacterial responsiveness, due to changes in bacterial metabolism or growth rate. The fact that *S. aureus* survives professional and even nonprofessional mammalian phagocytes has been well described by several reports.[9,10] However, the intracellular location of the bacteria might explain the slow response to antibiotic as they might be protected from the effects of antibiotics.[11]

In general, intracellular antimicrobial activity is markedly impaired compared with the activity seen in broth or the extracellular milieu,[12–14]

although we know of situations in which the opposite is true. Antibiotic treatments should therefore be optimized not only toward the extracellular forms of *S. aureus* but also toward the intracellular forms. Some reports[15] indicated that quinolone accumulated by macrophages are found in the cytosol after cell fractionation but function readily against intracellular *S. aureus*, which suggests that they can access the phagolysosomal compartment in infected cells. For this study, we selected typical representatives of antibiotics with known activities against *S. aureus* and included in commonly used guidelines for the handling of staphylococcal infections. In this context, the different quinolone, sitafloxacin (STX), levofloxacin (LVX) and moxifloxacin (MXF), were selected for the following studies. The three different quinolone exhibited strong anti-*S. aureus* activity *in vitro*, and were also expected to be useful for the clinical treatment of *S. aureus*.[16] For these studies, the direct assessment of antibiotic activity in the pertinent models is warranted, and several *in vitro* models with either human or animal cells have been developed to study the extracellular and intracellular activities of antibiotics.[12,14] Therefore, we selected and used the cell line of RAW264.7 macrophages, because they present many of the characteristics of human

macrophages while forming a homogeneous and reproducible population.[17] We used this cell line to analyze the potential relationship between the accumulation of antibiotics in cells and intracellular activity. In addition, a corresponding *in vivo* model has been developed to study the extracellular and intracellular activities of antibiotics. In conclusion, the present study is one of the first systematic attempts to examine the activity of STX, LVX and MXF against extracellular and intracellular *S. aureus in vitro* (in broth and cell culture) and *in vivo* (in a murine peritonitis model).

MATERIALS AND METHODS
Bacterial strains, antimicrobial agents and sources of other products
The study used the following *S. aureus* strains: ATCC29213, ATCC25923, ATCC43300 (American Type Culture Collection (ATCC), Manassas, VA, USA). Drugs used were as follows: STX (BrightGene Bio-Medical Technology Co., Jiangsu, China); MXF (Bayer Schering Pharma. Co., Berlin, Germany); LVX (LuKang cisen Pharmaceutical Co., Shandong, China). The microbiological medium was obtained from NIFDC (National Institute for Food and Drug Control, Beijing, China). The cell culture medium and newborn bovine serum (NBC) were purchased from Gibco Biocult (Paisley, Scotland, UK).

Cell, cell culture and assays for cell viability
The murine macrophage cell line RAW264.7[18.] was obtained from the ATCC, cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and 10% NBCS, and maintained in a 37 °C humidified incubator containing 5% CO_2. These cells were permissive toward a large number of intracellular bacteria. Viability upon exposure to antibiotics (at the maximal concentration tested and for up to 24 h) was assessed by MTT (3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide) assay, which was used essentially that method of Mosmann.[19] Briefly, cells were plated in 96-well plates with 100 μl of cells which had previously been resuspended to 1×10^5 cfu ml^{-1}. The cells were left to adhere overnight, then exposed to STX, LVX and MXF (256 mg l^{-1}) in media containing 10% NBCS, and returned to the incubator for 24 h. Subsequently, MTT reagent (5 g l^{-1} in sterile PBS) was added to the wells. Cells were returned to the incubator for 4 h whereupon medium was removed and DMSO was added, and OD was assessed at 490 nm. The results were measured at the end of the experiments (more than 90% of the total cell content).

In vitro susceptibility studies
MICs were determined according to the recommendations of the Clinical and Laboratory Standard Institute (2008). MBCs were defined as the lowest concentration of each drug causing >99% reduction in growth. MICs and MBCs were measured at pH 5.4 and 7.4 by microdilution, with the bacterial suspension set at $1 \sim 5 \times 10^5$ cfu ml^{-1} and by using as the endpoint the drug concentration that caused a 5-log decrease in the original inoculum. Mutant prevention concentration (MPC)[20] was defined as the lowest drug concentration that prevented bacterial colony formation from a culture containing >10^{10} bacteria. The determination was similar to that for MIC, except that >10^{10} cells were tested at high drug concentrations, inoculated plates were incubated for 72 h, and colonies were counted at 24 h intervals until colony numbers became constant. The MPCs were identical when exponentially growing bacterial cultures, rather than stationary-phase cells, were applied to agar plates.

Cell infection and assessment of antibiotic intracellular susceptibility breakpoint
All experiments were conducted with RAW264.7. macrophages. Bacterial cultures exhibiting logarithmic growth were centrifuged at 3000 r.p.m. for 15 min, and the sedimented bacteria were resuspended in DMEM supplemented with 10% NBCS, and incubated for 30 min at 37 °C to allow for opsonization. This suspension was then used to replace the culture medium of macrophages, and infection/phagocytosis was achieved by incubating macrophages with bacteria for 1 h (0.5:1 bacteria/macrophage ratio). Extracellular bacteria were then eliminated by washing in PBS (before the first washing, the

cells were incubated for 1 h in DMEM supplemented by 200 mg l^{-1} linezolid kill *S. aureus*). The culture medium samples were investigated for extracellular bacteria by agar plate assays. The infected macrophage samples were subjected for 24 h to a range of quinolone (STX, LVX and MXF) at concentrations from 0.03125 to 512 mg l^{-1}. Finally, the medium was decanted, and the cell layer was quickly washed twice with PBS, cells were collected by scraping in 0.2 ml of distilled water, and intracellular bacteria were enumerated by agar plate assays with appropriately diluted samples. In this experiment, linezolid was added at its MIC value during the whole incubation period to prevent the extracellular growth of bacteria.

Determination of cellular quinolone accumulation
Cells (infected or uninfected) were incubated with quinolone at a final concentration of 4 mg l^{-1} for 30, 180, 300 min. Then the medium was decanted, and the cell layers were quickly washed twice with PBS (this effectively removes all of the drugs not tightly cell bound, and we checked for the absence of detectable quinolone in the final wash). Cells were collected by scraping in 0.5 ml of distilled water and sonicated (10 s, 400 w) to achieve homogeneity. A part of each sample was withheld and used for determination of the total protein content (bicinchoninic acid method; enhanced BCA protein assay kit, Beyotime, Jiangsu, China). The remainder part was centrifuged at 18 000 r.p.m. for 10 min, and 50 μl of the supernatant was used for chromatography (SHIMADZU chromatograph equipped with a UV detector (Shimadzu Co., Kyoto, Japan) (set at 295 nm for STX, 296 nm for LVX and MXF, 1.0 ml min^{-1})), using a C18 column in conjunction with a precolumn, a mobile phase made of 22% acetonitrile and 78% 100 mM Na_2HPO_4 buffer (pH 3.0) for STX (the limit of detection was ∼0.3 ng, R^2=0.9990),[21] a mobile phase of 20% acetonitrile and 80% 20 mM Na_2HPO_4 buffer (pH 3.0) for LVX (the limit of detection was ∼0.16 ng, R^2=0.9984),[22] and a mobile phase of 22% acetonitrile and 78% 10 mM Na_2HPO_4 buffer (pH 2.7) for MXF (the limit of detection was ∼0.16 ng, R^2=0.9984).[23]

In vitro extracellular and intracellular concentration- and time-kill studies
For extracellular concentration- and time-kill studies, bacteria at a density of 5×10^5 cfu ml^{-1} were exposed to quinolone (STX, LVX or MXF) concentrations that varied over a wide range (0.01-fold. to 100-fold the MPC, to obtain a full description of the pharmacological response) in broth, Incubation at 37 °C for 24 h by plate assays with appropriately diluted samples. Quantification of cfu was performed with MPC after 0, 6 and 24 h of incubation for time-kill studies. Intracellular concentration-kill studies were performed with phagocytized bacteria, opsonized *S. aureus* was added to the cell culture medium at a 0.5:1 bacteria/macrophage ratio for 1 h. Adherent, non-phagocytized bacteria were eliminated by washing and a short incubation (1 h) in Hanks buffer containing linezolid (200 mg l^{-1}), and thereafter, the cells were incubated with quinolones for up to 24 h over a wide range of extracellular concentrations (In this period, linezolid was also added at its MIC value). Then the medium was decanted, and the cell layer were quickly washed twice with PBS, cells were collected by scraping in 0.2 ml of distilled water, and intracellular bacteria were enumerated by plate assays with appropriately diluted samples. The numbers of cell-associated cfu were measured at time zero and the end of the incubation. Quantitation of cfu was performed with MPC after 0, 6 and 24 h of incubation for time-kill studies.

In vivo extracellular and intracellular concentration- and time-kill studies
Mouse peritonitis model. ICR mice (weight: 25–30 g) were used throughout the studies. The mice had free access to chow and water. The mice were inoculated by i.p. injection of 0.5 ml bacterial suspension containing 5×10^6 cfu ml^{-1} and 5% (wt·vol^{-1}) mucin. After killing the mice, peritoneal washes were performed by injecting 2 ml of Hanks balanced salt solution i.p. and then massaging the abdomen and opening the peritoneum to collect cells and bacteria.

(1) Determination of maximum drug concentration in serum (Cmax): Uninfected serum samples were obtained at intervals from mice injected

s.c. with the test drugs at $100 \, mg \, kg^{-1}$. Drug concentrations were determined from the diameter of zones of growth inhibition around disks saturated with sample solutions, which were placed onto agar plates inoculated with *Bacillus subtilis* as an indicator bacterium.[24]

(2) Extracellular and intracellular dose-kill studies: Mice were treated s.c. with five different single doses of STX, LVX or MXF (200, 100, 50, 20, $10 \, mg \, kg^{-1}$; four mice per group) 2 h after inoculation, and samples were obtained after 4 h of drugs exposure. Untreated control groups were included.

(3) Extracellular and intracellular time-kill studies: Mice were treated with one or three doses of STX, LVX or MXF ($100 \, mg \cdot kg^{-1}$) and received the first dose 2 h after inoculation (four mice per group). The three-dose regimen was administered every 8 h. The mice were sampled 19 h after treatment onset. Untreated control groups were included, but only for 6 h time point post infection. At this point, untreated mice met the clinical signs of irreversible bacteremia and were euthanized.

(4) Separation of extracellular and intracellular *S. aureus* in the peritoneal wash: The peritoneal fluid was collected from each mouse. The total amount of bacteria was quantified before any other procedure was performed. A volume of 0.6 ml of the pooled samples was transferred to tubes and the tubes were centrifuged at 300 r.p.m. and 25 °C for 5 min. The extracellular bacteria were still suspended, while the cells were sedimented. The extracellular bacteria in the supernatant were quantified. Then, the samples were centrifuged at 3000 r.p.m. and 25 °C for 10 min, and the sedimentated cells were resuspended in Hanks balanced salt solution with $200 \, mg \, l^{-1}$ of linezolid. The suspension was incubated 37 °C for 1 h to kill the remaining extracellular bacteria. A cell-free bacterial suspension was run in parallel as a control for extracellular killing. The linezolid was then removed by washing the samples twice with 2.0 ml Hanks balanced salt solution. The samples were finally resuspended in 1.5 ml of sterile water for cell lysis, and the intracellular bacterial count was quantified.

Curve fitting and statistical analyses
Curve fitting was performed using a sigmoidal dose–response equation (Graph Pad Prism version 5.01, Graph Pad Software, San Diego, CA, USA) to obtain, for each condition, numeric values of two key descriptors: (i) the decrease in the number of cfu for an infinitely large concentration of antibiotic (relative maximal efficacy (Emax; in log_{10} cfu units)); (ii) the concentration of antibiotic resulting in no apparent bacterial growth compared with the original inoculum (static concentration (Cs; in $mg \, l^{-1}$ or multiples of MPC)). Statistical analyses were performed using Graph Pad Instat version 3.06 (Graph Pad Software).

RESULTS
In vitro extracellular and intracellular susceptibility of quinolone
In the test of *in vitro* antimicrobial activities, the MICs of the three different quinolones against clinical isolates of *S. aureus* are shown in Table 1. The order was STX ($0.015–0.031 \, mg \, l^{-1}$) > MXF ($0.031–0.063 \, mg \, l^{-1}$) > LVX ($0.25–0.5 \, mg \, l^{-1}$). For the anti-*S. aureus* ATCC25923, 29213, 43300 activities of the quinolone in broth, the following results were obtained as shown in Table 2. First, the MIC was ranked in the order of STX ($0.015–0.031 \, mg \, l^{-1}$) > MXF ($0.031–0.063 \, mg \, l^{-1}$) > LVX ($0.25–0.5 \, mg \, l^{-1}$). Second, the order of MBC was STX, MXF ($0.063–0.25 \, mg \, l^{-1}$) > LVX ($1–4 \, mg \, l^{-1}$). Third, for MPC, the order was STX ($0.031–0.125 \, mg \, l^{-1}$) > MXF ($0.125–1 \, mg \, l^{-1}$) > LVX ($0.5–8 \, mg \, l^{-1}$). Overall, based on these antimicrobial parameters, the *in vitro* activities of STX was somewhat greater than that of MXF and was much greater than that of LVX. In cell culture medium (DMEM), anti-*S. aureus* activities of the quinolone were similar compared with that in broth.

Furthermore, an attempt to approach what could be defined as an intracellular susceptibility breakpoint, was used to correlate the level of intracellular activity with the MIC. However, the maximal relative

Table 1 STX, LVX and MXF activity with clinical isolates of *S. aureus*

	Sitafloxacin		Levofloxacin		Moxifloxacin	
S. aureus strain	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA
MIC_{50} ($mg \, l^{-1}$)[a]	0.063	0.5	1	8	0.25	4
MIC_{90} ($mg \, l^{-1}$)[b]	1	2	4	16	4	32

Abbreviations: LVX, levofloxacin; MXF, moxifloxacin; STX, sitafloxacin.
[a]MIC_{50} drug concentration at which 50% of isolates are inhibited.
[b]MIC_{90} drug concentration at which 90% of isolates are inhibited.

intracellular activity of the three different quinolone is considerably less active than its extracellular activity (as measured in broth). The following results were obtained as shown in Table 3 and the order of minimum bactericidal concentration was STX ($32–64 \, mg \, l^{-1}$) > MXF, LVX ($>512 \, mg \, l^{-1}$). In preliminary conclusion, the extra- and intracellular activities of STX were better than that of LVX and MXF.

Validation of the intracellular model
The model needed one antibiotic in the medium to prevent the extracellular growth of the bacteria released from macrophages 6 to 8 h after phagocytosis. On the basis of some reports,[25] linezolid was added to the culture medium to prevent this contamination. We found that a linezolid concentration as low as $0.01\times$ its MIC (0.5, 1 and $0.25 \, mg \, l^{-1}$ for *S. aureus* ATCC25923, ATCC29213 and ATCC43300, respectively) reduced the extracellular contamination to a negligible level, although it still allowed a marked increase in the number of cell-associated cfu. A further increase in the extracellular concentration of linezolid to its MIC allowed extracellular contamination to go to undetectable levels. Because of all those uncertainties in the true level of intracellular growth of *S. aureus* and the potential impact of even low concentrations of linezolid, intracellular activities were therefore expressed as the difference from the controls which meant that use of linezolid could be allowed in the controls. The model is nevertheless suitable if activity is defined as a reduction in cfu in comparison with the original inoculum.

Cellular accumulation of quinolone
Figure 1 shows the time profiles of the accumulation of STX, MXF and LVX by uninfected and infected cells over a 5 h period. The accumulation was maximal after only 30 min of incubation and remained almost constant thereafter. Marked differences were observed among drugs, with the following ranking: MXF > STX > LVX. Differences between uninfected and infected cells were small and not significant.

In vitro extracellular and intracellular concentration- and time-kill studies
In a series of experiments, time-kill curves were performed with STX, LVX and MXF for the three different *S. aureus* in broth and after phagocytosis by mouse RAW264.7 macrophages, using a fixed concentration of each antibiotic corresponding to its MPC (total drug). The results are presented in Figure 2. In broth, STX was characterized by a very great bactericidal effect, reaching a $6 log_{10}$ units decrease in about 24 h, followed by LVX and MXF. When the intracellular activity is considered, all antibiotics showed a marked decrease in the rate of their antibacterial effect. Less than a $1 log_{10}$ unit decrease in the bacterial counts was observed at concentrations above the extracellular MPC after 24 h. As a result, the STX bactericidal effect was greater than that of MXF and LVX (less than a $1 log_{10}$ unit decrease, or no progression overtime after 24 h) against *S. aureus* ATCC25923, 29213, but LVX effect was better than STX and MXF against *S. aureus*

Table 2 MIC, MBC and MPC of test quinolones for *S. aureus* in broth at pH 7.4

Strain no.	ATCC25923			ATCC29213			ATCC43300				
Concentration (mg l^{-1})	MIC	MBC	MPC	MIC	MBC	MPC	MIC	MBC	MPC	Cmax[a]	Dosage and route[b]
Sitafloxacin	0.031	0.125	0.031	0.015	0.063	0.125	0.031	0.25	0.125	1.86	200 mg p.o.
Levofloxacin	0.5	1	0.4	0.25	4	4	0.5	1	8	4	400 mg p.o.
Moxifloxacin	0.063	0.125	0.125	0.063	0.063	1	0.031	0.25	0.125	4	400 mg p.o.

Abbreviation: MPC, mutant prevention concentration.
[a]Concentrations corresponding to the peak concentrations of the respective antibiotics in serum after administration of currently used and/or approved doses for humans, as indicated in Keating,[16] Barcia-Macay *et al.*[25] and Zhao and Drlica.[26]
[b]p.o., oral.

Table 3 The MBC of test quinolones against intracellular *S. aureus*

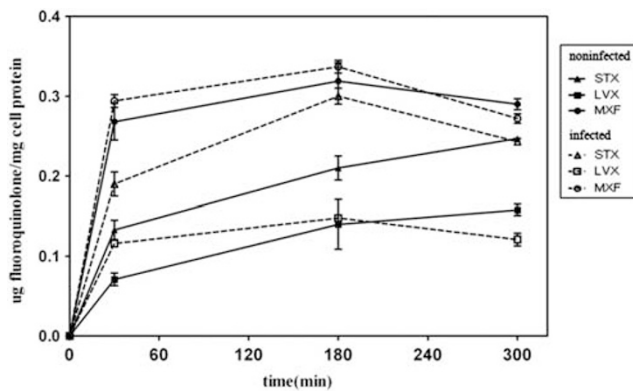| Strain no.	ATCC25923	ATCC29213	ATCC43300
Sitafloxacin (mg l^{-1})	32	32	64
Levofloxacin (mg l^{-1})	>256	>256	>256
Moxifloxacin (mg l^{-1})	>256	>256	>256

Figure 1 Cellular accumulation of quinolones upon incubation of uninfected (closed symbols and continuous lines) and infected (infected *S. aureus* ATCC25923, open symbols and broken lines) RAW264.7 macrophages exposed to STX (triangles), LVX (squares) or MXF (circles) at a fixed extracellular concentration (4 mg·l^{-1}) for the time periods indicated. Each value is the mean of three independent experiments (mean ± s.e.m.; $n=3$).

ATCC43300. In a second series of experiments, Figure 3 and Table 4 show the extra- and intracellular concentrations and response of STX, LVX and MXF against the three different *S. aureus* in broth and in RAW264.7 macrophages, when tested over a wide range of concentrations (0.01-fold to100-fold the MPC) for 24 h. The maximal relative efficacy (Emax) of all compounds was considerably reduced intracellularly when compared with the extracellular values. All drugs displayed similar relative potencies against extracellular bacteria; their relative efficacies (Emax) decreased by ∼7log$_{10}$ cfu ml^{-1}, close to the limit of detection. Against intracellular bacteria, all drugs had significant decreases in their relative efficacies (Emax), but these decreases were roughly similar (Emax against intracellular bacteria/ Emax against extracellular bacteria ratios, 0.109 (minimum) to 0.499 (maximum)). As for bacteria phagocytized by RAW264.7 macrophages, STX was the most active agent against *S. aureus* when considering its Emax, followed by LVX and MXF. The static concentration (Cs) of STX was considerably lower than that of LVX and MXF. In the cell culture model, the extracellular kill curves were

measured in broth without cells, which offers optimal growth conditions for the bacteria.

Determination of Cmax
Results showed the pharmacokinetics of STX, LVX and MXF given to mice s.c. at 100 mg kg^{-1}. Cmax values of the quinolone in mouse blood were determined as follows: STX, 11.18 mg l^{-1}; LVX, 11.58 mg l^{-1}; and MXF, 15.63 mg l^{-1}. Cmax values of MXF were significantly greater than those of STX and LVX. Using these pharmacokinetic parameters, next the time-kill study of quinolone against intracellular *S. aureus* residing within mouse peritoneal macrophages were examined when the quinolone were added at the Cmax.

In vivo* effect of one dose and three doses on extracellular and intracellular *S. aureus
Time-kill curves displaying the changes cfu in the peritoneums of mice over time after treated with quinolone in relation to the number of doses given are shown in Figure 4. LVX and MXF did not show an effect by the number of doses on the total, extracellular, or intracellular colony counts. In contrast, the infection outcome was highly affected by the number of doses given in the mice treated with STX.

In total, a decrease in the colony counts of approximately 2–3log$_{10}$ units during the 19 h of treatment was estimated for the mice receiving one and three doses of LVX and MXF, but mice receiving three doses of STX estimated a decrease in the colony counts of approximately 4log$_{10}$ units. In extracellular conditions, a reduction of approximately 2log$_{10}$ units appeared with in the first 4 h of treatment for mice treated with LVX and MXF, and a reduction of 1log$_{10}$ unit occurred for the following 15 h. But a reduction of 3log$_{10}$ units appeared for mice receiving one dose of STX during the 4 h of treatment, and a reduction of more than 5log$_{10}$ units occurred for the mice receiving three dose. In intracellular conditions, a reduction of approximately 2log$_{10}$ units appeared with in the first 4 h of treatment for mice treated with LVX and MXF, and no regrowth appeared for the following 15 h, irrespective of the number of doses. Whereas for mice receiving one dose of STX a reduction of 5log$_{10}$ units appeared during the 4 h of treatment, an increase in the colony count of more than 2.5log$_{10}$ units was observed compared with the bacterial level at the start of treatment for the subsequent 15 h. However, a reduction of more than 5log$_{10}$ units occurred for the mice receiving three doses of STX.

***In vivo* extracellular and intracellular dose–response relationships**
Figure 5 shows the full pharmacological response of extracellular and intracellular isolates of strains to quinolone at 4 h when they were exposed to a wide range of extracellular concentrations (10, 20, 50, 100, 200 mg kg^{-1}). Significant dose–response correlations were recorded both for the total count and when the counts were separated
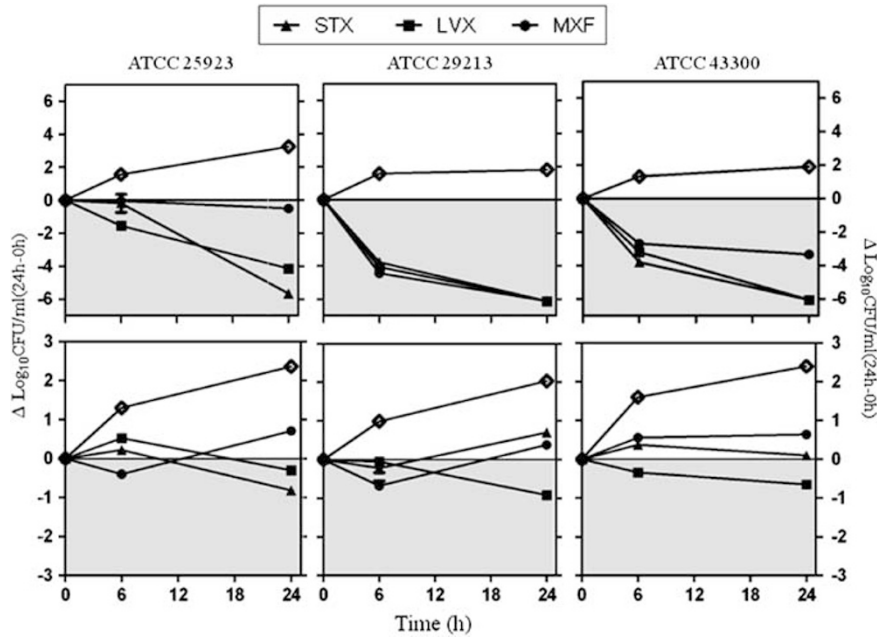
Figure 2 *In vitro* time-kill curves of STX, LVX and MXF corresponding to its MPC (total drug) against three different *S. aureus.* Activity against the extracellular form of the bacteria measured in broth (upper panels), activity against the intracellular form of the bacteria measured in RAW264.7 macrophages (lower panels). The gray shades display decreased colony counts compared with the starting bacterial count, and the white shades display increased colony counts compared with the starting bacterial count. The graphs showed the activity as changes in the numbers of cfu (Δlogcfu, means $\pm$ s.d.; $n=3$). Cells incubated with MIC of linezolid served as controls for all subsequent experiments.
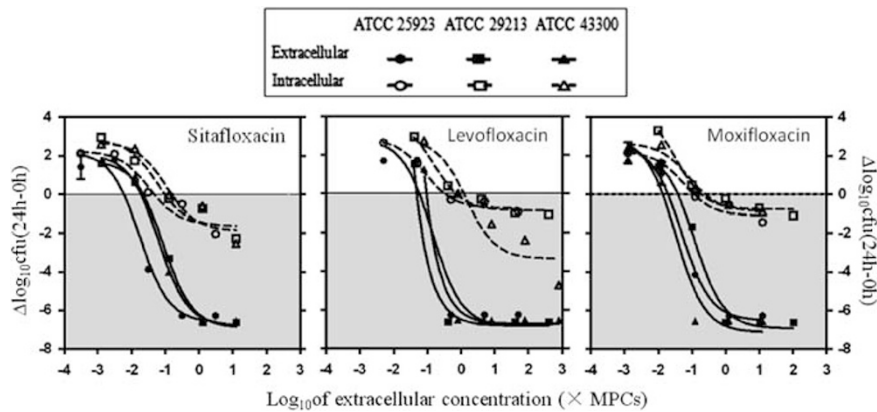

Figure 3 Concentration–response curves of three different extracellular (in broth) and intracellular (in RAW264.7 macrophages) *s. aureus* exposed to STX, LVX and MXF for 24 h.The ordinate showed the change in the number of cfu (Δlogcfu, means $\pm$ s.d.; $n=3$) per ml of culture medium (extracellular, filled symbols; intracellular, open symbols) compared with the initial inoculum (broken line at 0). The abscissa showed the antibiotic concentration (total drug) expressed as multiples of the extracellular MPC of each antibiotic for the corresponding strain. The gray shades display decreased colony counts compared with the starting bacterial count, and the white were as display increased colony counts compared with the starting bacterial count. The data were used to fit sigmoidal functions (Hill's equation; slope factor=1). The goodness of fit (R^2) and key pharmacodynamic parameters derived from each function (E_{max} and C_s) for each condition are shown in Table 4.[29]

into the extra- and intracellular compartments. In total, a reduction of probably 1log$_{10}$ unit appeared within 4 h of treatment for mice treated with LVX and MXF, and a reduction of approximately 2.5log$_{10}$ units occurred for mice treated with STX. In the extracellular compartment, a decrease in the colony counts of approximately 1–2log$_{10}$ units during the 4 h of treatment with LVX and MXF, and a reduction of probably 3log$_{10}$ units occurred for mice treated with STX. In intracellular, an increase of probably 1log$_{10}$ unit appeared for mice treated with LVX and MXF. Nevertheless, STX showed a Emax of a 0.5log$_{10}$ unit decrease, despite the impairment of its intracellular antibiotic activity.

DISCUSSION
In the present study, based on MIC, MBC and MPC, the *in vitro* activities of STX were regarded as somewhat greater than that of MXF and much greater than that of LVX. STX showed maximal extra-cellular activity as long as its MPC for the three different *S. aureus* (ATCC25923, 29213, 43300) did not exceed 0.5 mg l^{-1}. Because no resistant colonies were recovered even when $>10^{10}$ cells were plated, and resistant mutants were selected exclusively within a concentration range (mutant selection window)[26] that extended from the point where growth inhibition begins, approximated by the MIC, up to the

Table 4 Pertinent regression parameters (with 95% CI) and statistical analysis of the concentration–response curves illustrated in Figure

	Extracellular			Intracellular			
Strain no. and antibiotic	E_{max} (95% CI), log cfu[a]	$C_s, \times MPC$[c]	R^2	E_{max} (95% CI), log cfu	$C_s, \times MPC$	R^2	P-value[c]
ATCC25923							
Sitafloxacin	−6.603 (−7.269 to −5.936)	0.01	0.972	−1.557 (−2.061 to −1.052)	0.05	0.929	<0.001
Levofloxacin	−6.845 (−7.921 to −5.770)	0.06	0.928	−0.747 (−0.910 to −0.583)	0.33	0.984	<0.001
Moxifloxacin	−6.505 (−6.838 to −6.172)	0.02	0.993	−1.094 (−1.446 to −0.743)	0.12	0.944	<0.001
ATCC29213							
Sitafloxacin	−6.896 (−7.086 to −6.706)	0.02	0.998	−1.633 (−2.127 to −1.139)	0.10	0.939	<0.001
Levofloxacin	−6.830 (−7.096 to −6.564)	0.06	0.991	−0.871 (−1.064 to −0.677)	0.70	0.977	<0.001
Moxifloxacin	−6.918 (−7.256 to −6.579)	0.04	0.988	−0.754 (−0.955 to −0.553)	0.19	0.978	<0.001
ATCC43300							
Sitafloxacin	−6.791 (−7.048 to −6.535)	0.02	0.996	−1.869 (−2.488 to −1.250)	0.16	0.923	<0.001
Levofloxacin	−6.740 (−6.993 to −6.487)	0.12	0.991	−3.363 (−4.207 to −2.518)	1.20	0.877	<0.001
Moxifloxacin	−7.115 (−8.147 to −6.084)	0.01	0.937	−0.859 (−1.132 to −0.585)	0.25	0.971	<0.001

Abbreviations: CI, confidence interval; MPC, mutant prevention concentration.
[a]E_{max}, Maximal relative efficacy, decreased in logcfu after 24 h compared with original inoculum (t=0 h) and extrapolated for an infinitely large antibiotic concentration.
[b]Cs, Static concentration, concentration (in ×MPC) resulting in no apparent bacterial growth at 24 h compared with the initial inoculum (t=0 h), as determined by graphical interpolation using the Hill's equation.
[c]P-values determined by analysis of covariance for extracellular versus intracellular concentrations between all drugs.
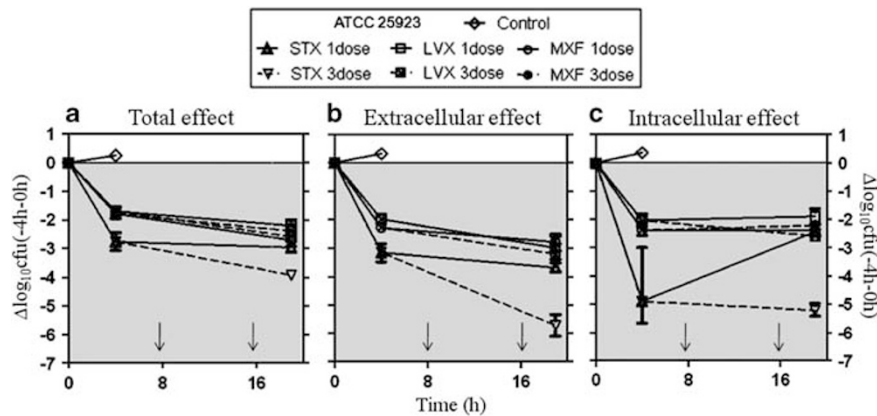

Figure 4 *In vivo* time-kill curves showing the change in the numbers of cfu in the peritoneums of mice (Δlogcfu, mean ± range; n=4) compared with the number of doses of STX, LVX and MXF (100 mg kg^{-1}). (**a**) Change in the total count; (**b**) change in extracellular count; (**c**) change in intracellular count. The ordinate showed the change in the number of cfu. The arrows at the abscissa indicated the times of dosing the second and third doses of drugs. The gray shades display decreased colony counts compared with the starting bacterial count, and the white shades display increased colony counts compared with the starting bacterial count.

MPC. Therefore, MPC was used to the concentration- and time-kill studies.

In the *in vitro* studies, the model was suitable as a tool for the first screening of intracellular activity and had the advantage of being able to study a specific constellation of drug and cell types. Our results showed that the intracellular activity of new antibiotics should be addressed early on during the selection and development process, whereas the *in vivo* model should also be used when extended knowledge of drug efficacy and PK/PD relationships are needed. The two models therefore complemented each other very well and might be used to obtain important knowledge of the extra- and intracellular activities of both potentially new antibiotics and well-established antibiotics such as STX.

In this cell culture model, the RAW264.7 cells displayed poor intrinsic defenses against intracellular infection. The antibiotic

concentration and the number of cells remained fairly constant, which allowed exploration of the effect of antibiotics over a wide range of known concentrations. This model therefore offered valuable information concerning the specific extra- and intracellular activities of the drug by excluding other parameters that could affect the antimicrobial activity. Our work demonstrated that the maximal relative intracellular activity (Emax) of quinolone is considerably lower than its extracellular activity (measured in broth). First, all quinolone studied here, show concentration-dependent effects (for the three molecules tested in detail, we even observed typical pharmacological dose–response curves with the classical basic properties of threshold, slope, and maximal effects upon increasing concentration, irrespective of their specific modes of action; see Figure 3, Table 4). This definitely helps to provide an understanding of why contradictory results are reported when only narrow ranges
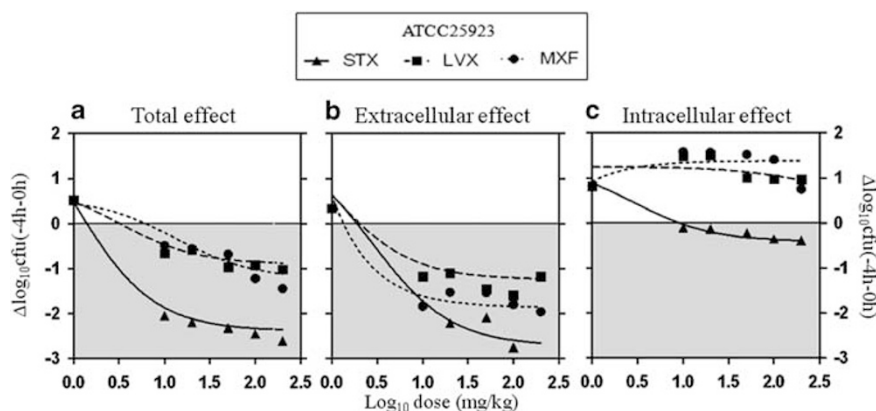
Figure 5 Dose–response curves for STX, LVX and MXF showing the change in the numbers of $\log_{10}$ cfu in the peritoneal wash in mice after 4 h of treatment in relation to dose. (**a**) Change in the total count; (**b**) change in extracellular count; (**c**) change in intracellular count. The ordinate showed the change in the number of cfu (Δlogcfu, means; $n=4$). The dark gray were as display decreases in colony counts compared with the starting level of bacteria, and the light gray were as display increases in colony counts compared with the starting level of bacteria.

of extracellular concentrations are explored. But all drugs did not show time-dependent effects when they were tested at MPC (Figure 2). These studies suggested that the activities of three different quinolone were related with their concentrations. However, despite a higher intrinsic activity and a higher level of accumulation, intracellular activities of LVX and MXF against *S. aureus* were not better than STX *in vitro*.

Although the *in vitro* model could help in rationalizing the treatment of staphylococcal infections, the clinical significance of the present data remains conjectural, mainly because the *in vitro* model used here suffers from several limitations that have been discussed elsewhere.[17,27–29] However, the *in vitro* models could not easily simulate drug pharmacokinetics/pharmacodynamics as they exist in animals and could not evaluate the interaction between a fully functional immune system and antibiotic treatment.

Nevertheless, the *in vivo* model had the advantage of allowing the study conducted in a whole-body system, for example, it included a fully functional immune system, so whole-body drug kinetics were occurring, and the intracellular infection was dynamic. Both the *in vivo* and the *in vitro* models used to assess intracellular antibacterial effect had their own advantages, disadvantages, and limitations. Therefore, it was necessary that both the pharmacodynamic evaluation of the extra- and intracellular activities of STX, LVX and MXF against *S. aureus* were carried out *in vivo*.

For *in vivo* studies, some methodological pitfall should be considered. (i) Loss of bacteria happened along with the *in vivo* separation assay. The sum of extra- and intracellular bacterial counts were always smaller than before the operation. To isolate the cells and the intracellular bacteria from the extracellular bacteria, the cell suspension required centrifugation. Cell lysis might also occur during the separation assay, which could confuse the separation of extra- and intracellular bacteria. However, cell lysis could be restrained by careful sample handling during the whole separation assay. Because of the above reasons, the comparison can be conducted among the samples that had undergone the same isolation procedure. Besides, the counts should be evaluated separately, whether the total, extracellular, or intracellular bacterial counts. (ii) After 2 h of infection, the variation of intracellular bacterial counts was more significant than the total and extracellular counts. This indicated that the number of intracellular bacteria was not static. and the significant variation was due to both

the potential intracellular bacterial growth and internalized extracellular bacteria because of phagocytosis. As the phagocytosis and cell lysis continue throughout the whole infectious process, the dynamic nature of intracellular bacterial counts should be considered. Besides, the migration of new cells to the infection site occurred in the whole process.

Compared with the usual clinical dose ($10\,\mathrm{mg\,kg^{-1}}$ per adult), the test quinolone applied to mice in the present study was far higher ($100\,\mathrm{mg\,kg^{-1}}$). Therefore, animal experiments are absolutely necessary to make a precise comparison of the therapeutic activities of the three quinolone. The results of the dose–response study with STX, LVX and MXF (Figure 5) give insight to the dose–response relationship in extra- and intracellular study. The curves show that the dose needed in intracellular compartment is higher than in extracellular compartment to obtain the same effect.

The results for STX (Figure 4) showed that the difference in dose can affect the intracellular activity-time profile of an antibiotic. The difference in effects on the animals that received a single dose could be accounted by whether the colony counts were obtained after 4 or 21 h. On the basis of the results alone, we concluded that STX was superior to LVX and MXF intracellularly after the administration of one and three doses. Therefore, in intracellular activity studies, the timing of treatment onset should be carefully designed. However, the extra-and intracellular activity of STX against *S. aureus* was greater than LVX and MXF *in vivo*.

In conclusion, STX showed a higher relative antibiotic potency than LVX and MXF (lower values for the Emax and Cs pharmacodynamic parameters). Meanwhile, when used at the same weight concentration, the intracellular antimicrobial activity of STX was obviously superior over LVX and MXF. STX displayed significant activity against *S. aureus* isolates and could be very useful where not only eradication of extracellular forms is required, but also significant reduction of the intracellular bacteria is desirable. In the circumstances that intracellular component is required to combat infections, STX can be very useful.

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