

## Activity of moxifloxacin against intracellular community-acquired methicillin-resistant *Staphylococcus aureus*: comparison with clindamycin, linezolid and co-trimoxazole and attempt at defining an intracellular susceptibility breakpoint

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**Background:** Co-trimoxazole, clindamycin and linezolid are used to treat community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections, but little is known about intracellular activity. Moxifloxacin is active against intracellular methicillin-susceptible *S. aureus* (MSSA), but CA-MRSA has not been studied.

**Methods:** We used 12 clinical CA-MRSA, 1 MSSA overexpressing *norA* and 2 hospital-acquired MRSA (moxifloxacin MICs: 0.03 to 4 mg/L). Activity was assessed in broth and after phagocytosis by THP-1 macrophages or keratinocytes (concentration-dependent experiments [24 h of incubation] to determine relative potencies [ $EC_{50}$ ], static concentrations [ $C_s$ ] and maximal relative efficacies [ $E_{max}$  (change in  $\log_{10}$  cfu compared with initial inoculum)] and time-dependent experiments [0–72 h] at human  $C_{max}$ ).

**Results:** Concentration-dependent experiments: in broth,  $EC_{50}$  and  $C_s$  were correlated with the MIC for all antibiotics, but moxifloxacin achieved significantly ( $P < 0.01$ ) greater killing (more negative  $E_{max}$ ) than the comparators; and in THP-1 cells and keratinocytes, moxifloxacin acted more slowly but still reached a near bactericidal effect (2 to 3  $\log_{10}$  cfu decrease) at 24 h with unchanged  $EC_{50}$  and  $C_s$  as long as its MIC was  $\leq 0.125$  mg/L (recursive partitioning analysis). Clindamycin and linezolid were static, and co-trimoxazole was unable to suppress the intracellular growth of CA-MRSA. At human  $C_{max}$  in broth, moxifloxacin killed more rapidly and more extensively ( $\geq 5 \log_{10}$  cfu decrease at 10 h) than clindamycin (4  $\log_{10}$  cfu at 48 h) or co-trimoxazole and linezolid (1–2  $\log_{10}$  cfu at 72 h).

**Conclusions:** Moxifloxacin is active against both extracellular and intracellular CA-MRSA if the MIC is low, and is more effective than clindamycin, co-trimoxazole and linezolid.

**Keywords:** THP-1 macrophages, keratinocytes, Hill equation, maximal relative efficacy, relative potency, static concentration, bactericidal effect

### Introduction

The fast emergence of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) as a causative agent of infections in young patients<sup>1,2</sup> and other individuals without healthcare-related risk factors raises alarming concerns. These isolates are now also recovered in hospitals and nursing homes.<sup>3–5</sup> CA-MRSA causes not only apparently minor skin and soft-tissue infections, but also bacteraemia, endocarditis,

osteomyelitis or severe necrotizing pneumonia that rapidly progress to death.<sup>6</sup> Beyond its extreme virulence, the ability of *S. aureus* to easily adhere to and invade eukaryotic cells<sup>7</sup> creates a potential additional therapeutic challenge.<sup>8</sup> Intracellular forms of *S. aureus* may indeed be responsible for the high frequency of infection relapses and recurrences observed in clinics.<sup>4</sup>

*In vitro* models<sup>9–12</sup> have shown that most antistaphylococcal antibiotics are considerably less active against the intracellular forms of *S. aureus* than anticipated from their intrinsic activity

and their level of accumulation in cells. Thus, only the novel lipopeptides telavancin and oritavancin, quinupristin/dalfopristin and moxifloxacin have been shown to yield a truly bactericidal effect in these models when tested against a fully susceptible strain.<sup>10,13,14</sup> In the present study, we assessed the activity of moxifloxacin against the intracellular forms of a series of clinical isolates of CA-MRSA. Because resistance of MRSA to fluoroquinolones is of concern,<sup>15–20</sup> we used strains with increased MICs of moxifloxacin to better delineate its actual usefulness against intracellular bacteria with respect to *in vitro* conventional susceptibility testing data and current clinical breakpoints.<sup>21–23</sup> As the current recommended empirical treatment of suspected CA-MRSA infections includes co-trimoxazole (trimethoprim/sulfamethoxazole), clindamycin and linezolid,<sup>24</sup> these antibiotics were included for comparison.

## Materials and methods

### Materials

The following antibiotics were obtained as microbiological standards from their corresponding manufacturers: ciprofloxacin and moxifloxacin from Bayer Healthcare, Leverkusen, Germany; and linezolid from Pfizer

Inc., New York, NY, USA. Clindamycin, trimethoprim and sulfamethoxazole were purchased from Sigma-Aldrich (St Louis, MO, USA) and trimethoprim and sulfamethoxazole were mixed at a 1:20 (w/w) ratio as in the registered clinical preparation (co-trimoxazole). Culture media and sera were from Invitrogen Corporation (Carlsbad, CA, USA) and Becton Dickinson (Franklin Lakes, NJ, USA) and other reagents were from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).

### Bacterial strains

The strains used in this study are presented in Table 1, with information on their origin as well as on their particular resistance characteristics: 12 clinically defined CA-MRSA (corresponding to the definition proposed by Millar *et al.*<sup>25</sup>) selected to cover a range of MICs spanning from below to close to or above the current clinical susceptibility breakpoints set by the US CLSI<sup>21</sup> and the European Committee for Antibiotic Susceptibility Testing (EUCAST);<sup>22</sup> 1 methicillin-susceptible *S. aureus* (MSSA) overexpressing *norA*; and 2 MRSA strains with full resistance to moxifloxacin. The fully susceptible ATCC 25923 strain [Panton-Valentine leucocidin (PVL)-positive;<sup>26</sup> confirmed for the strain used here by the same technique] was used as an internal control and for comparison with previous studies.<sup>27,28</sup> The MRSA phenotype of each strain was confirmed by detection of the *mecA* gene by PCR, and the staphylococcal cassette chromosome *mec* (SCC*mec*) subgroup was established as previously described.<sup>29,30</sup>

**Table 1.** Strains used in this study (resistance phenotype, origin, SCC*mec* subgroup<sup>60</sup> and MICs of moxifloxacin and ciprofloxacin)

| Strain no.      | Resistance phenotype <sup>a</sup>   | Origin <sup>b</sup> | SCC <i>mec</i> group <sup>c</sup> | MIC (mg/L) <sup>d</sup> |                        |
|-----------------|---|---------------------|-----------------------------------|-------------------------|------------------------|
|                 |   |                     |                                   | MXF                     | CIP                    |
| ATCC 25923      | MSSA/PVL+ <sup>26</sup>   | ATCC                | NA                                | 0.03                    | 0.125                  |
| N4090440        | CA-MRSA/PVL+  | 28                  | IVa                               | 0.015                   | 0.25                   |
| STA44           | CA-MRSA/PVL+  | 61                  | V                                 | 0.015                   | 0.5                    |
| STA268          | CA-MRSA/PVL+  | 61                  | V                                 | 0.015                   | 0.5                    |
| CHU1            | CA-MRSA/PVL+  | 61                  | V                                 | 0.03                    | 0.5                    |
| N4042228        | CA-MRSA/PVL+  | 28                  | IVa                               | 0.03                    | 0.25                   |
| NRS192          | CA-MRSA/PVL+  | NARSA               | IVa                               | 0.03                    | 0.5                    |
| SA1             | MSSA – <i>norA</i> overexpression/PVL+                                    | 62                  | NA                                | 0.06                    | <b>4.0</b>             |
| NRS384 (USA300) | CA-MRSA/PVL+  | NARSA               | IVa                               | 0.06                    | 0.5                    |
| MEH22256        | CA-MRSA/PVL+  | NUS                 | IVa                               | 0.06                    | 1.0                    |
| NRS386 (USA700) | CA-MRSA/PVL–  | NARSA               | IVa                               | 0.125                   | <b>4.0</b>             |
| SA069           | CA-MRSA/ <i>gyrA-grlA</i> (single)/PVL–                                   | HMC                 | IV                                | <b>1.0</b>              | <b>64</b>              |
| HMC551          | CA-MRSA <sup>e</sup> /PVL–  | HMC                 | IVa                               | <b>2.0</b>              | <b>32</b>              |
| KKHII-7924      | CA-MRSA/PVL–  | NUS                 | IVc                               | <b>2.0</b>              | <b>64</b>              |
| SA873           | HA-MRSA/h-VISA/PVL–   | HMC                 | II                                | <b>4.0</b>              | <b>128<sup>f</sup></b> |
| SA481           | HA-MRSA <sup>g</sup> / <i>gyrA</i> (single) and <i>grlA</i> (double)/PVL– | HMC                 | II                                | <b>4.0</b>              | <b>256</b>             |

<sup>a</sup>CA-MRSA, community-acquired MRSA (as per clinical record); HA-MRSA, hospital-acquired MRSA (as per clinical record); h-VISA, hetero-VISA; PVL+/-, Panton-Valentine leucocidin producer/non-producer (assessed by PCR); *norA*, gene encoding norfloxacin efflux transporter [member of the major facilitator superfamily ('MFS') transporters]; *gyrA*, *grlA* and *gyrB* (single or double), mutation(s) in the corresponding subunits of DNA gyrase.

<sup>b</sup>ATCC, American Tissue Culture Collection (Manassas, VA, USA); NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus* (operated by Eurofins Medinet, Hendon, VA, USA); HMC, Hershey Medical Center, PA, USA (this study); NUS, National University of Singapore (Dr L. Y. Hsu).

<sup>c</sup>NA, not applicable.

<sup>d</sup>MXF, moxifloxacin; CIP, ciprofloxacin. Figures in bold and italic and in bold indicate MIC values that exceed the 'susceptible' (S) and 'resistant' (R) clinical breakpoints of EUCAST<sup>22</sup> (moxifloxacin,  $S \leq 0.5$  mg/L/R  $> 1$  mg/L; ciprofloxacin,  $S \leq 1$  mg/L/R  $> 1$  mg/L); for comparison, the current clinical breakpoints of the CLSI are: moxifloxacin,  $S \leq 0.5$  mg/L/I = 1 mg/L/R  $\geq 2$  mg/L; and ciprofloxacin,  $S \leq 1$  mg/L/I = 2 mg/L/R  $\geq 4$  mg/L.

<sup>e</sup>Isolated in hospital, but with SCC*mec* cassette suggesting a community origin.<sup>63</sup>

<sup>f</sup>Macrobroth dilution method.

<sup>g</sup>CA-MRSA by clinical definition, but showing genotype of HA-MRSA (SCC*mec* II, PVL–).

## Susceptibility testing and dose-effect relationship in broth

MICs were determined in Mueller–Hinton (MH) broth by the microdilution method according to CLSI recommendations.<sup>23</sup> Dose–kill curve studies were performed as described previously.<sup>9</sup> For fluoroquinolone antibiotics, 24 h samples were also incubated in the presence of MH broth supplemented with 25 g/L activated charcoal (to minimize antibiotic carry-over), but no significant differences in antibiotic activity were observed between activated charcoal-treated and untreated samples. Bactericidal activity was defined as a reduction of 99.9% ( $\geq 3 \log_{10}$  cfu decrease) of the total count from the corresponding original inoculum.<sup>21</sup>

## Cells lines and assessment of cell viability

Experiments were performed with: (i) THP-1 cells (ATCC TIB-202), a human myelomonocytic cell line displaying macrophage-like activity and maintained in our laboratory as previously described;<sup>31</sup> and (ii) human skin keratinocytes, obtained as Primary Human Keratinocytes (catalogue no. 12332-011; Invitrogen s.a., Merelbeke, Belgium) and cultivated in BD Biocoat™ Collagen type IV-coated flasks and defined Keratinocyte-SFM medium (as described by the cell supplier's instructions). The cell viability was assessed by the trypan blue exclusion assay after exposure to the highest concentration of each of the antibiotics. No significant difference was detected between treated and control cells (<10% stained cells). No significant change in cell viability was observed either for cells infected with MSSA or CA-MRSA (<15% stained cells).

## Cell infection and assessment of the intracellular activity of antibiotics

Cell infection was performed as described previously.<sup>10,28,30</sup> The changes in the number of cfu from the post-phagocytosis inoculum (set at  $1.0$  to  $3.0 \times 10^6$  cfu/mg of cell protein) were taken as the response to the antibiotics and plotted as a function of the extracellular concentration of antibiotic (see Barcia-Macay et al.<sup>10</sup> and Lemaire et al.<sup>28</sup> for more details).

## Curve fitting and statistical analyses

Curve fittings were made using the Hill's equation (GraphPad Prism® version 4.03, GraphPad Software, San Diego, CA, USA) to obtain, for each condition, numeric values of four key descriptors: (i) the increase in the number of cfu for an infinitely low antibiotic concentration [relative minimal efficacy ( $E_{\min}$ ; in  $\log_{10}$  cfu units)] compared with the original post-phagocytosis inoculum; (ii) the decrease in the number of cfu for an infinitely large concentration of antibiotic [relative maximal efficacy ( $E_{\max}$ ; in  $\log_{10}$  cfu units)]; (iii) the concentration of antibiotic yielding a response halfway between  $E_{\min}$  and  $E_{\max}$  [relative potency ( $EC_{50}$ ; in mg/L or in multiples of MIC)]; and (iv) the concentration of antibiotic resulting in no apparent bacterial growth compared with the original inoculum [static concentration ( $C_s$ ; in mg/L or in multiple of MIC)]. Statistical analyses were performed using GraphPad InStat® version 3.06 (GraphPad Software). Recursive partitioning analysis was performed using JMP version 8.0.1 from the SAS Institute, Cary, NC, USA.

## Results

### Susceptibility testing

Table 1 shows the MICs of moxifloxacin and ciprofloxacin for each strain studied. For moxifloxacin, these ranged from 0.015 to 0.125 mg/L for strains without mutation in DNA gyrase (all these strains can be categorized as susceptible according to

EUCAST<sup>22</sup> and the CLSI<sup>21</sup>), 1.0 mg/L for one strain with a single DNA gyrase mutation (categorized as intermediate) and 2 mg/L for two confirmed CA-MRSA strains (categorized as resistant by EUCAST but intermediate by CLSI criteria). The two healthcare-associated (HA)-MRSA strains were fully resistant (MIC=4 mg/L; a double mutation was documented for one them). The strain SA1 overexpressing *norA* was as susceptible to moxifloxacin as all other non-mutated strains. The MICs of ciprofloxacin were, on average, 3–4  $\log_2$  dilutions higher than those of moxifloxacin for all non-mutated strains, except for SA1 and NRS386 (USA700). All strains with mutations showed high-level resistance to ciprofloxacin. The strains ATCC 25923 and NRS192 were also tested for susceptibility to the other antibiotics used in this study, with the following results: clindamycin, 0.06 and 0.125 mg/L; co-trimoxazole, 1 and 1–2 mg/L; linezolid, 1–2 and 1–2 mg/L; and rifampicin, 0.03 and 0.06 mg/L ( $n = 4$  for most antibiotic/strain combinations; if values differed by 1  $\log_2$  dilution, both are given; there were no values differing by more than 1  $\log_2$  dilution).

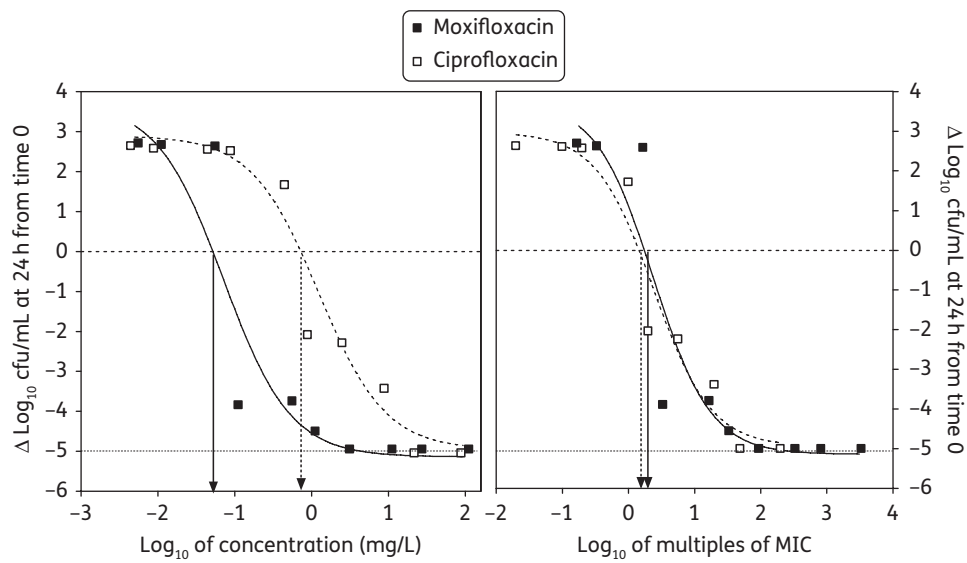
### Fixed timepoint (24 h) pharmacodynamic studies

#### Moxifloxacin versus ciprofloxacin for strain NRS192 (CA-MRSA) in broth

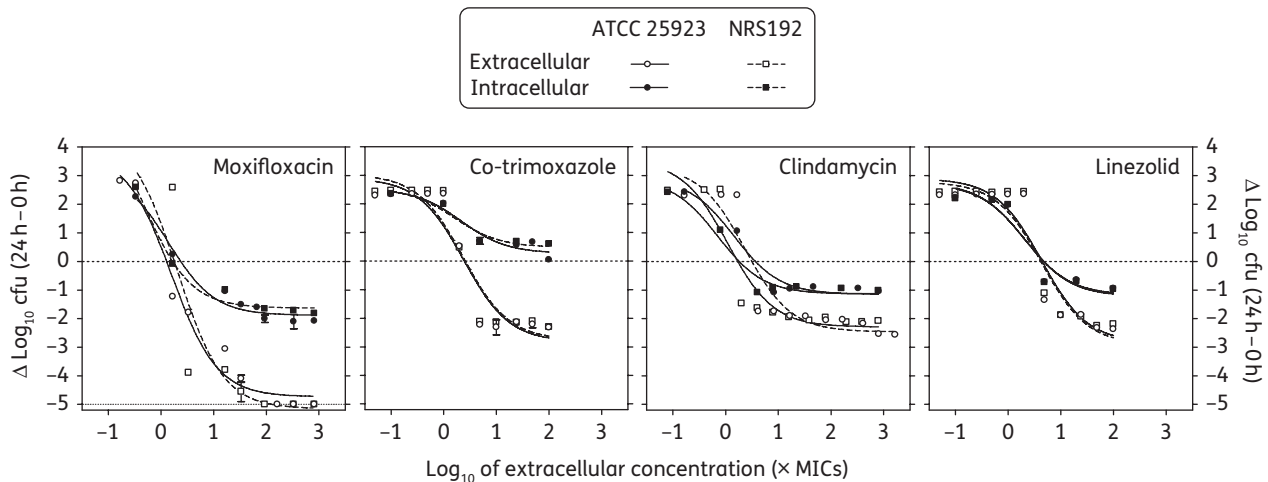
Since moxifloxacin appeared markedly more active than ciprofloxacin by conventional MIC testing for all strains tested, we examined whether this would translate into differences in pharmacodynamic properties between the two antibiotics. To this effect, we exposed one strain of CA-MRSA (NRS192) for 24 h in broth to a wide range of concentrations (ranging from  $0.3 \times$  to  $200 \times$  the MIC). Data presented in Figure 1 show that whereas moxifloxacin and ciprofloxacin had different relative potencies ( $EC_{50}$ ) and static concentrations ( $C_s$ ) when expressed as weight concentrations (mg/L), the responses were almost undistinguishable when data were plotted as a function of multiples of the MIC, indicating that the MIC was the only driving factor in this context.

#### Moxifloxacin versus co-trimoxazole, clindamycin and linezolid for strains ATCC 25923 (MSSA) and NRS192 (CA-MRSA) in broth, THP-1 macrophages and human keratinocytes

In a second series of experiments, moxifloxacin was compared with co-trimoxazole, clindamycin and linezolid using both the MSSA strain ATCC 25923 and a typical CA-MRSA strain (NRS192), comparing both extracellular (broth) and intracellular activities. Figure 2 shows the results of the concentration–effect studies using multiples of MIC as a basis of comparison. Numerical values (including conversion of the  $C_s$  values into mg/L) are presented in Table 2. In broth, all antibiotics showed a static effect ( $C_s$ ) close to their MIC and a similar relative potency ( $EC_{50}$ ) when expressed in multiples of the MIC. However, marked differences were observed concerning their relative efficacies ( $E_{\max}$ ). Moxifloxacin achieved an almost complete eradication (about  $5 \log_{10}$  cfu/mL decrease; close to the limit of detection), whereas none of the three other antibiotics were bactericidal (less than  $3 \log_{10}$  cfu/mL decrease). The activity of all antibiotics was markedly decreased when tested



**Figure 1.** Concentration–effect relationships of CA-MRSA strain NRS192 in broth after 24 h of incubation with moxifloxacin (filled symbols) or ciprofloxacin (open symbols). The ordinate shows the change in the number of cfu/mL compared with the original inoculum (broken horizontal line at 0). The broken line at  $-5$  corresponds to the limit of quantification. The abscissa shows the antibiotic concentration expressed as the  $\log_{10}$  of its weight values (mg/L; left-hand panel) or as the  $\log_{10}$  of the multiples of the MIC (right-hand panel). Values shown are means  $\pm$  SD;  $n=3$  determinations in a single experiment; most SD bars are smaller than the symbols. Data were used to fit Hill equations [slope factor=1;  $R^2=0.87$  (lowest) to 0.96 (highest)] and deriving the pertinent key pharmacodynamic properties for moxifloxacin versus ciprofloxacin, namely: (i)  $E_{max}$  (relative efficacy, in  $\log_{10}$  cfu units compared with the original inoculum),  $-5.14$  [95% confidence interval (CI),  $-6.62$  to  $-3.67$ ] versus  $-4.96$  (95% CI,  $-6.21$  to  $-3.70$ ); (ii)  $EC_{50}$  [relative potency; concentration causing a reduction of the inoculum halfway between  $E_{min}$  (change in cfu for an infinitely low antibiotic concentration) and  $E_{max}$ ],  $0.07$  mg/L (95% CI,  $0.02$  to  $0.32$ ) and  $2.47 \times$  MIC (95% CI,  $0.43$  to  $14.11$ ) versus  $1.24$  mg/L (95% CI,  $0.54$  to  $2.87$ ) and  $3.01 \times$  MIC (95% CI,  $1.07$  to  $8.4$ ); and (iii)  $C_s$  (concentration resulting in no apparent bacterial growth),  $0.05$  mg/L and  $1.83 \times$  MIC (vertical arrowed continuous lines) versus  $0.73$  mg/L and  $1.56 \times$  MIC (vertical arrowed broken lines).



**Figure 2.** Concentration–response curves of extracellular (broth) and intracellular (THP-1 macrophages) *S. aureus* ATCC 25923 (MSSA) and NRS192 (CA-MRSA) exposed to moxifloxacin, co-trimoxazole, clindamycin and linezolid for 24 h. The ordinate shows the change in the number of cfu (means  $\pm$  SD;  $n=3$  determinations in a single experiment; most SD bars are smaller than the symbols) per mL of culture medium (extracellular; open symbols) or per mg of cell protein (intracellular; filled symbols) compared with the initial inoculum (broken line at 0). The broken line at  $-5$  in the left-hand panel corresponds to the limit of quantification. The abscissa shows the antibiotic concentration (total drug) expressed as multiples of the MIC of each antibiotic for the corresponding strain [moxifloxacin,  $0.03$  mg/L for both strains; co-trimoxazole,  $1$  mg/L for both strains; clindamycin,  $0.06$  (ATCC 25923) and  $0.125$  mg/L (NRS192); and linezolid,  $1$  mg/L for both strains]. The data were used to fit sigmoidal functions (Hill equation; slope factor=1).<sup>64</sup> The goodness of fit ( $R^2$ ) and key pharmacodynamic parameters derived from each function ( $E_{max}$ ,  $EC_{50}$  and  $C_s$ ) for each condition are shown in Table 2.

**Table 2.** Pertinent regression parameters (with 95% confidence intervals) and statistical analysis of the concentration–response curves of moxifloxacin versus comparators against ATCC 25923 (MSSA) and NRS192 (CA-MRSA) in broth (extracellular bacteria) and in THP-1 cells (phagocytized bacteria) as illustrated in Figure 2

| Condition and antibiotic | ATCC 25923 (MSSA)          |                         |                                |       | NRS192 (CA-MRSA)           |                         |                                |       |
|--------------------------|----------------------------|-------------------------|--------------------------------|-------|----------------------------|-------------------------|--------------------------------|-------|
|                          | $E_{max}^a$                | $EC_{50}^b$             | $C_s^c$                        | $R^2$ | $E_{max}^a$                | $EC_{50}^b$             | $C_s^c$                        | $R^2$ |
| Broth                    |                            |                         |                                |       |                            |                         |                                |       |
| moxifloxacin             | −4.79 a;x (−5.35 to −4.23) | 1.58 a;x (0.76 to 3.29) | ×MIC<br>~1.29<br>mg/L<br>~0.04 | 0.98  | −5.12 a;x (−6.72 to −3.52) | 2.04 a;x (0.23 to 18.0) | ×MIC<br>~1.78<br>mg/L<br>~0.05 | 0.85  |
| co-trimoxazole           | −2.83 b;x (−3.91 to −1.75) | 2.39 a;x (0.80 to 7.18) | ×MIC<br>~2.45<br>mg/L<br>~2.4  | 0.92  | −2.77 b;x (−3.81 to −1.73) | 2.34 a;x (0.82 to 6.67) | ×MIC<br>~2.45<br>mg/L<br>~2.45 | 0.93  |
| clindamycin              | −2.46 b;x (−3.19 to −1.74) | 2.34 a;x (0.72 to 7.63) | ×MIC<br>~3.16<br>mg/L<br>~0.2  | 0.88  | −2.29 b;x (−2.99 to −1.59) | 1.10 a;x (0.35 to 3.47) | ×MIC<br>~3.16<br>mg/L<br>~0.4  | 0.89  |
| linezolid                | −2.92 b;x (−4.23 to −1.62) | 4.34 a;x (1.34 to 14.1) | ×MIC<br>~4.07<br>mg/L<br>~4.1  | 0.91  | −2.85 b;x (−4.12 to −1.58) | 4.31 a;x (1.38 to 13.4) | ×MIC<br>~4.37<br>mg/L<br>~4.4  | 0.92  |
| THP-1 cells              |                            |                         |                                |       |                            |                         |                                |       |
| moxifloxacin             | −1.96 a;y (−2.27 to −1.64) | 1.40 a;x (0.47 to 4.16) | ×MIC<br>~2.34<br>mg/L<br>~0.07 | 0.97  | −1.69 a;y (−2.04 to −1.33) | 0.50 a;x (0.09 to 2.84) | ×MIC<br>~1.62<br>mg/L<br>~0.05 | 0.98  |
| co-trimoxazole           | 0.26 b;y (−0.29 to 0.81)   | 2.16 a;x (0.22 to 21.1) | ×MIC<br>>100<br>mg/L<br>>100   | 0.94  | 0.48 b;y (−0.22 to 1.17)   | 1.55 a;x (0.05 to 44.2) | ×MIC<br>>100<br>mg/L<br>>100   | 0.94  |
| clindamycin              | −1.15 c;y (−1.68 to −0.62) | 1.45 a;x (0.39 to 5.35) | ×MIC<br>~3.72<br>mg/L<br>~0.22 | 0.96  | −1.14 a;x (−1.68 to −0.59) | 0.71 a;x (0.19 to 2.67) | ×MIC<br>~1.82<br>mg/L<br>~0.23 | 0.96  |
| linezolid                | −1.19 c;y (−2.41 to 0.03)  | 2.04 a;x (0.17 to 23.9) | ×MIC<br>~4.79<br>mg/L<br>~4.8  | 0.93  | −1.23 a;y (−2.49 to 0.02)  | 2.13 a;x (0.17 to 25.9) | ×MIC<br>~4.79<br>mg/L<br>~4.8  | 0.92  |

Statistical analysis (using raw values for  $E_{max}$  and log-transformed values for  $EC_{50}$ ): analysis per column, values ( $E_{max}$  or  $EC_{50}$ ) with different first lowercase letters (a, b, c) are significantly different ( $P < 0.05$ ) from each other within the same condition group [broth or THP-1 cells (one-way ANOVA with Tukey post test for multiple comparisons)] and values ( $E_{max}$  or  $EC_{50}$ ) with different last lower case letters (x, y, z) are significantly different ( $P < 0.05$ ) from each other when comparing broth with THP-1 cells (unpaired, two-tailed  $t$ -test); and analysis per row, there was no statistically significant differences when comparing values ( $E_{max}$  or  $EC_{50}$ ) between ATCC 25923 and NRS192 strains (unpaired, two-tailed  $t$ -test; all comparisons with  $P > 0.05$ ).

<sup>a</sup>Maximal relative efficacy; cfu decrease (in  $\log_{10}$  units) at 24 h from the original, post-phagocytosis inoculum (per mL for broth or per mg of cell protein for cells), as extrapolated for an infinitely large antibiotic concentration using the Hill equation (slope factor=1).

<sup>b</sup>Relative potency; concentration (in multiples of MIC; total drug) causing a reduction of the inoculum at 24 h halfway between the minimal ( $E_{min}$ ) and the maximal ( $E_{max}$ ) values derived from the Hill equation.

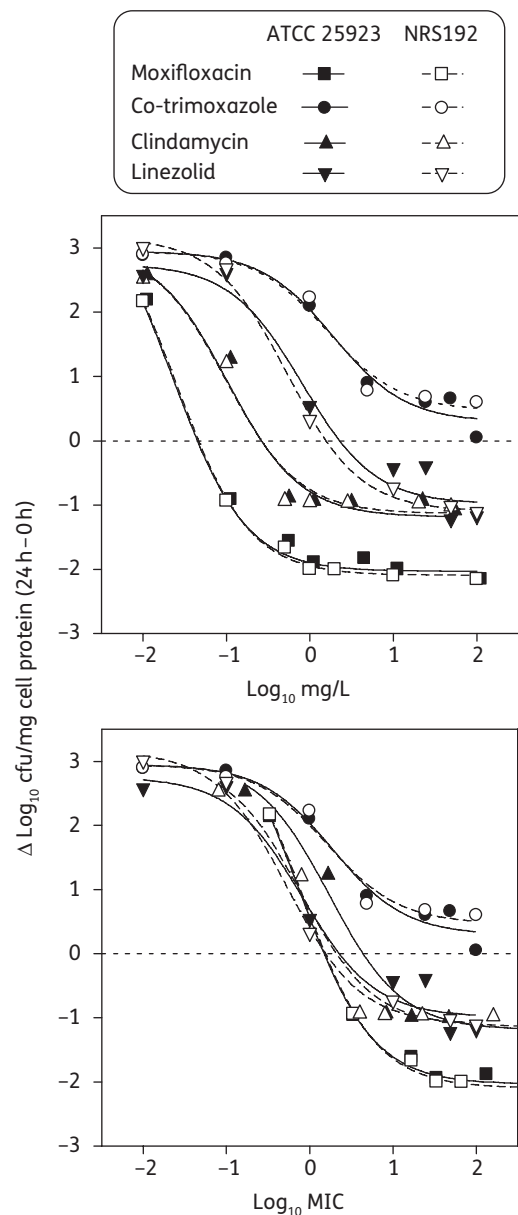
<sup>c</sup>Concentration [multiples of the MIC (upper row) and mg/L (lower row); total drug] resulting in no apparent bacterial growth at 24 h compared with the initial inoculum (time=0 h), as determined by graphical interpolation using the Hill equation.

against the intracellular forms of both ATCC 25923 and NRS192 using THP-1 macrophages. With the exception of co-trimoxazole, however, the changes did not affect the relative potencies ( $EC_{50}$ ) or the static concentrations ( $C_s$ ), but rather the maximal relative efficacies ( $E_{max}$ ) of the antibiotics. Although moxifloxacin was not truly bactericidal (3  $\log_{10}$  cfu decrease), its  $E_{max}$  was nevertheless the most important (most negative) of all antibiotics tested. For co-trimoxazole, the loss of activity was such that no static concentration could be determined against intracellular *S. aureus*. In all these experiments, no difference in the responses was seen between ATCC 25923 and NRS192.

Figure 3 shows the results of concentration–effect studies of moxifloxacin, co-trimoxazole, clindamycin and linezolid with the strains ATCC 25923 and NRS192 after phagocytosis by human keratinocytes [presented both as a function of the weight concentrations (mg/L; upper diagram) and of the multiples of the respective MICs (lower diagram)]. Numerical data and statistical analyses are presented in Table 3. As for bacteria phagocytized by THP-1 macrophages, moxifloxacin was the most active agent when considering its  $E_{max}$ , followed by clindamycin and linezolid. As in THP-1 macrophages, co-trimoxazole did not achieve a static effect towards *S. aureus* phagocytized by human keratinocytes. No significant difference in the responses was seen between the ATCC 25923 and NRS192 strains in all these experiments. When examining the responses as a function of equipotent antibiotic concentrations (multiples of MIC), no significant difference was noted between moxifloxacin, clindamycin and linezolid with respect to their relative potencies ( $EC_{50}$ ) and static concentrations ( $C_s$ ), suggesting that, as for the other conditions described so far, MIC was the driving factor regarding these parameters for these antibiotics. In contrast, co-trimoxazole showed a significantly lower relative potency (higher value of  $EC_{50}$ ) compared with the other antibiotics, even when expressed as a multiple of their MIC, further documenting its intrinsically weaker activity against intracellular *S. aureus*.

#### Moxifloxacin against CA-MRSA strains with differing susceptibilities after phagocytosis by THP-1 macrophages

Since the experiments described so far suggested that MIC is the driving force in the intracellular activity of moxifloxacin, with respect to its relative potency and static concentration, we performed systematic concentration–effect experiments using strains with increasing MICs. The results are shown in Figure 4(a) for eight strains selected for displaying MICs  $\leq 0.06$  ( $n = 3$ ),  $=0.125$  ( $n = 1$ ),  $=1$  mg/L ( $n = 1$ ) and  $\geq 2$  mg/L ( $n = 3$ ), respectively, with the data plotted as a function of the extracellular weight concentration (mg/L). The strains with an MIC  $\leq 0.06$  mg/L showed an essentially indistinguishable behaviour, with static concentrations ( $C_s$ ) around the same value and maximal relative efficacies ( $E_{max}$ ) around  $-2 \log_{10}$  cfu/mg protein. Strains with higher MICs showed a progressive shift of both the  $C_s$ , which increased, and of the  $E_{max}$ , which decreased (less negative  $E_{max}$  values). This culminated with the HA-MRSA strain SA481, for which the  $E_{max}$  was decreased to the point where the antibiotic became static [no reduction of the inoculum



**Figure 3.** Concentration–response curves of intracellular *S. aureus* ATCC 25923 (MSSA; filled symbols) and NRS192 (CA-MRSA; open symbols) phagocytized by human keratinocytes and exposed to moxifloxacin, co-trimoxazole, clindamycin or linezolid for 24 h. The ordinate shows the change in the number of cfu (means  $\pm$  SD;  $n = 3$  determinations in a single experiment; most SD bars are smaller than the symbols) per mg of cell protein compared with the initial inoculum (time = 0 h; broken line) and the abscissa the antibiotic concentration (total drug) expressed as mg/L (upper panel) or multiples of the MIC for the corresponding strain [lower panel: moxifloxacin, 0.03 mg/L for both strains; co-trimoxazole, 1 mg/L for both strains; clindamycin, 0.06 (ATCC 25923) and 0.125 mg/L (NRS192); linezolid, 1 mg/L for both strains]. The data were used to fit sigmoidal functions (Hill equation; slope factor = 1).<sup>64</sup> The goodness of fit ( $R^2$ ) and key pharmacodynamic parameters derived from each function ( $E_{max}$ ,  $EC_{50}$  and  $C_s$ ) for each antibiotic–strain combination are shown in Table 3.

**Table 3.** Pertinent regression parameters (with 95% confidence intervals) and statistical analysis of the concentration–response curves to moxifloxacin, co-trimoxazole, clindamycin and linezolid against ATCC 25923 (MSSA) and NRS192 (CA-MRSA) in keratinocytes (phagocytized bacteria) as illustrated in Figure 3

| Antibiotic     | ATCC 25923 (MSSA)           |  |                |       | NRS192 (CA-MRSA)            |  |                |       |
|----------------|-----------------------------|--|----------------|-------|-----------------------------|--|----------------|-------|
|                | $E_{\max}^a$                | $EC_{50}^b$  | $C_s^c$        | $R^2$ | $E_{\max}^a$                | $EC_{50}^b$  | $C_s^c$        | $R^2$ |
| Moxifloxacin   | −2.03 a<br>(−2.22 to −1.84) | ×MIC 0.77 a (0.36 to 1.61)<br>mg/L 0.02 a (0.01 to 0.05) | ~1.48<br>~0.04 | 0.99  | −2.09 a<br>(−2.21 to −1.97) | ×MIC 0.83 a (0.54 to 1.29)<br>mg/L 0.03 a (0.02 to 0.04) | ~1.55<br>~0.05 | 0.99  |
| Co-trimoxazole | 0.29 b<br>(−0.12 to 0.71)   | ×MIC 1.90 a (0.66 to 5.46)<br>mg/L 1.90 b (0.66 to 5.46) | >100<br>>100   | 0.97  | 0.47 b<br>(−0.11 to 1.05)   | ×MIC 1.61 b (0.40 to 6.51)<br>mg/L 1.6 b (0.40 to 6.51)  | >100<br>>100   | 0.97  |
| Clindamycin    | −1.19 c<br>(−1.66 to −0.71) | ×MIC 1.73 a (0.59 to 5.02)<br>mg/L 0.10 c (0.04 to 0.30) | ~4.37<br>~0.26 | 0.97  | −1.13 c<br>(−1.61 to −0.65) | ×MIC 0.79 a (0.26 to 2.38)<br>mg/L 0.10 c (0.03 to 0.30) | ~2.14<br>~0.26 | 0.97  |
| Linezolid      | −0.99 c<br>(−1.56 to −0.41) | ×MIC 0.81 a (0.21 to 3.07)<br>mg/L 0.81 b (0.21 to 3.07) | ~2.26<br>~2.24 | 0.97  | −1.10 c<br>(−1.39 to −0.80) | ×MIC 0.54 a (0.31 to 0.95)<br>mg/L 0.54 d (0.31 to 0.95) | ~1.55<br>~1.55 | 0.99  |

Statistical analysis (using raw values for  $E_{\max}$  and log-transformed values for  $EC_{50}$ ): analysis per column; values ( $E_{\max}$  or  $EC_{50}$ ) with different lowercase letters (a, b, c) are significantly different ( $P < 0.05$ ) from each other (one-way ANOVA with Tukey post test for multiple comparisons); and analysis per row; there was no statistically significant differences when comparing values ( $E_{\max}$  or  $EC_{50}$ ) between ATCC 25923 and NRS192 strains (unpaired, two-tailed *t*-test; all comparisons with  $P > 0.05$ ).

<sup>a</sup>Relative efficacy; cfu decrease (in  $\log_{10}$  units) at 24 h from the original, post-phagocytosis inoculum, as extrapolated for an infinitely large antibiotic concentration using the Hill equation (slope factor=1).

<sup>b</sup>Relative potency; concentration [in multiples of MIC (upper row) and in mg/L (lower row); total drug] causing a reduction of the inoculum at 24 h halfway between the minimal ( $E_{\min}$ ) and the maximal ( $E_{\max}$ ) values derived from the Hill equation.

<sup>c</sup>Concentration [multiples of the MIC (upper row) and mg/L (lower row); total drug] resulting in no apparent bacterial growth at 24 h compared with the initial inoculum (time=0 h), as determined by graphical interpolation using the Hill equation.

below the initial, post-phagocytosis value, even at the highest concentration tested (100 mg/L)]. These experiments were then extended to all the other strains shown in Table 1, and the corresponding  $E_{\max}$  and  $\log_{10} C_s$  values (calculated from the corresponding Hill equations) of all 15 strains are shown in Figure 4(b). Recursive partitioning analysis of these data suggested a dichotomous split at an MIC of 0.125 mg/L between strains with a lower MIC for which moxifloxacin showed a large maximal relative efficacy ( $E_{\max} = -1.87 \pm 0.28 \log_{10}$  cfu/mg protein) and a low static concentration ( $C_s = 0.11 \pm 0.05$  mg/L) versus those with a larger MIC for which the maximal efficacy was considerably reduced ( $E_{\max} = -0.66 \pm 0.57 \log_{10}$  cfu/mg protein) and the static concentration considerably increased ( $C_s = 19.9 \pm 23.9$  mg/L).

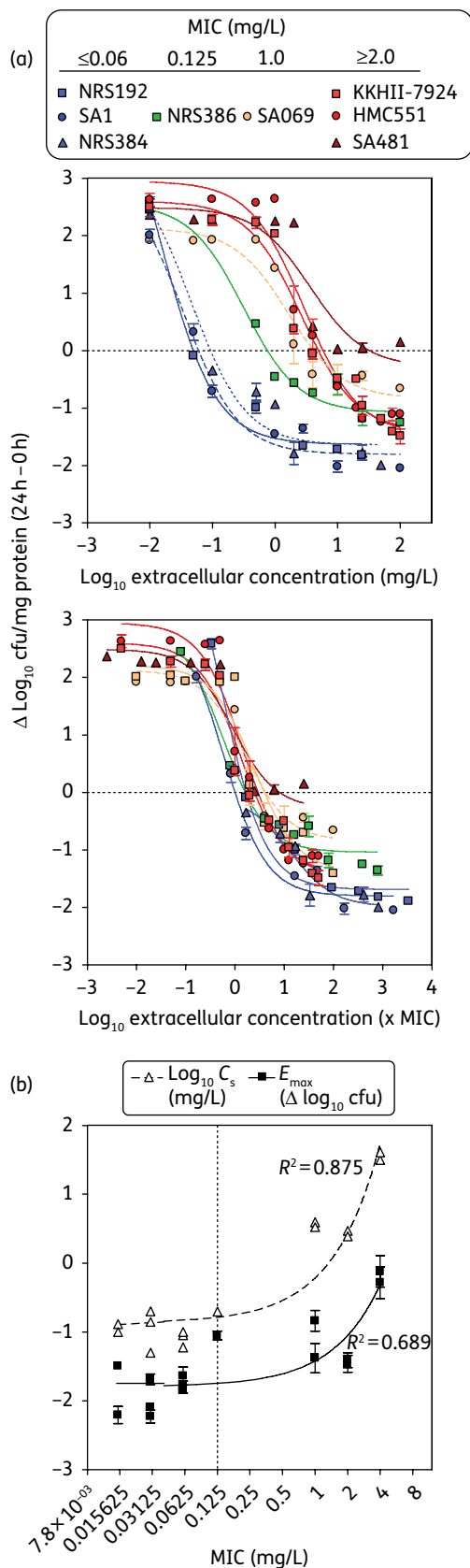
### Time-dependent studies

In the latter series of experiments, time–kill curves were performed with moxifloxacin, co-trimoxazole, clindamycin and linezolid for both the ATCC 25923 and the NRS192 strains in broth and after phagocytosis by human THP-1 macrophages, using a fixed concentration of each antibiotic corresponding to its human  $C_{\max}$  (total drug). The results are presented in Figure 5 for strain NRS192. In broth, moxifloxacin was characterized by a very fast bactericidal effect, reaching a  $3 \log_{10}$  cfu/mL decrease in about 2.5 h. Clindamycin achieved a similar killing effect in about 24 h, whereas the decrease in cfu was limited to about  $2 \log_{10}$  cfu/mL for co-trimoxazole and linezolid. In THP-1 macrophages, all antibiotics showed a marked decrease in the rate of their antibacterial effect. As a result, moxifloxacin's bactericidal effect was about 10× slower and, even at 72 h, only

marginally exceeded a  $2 \log_{10}$  cfu/mg protein decrease. Clindamycin and linezolid were essentially static (less than a  $1 \log_{10}$  cfu/mg protein decrease, with no progression over time after 24 h). Co-trimoxazole was unable to control the intracellular growth of the bacteria. Very similar results were observed for the strain ATCC 25923.

### Discussion

Worldwide and rapid emergence of resistance to ciprofloxacin among MRSA clinical isolates in the late 1980s<sup>32</sup> has led to the conclusion that fluoroquinolones are not a reasonable option in the empirical treatment of infections caused by these organisms. Thus, the European Summary of Product Characteristics (SmPC) of moxifloxacin states that the drug is not recommended for the treatment of MRSA infections.<sup>33</sup> In the USA, infections due to MRSA are not an approved indication for moxifloxacin.<sup>34</sup> Yet, at least one clinical study suggests that moxifloxacin can be efficacious in complicated skin and skin structure infections caused by MRSA<sup>35</sup> with an MIC<sub>90</sub> of 2 mg/L (cited by Dryden<sup>36</sup>). We see from the present study that CA-MRSA strains, defined by clinical criteria, may remain susceptible to moxifloxacin, with MICs as low or close to that of the fully susceptible laboratory strain ATCC 25923, with no impact of the overexpression of *norA* that affects ciprofloxacin and other hydrophilic fluoroquinolones.<sup>37</sup> However, target mutations clearly make the strains intermediate or resistant based on clinical susceptibility breakpoints. This was used here to try to correlate the level of intracellular activity with the MIC in an attempt to approach what could be defined as an intracellular susceptibility breakpoint. For strains considered susceptible based on their MIC, we also could compare moxifloxacin



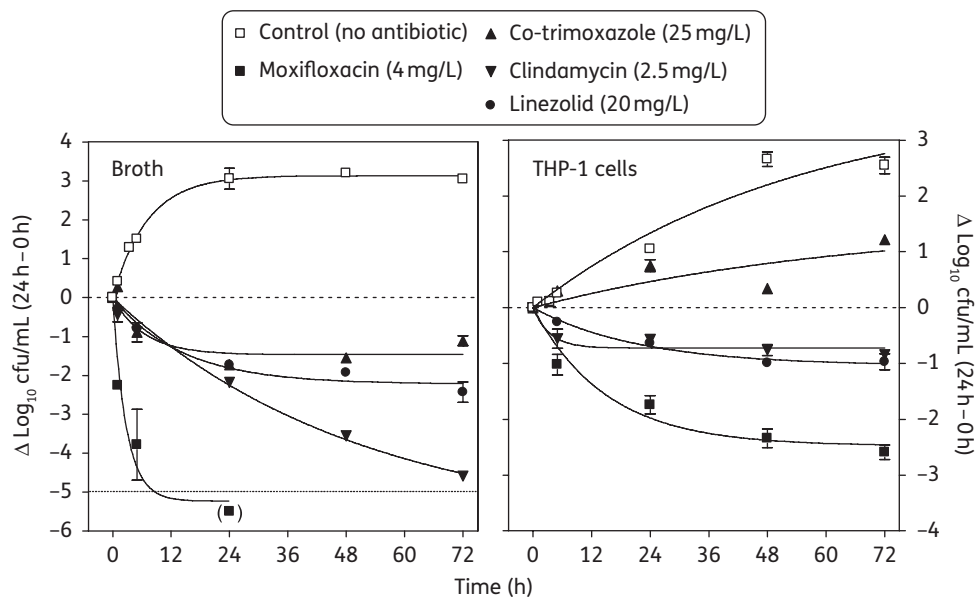
with three antibiotics commonly recommended for the treatment of infections caused by these organisms.

We first show that the activity of both moxifloxacin and the comparators towards the extracellular forms of the isolates tested is essentially driven by the value of their respective MICs as far as their relative potencies ( $EC_{50}$ ) and static concentrations ( $C_s$ ) are concerned. Moxifloxacin, however, shows a considerably larger maximal efficacy (lower  $E_{\max}$ ), allowing for a fast and apparently complete eradication at concentrations remaining clinically relevant. Our results are consistent with those obtained in an *in vitro* model simulating the epithelial lining fluid concentrations observed after conventional dosing of moxifloxacin and that showed a marked bactericidal effect of the drug against CA-MRSA isolates.<sup>38</sup> Conversely, they are divergent from those of Kaka *et al.*,<sup>39</sup> who performed time-kill studies with clinical isolates of CA-MRSA at quite similar concentrations to those used here. In their studies, co-trimoxazole was bactericidal at 24 h, clindamycin was bacteriostatic and moxifloxacin yielded a 3  $\log_{10}$  cfu/mL decrease at 4 h, with no further progression over time and, for one strain, regrowth at 48 h. We have no simple explanation for these differences that may relate to strain variability or differences in experimental conditions that need to be further explored.

Moving now to intracellular activity, we see that the activity of moxifloxacin is considerably impaired in terms of maximal relative efficacy ( $E_{\max}$ ), but not with respect to relative potency ( $EC_{50}$ ) or static concentration ( $C_s$ ). The data must be interpreted as indicating that while the bulk of the intracellular inoculum is as susceptible to moxifloxacin as the extracellular one, a small (1%) but measurable proportion is either in a refractive state or cannot be reached, whatever the extracellular concentration of the antibiotic (within the limits tested).<sup>31</sup> This situation of apparent refractive state or inaccessibility of part of the intracellular inoculum was already observed by us with the fully susceptible strain ATCC 25923.<sup>10</sup> The present study extends these

**Figure 4.** (a) Concentration-response curves of eight selected CA-MRSA strains {with MICs  $\leq 0.06$  [NRS192, SA1 and NRS384 (USA300)], =0.125 [NRS386 (USA700)], =1 mg/L (SA069) and  $\geq 2$  mg/L (KKHII-7924, HMC551 and SA481)} phagocytized by THP-1 cells exposed to moxifloxacin for 24 h. The graphs show the change in the number of cfu (means  $\pm$  SD;  $n=3$  determinations in a single experiment; most SD bars are smaller than the symbols) per mg of cell protein compared with the initial post-phagocytosis inoculum (time=0 h; broken line). The abscissa shows the antibiotic concentration (total drug) expressed as mg/L (upper panel) or multiples of the MIC for the corresponding strain (lower panel; see Table 1 for values). The data were used to fit sigmoidal functions (Hill equation; slope factor= $16^4$ ). (b) Relative maximal efficacies ( $E_{\max}$ ; filled squares) and  $\log_{10}$  of the static weight concentrations [ $C_s$  (mg/L); open squares] for all strains (15) shown in Table 1 after phagocytosis by THP-1 macrophages and exposure to moxifloxacin, as determined by concentration-response experiments similar to those illustrated in (a) (each dataset was used to calculate the corresponding parameters using the Hill equation). The abscissa shows the MICs for the corresponding strains (see Table 1). The curves correspond to linear regression lines between  $E_{\max}$  (continuous line) or  $\log_{10} C_s$  (broken line) taken as dependent variables versus MIC taken as an independent variable. The vertical broken line at an MIC value of 0.125 mg/L shows the optimized split value obtained by recursive partitioning analysis of  $E_{\max}$  and  $\log_{10} C_s$  versus each MIC category.





**Figure 5.** Time-dependent effects of fixed concentrations of moxifloxacin [4 mg/L (133× MIC)], co-trimoxazole [25 mg/L (25× MIC)], clindamycin [2.5 mg/L (20× MIC)] or linezolid [20 mg/L (20× MIC)] corresponding to their human  $C_{max}$  (total drug) against CA-MRSA strain NRS192 in broth (left-hand panel) or after phagocytosis by THP-1 macrophages (right-hand panel). The ordinates show the variation in the number of cfu per mL of broth (extracellular) or per mg of cell protein (THP-1 macrophages)  $\pm$ SD ( $n=3$ ; when not visible, the bars are smaller than the size of the symbols). The broken line at  $-5$  in the left-hand panel corresponds to the limit of quantification. The data were used to fit a one phase exponential association function [ $y=y_{max} \times (1 - e^{-kx})$ ] to calculate the parameters of the bacterial population changes over time (see the text).

observations in three main aspects. First, we show that the importance of this persistent inoculum increases if the MIC exceeds a threshold value. While this does not allow us to distinguish between the two hypotheses raised previously, it makes clear that the intracellular milieu may represent a niche where strains with reduced susceptibility will be protected. Once released from cells, these may contribute to increase the overall level of resistance. This will need careful exploration in future studies examining the effects of short-, medium- and long-term exposure to subinhibitory concentrations of moxifloxacin, somewhat similar to the method used to study the emergence of resistance of *Streptococcus pneumoniae* to fluoroquinolones, including moxifloxacin.<sup>40,41</sup> Second, it is striking that a similar level of persistence was also seen with the novel lipoglycopeptides telavancin and oritavancin, as well as for quinupristin/dalfopristin, two classes of antibiotics that, like moxifloxacin, show intense extracellular bactericidal effects.<sup>13,42</sup> Thus, we may actually deal here with a general limit of antibiotic action against intracellular *S. aureus*, the mechanisms of which require further exploration. Third, we see that the nature of the host cell (macrophages versus keratinocytes) is unimportant in this context, as the level of persistence is similar in both cases. This may have to do with the subcellular localization of the bacteria, which appears to be phagolysosomal in both cases.<sup>28,42</sup> Studies with endothelial cells, where part of the phagocytized bacteria may escape lysosomes and reach the cytosol,<sup>43</sup> would be rewarding in this context.

This persistence of an intracellular inoculum is considerably more important for the three other antibiotics examined. This could have been anticipated for linezolid, which is bacteriostatic, and is actually consistent with our previous *in vitro* and *in vivo*

observations.<sup>11,44,45</sup> It is more surprising for clindamycin, which is clearly bactericidal against extracellular bacteria. Interestingly, the induction of a state of intracellular persistence of viable *S. aureus* in keratinocytes has been previously described in murine keratinocytes and fibroblasts exposed to clindamycin or linezolid.<sup>46</sup> The results are quite distressing for co-trimoxazole, which proved unable to prevent the intracellular growth of *S. aureus* at all concentrations and times tested, pointing to an intrinsic inability of this drug to control intracellular infection in the model used and for the strains studied.

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 previously.<sup>10,13,14,28</sup> Yet, the data may help in rationalizing the treatment of staphylococcal infections in those situations where there is a poor or slow response to therapy and/or a high level of recurrence in which intracellular persistence could play a critical role.<sup>7,8</sup> The question may be raised about the importance of intracellular survival for CA-MRSA, as these organisms produce cytolytic toxins such as PVL.<sup>47,48</sup> However, not all CA-MRSA carry the *lukS*-PV and *lukF*-PV genes for this toxin,<sup>19</sup> as observed in some of the strains studied here. Moreover, we did not see any significant decrease in the viability of the cells used in our study (all of human origin) after phagocytosis of either PVL-positive or PVL-negative CA-MRSA strains. Conversely, we know that the fully susceptible ATCC 25923 strain is PVL-positive.<sup>26</sup> This apparent resistance of the cells to the lytic action of PVL (for those strains that are PVL-positive) may result from a combination of several factors. First, bacteria are washed after collection and again after opsonization, and are diluted to a large extent when added to cells. This may remove most of the PVL present

in the medium, as was observed for other toxins.<sup>7</sup> Second, the production of PVL and other toxins is maximal at the stationary stage,<sup>49</sup> which is not reached for intracellular bacteria under our experimental conditions. Third, it is also known that extensive changes occur in the genome expression of *S. aureus* after phagocytosis, with ~350 and 700 genes up-regulated and down-regulated, respectively.<sup>50</sup> More specifically, the expression of several genes encoding for toxins affecting host cell integrity (such as  $\alpha$ -haemolysin) is reduced, probably to avoid premature host death. Intracellular *S. aureus* also shows a decreased expression of *agr* (accessory gene regulator),<sup>50</sup> known to regulate the synthesis of many virulence factors<sup>51</sup> including PVL.<sup>52</sup> Thus, the bacteria may actually protect its host so as to maintain itself in an environment where it escapes immune defences and is less susceptible to antibiotics. However, it is not known whether this is specific to the cells used here, and how this will translate to the *in vivo* situation.

In a context of practical usage of antibiotics, the present study shows that moxifloxacin will retain its maximal intracellular activity as long as its MIC for the causative organism does not exceed 0.125 mg/L. This suggests the definition of a potential intracellular susceptibility breakpoint that could be applied to the whole population of MRSA isolates, separating those for which the MIC of moxifloxacin is  $\leq 0.125$  mg/L, in which case the response will be maximal, from those for which the MIC of moxifloxacin is higher, and for which the response will become increasingly suboptimal along with the increase of MIC. Although this is based on only a limited number of isolates, it is interesting to note that this 'intracellular breakpoint' is actually close to the clinical breakpoint defined for bacteria growing in broth as determined by EUCAST and CLSI ( $\leq 0.5$  mg/L) for systemic infections. The difference (two dilutions) could essentially reflect the loss of maximal efficacy suffered by the drug when acting against intracellular bacteria. This may come as a surprise since fluoroquinolones, and moxifloxacin in particular, accumulate in eukaryotic cells.<sup>53</sup> Further studies will need to examine which factors are responsible for this apparent loss of activity, but the data are consistent with our previous suggestions that cellular accumulation of antibiotics and intracellular activity are not necessarily correlated, whether *in vitro* or *in vivo*.<sup>27,54</sup> Our results also suggest that linezolid, clindamycin and co-trimoxazole will be poorly effective in acting against the intracellular inoculum. Further studies will need to establish whether this is indeed the case in comparison with moxifloxacin or other antibiotics with similar intracellular bactericidal activity.

Our data should not be taken as blindly advocating the use of moxifloxacin in infections caused by CA-MRSA. First, we clearly set a limit related to the susceptibility of the offending organism (MIC  $\leq 0.125$  mg/L). While most of our strains met this criterion, our study was not meant to provide epidemiological information and should not be interpreted out of context. Thus, even though CA-MRSA are often reported to be susceptible to moxifloxacin,<sup>55</sup> local patterns may be quite different (with only 15% of isolates showing an MIC  $\leq 0.5$  mg/L in at least one survey),<sup>56</sup> making actual documentation essential. We are also aware of the risk of emergence of resistance to moxifloxacin should its use against CA-MRSA become widespread. While this risk cannot be ignored, recent *in vitro* studies suggest that moxifloxacin may have a lower potential than other fluoroquinolones to select for resistance in *S. aureus*.<sup>57</sup> Moreover, this risk is probably also

shared by other antimicrobials, including clindamycin, doxycycline, linezolid and co-trimoxazole, as far as can be predicted from the measurement of their respective mutant prevention concentrations (MPCs) and an assessment of the percentage of the dosing interval during which serum concentrations fall within the mutant selection window.<sup>58</sup>

In conclusion, and based on all the evidence presented, the data suggest that moxifloxacin has the potential to display useful activity against CA-MRSA isolates where not only eradication of extracellular forms is required, but also significant reduction of the intracellular inoculum is desirable. This may represent an important asset when dealing with recurrent and persistent staphylococcal infections, such as complicated skin and soft-tissue infections or pulmonary infections where the intracellular component may play a critical role.<sup>50,59</sup>

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## Transparency declarations

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