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A mechanism-based pharmacokinetic/pharmacodynamic model allows prediction of antibiotic killing from MIC values for WT and mutants

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Objectives: *In silico* pharmacokinetic/pharmacodynamic (PK/PD) models can be developed based on data from *in vitro* time–kill experiments and can provide valuable information to guide dosing of antibiotics. The aim was to develop a mechanism-based *in silico* model that can describe *in vitro* time–kill experiments of *Escherichia coli* MG1655 WT and six isogenic mutants exposed to ciprofloxacin and to identify relationships that may be used to simplify future characterizations in a similar setting.

Methods: In this study, we developed a mechanism-based PK/PD model describing killing kinetics for *E. coli* following exposure to ciprofloxacin. WT and six well-characterized mutants, with one to four clinically relevant resistance mutations each, were exposed to a wide range of static ciprofloxacin concentrations.

Results: The developed model includes susceptible growing bacteria, less susceptible (pre-existing resistant) growing bacteria, non-susceptible non-growing bacteria and non-colony-forming non-growing bacteria. The non-colony-forming state was likely due to formation of filaments and was needed to describe data close to the MIC. A common model structure with different potency for bacterial killing (EC_{50}) for each strain successfully characterized the time–kill curves for both WT and the six *E. coli* mutants.

Conclusions: The model-derived mutant-specific EC_{50} estimates were highly correlated ($r^2 = 0.99$) with the experimentally determined MICs, implying that the *in vitro* time–kill profile of a mutant strain is reasonably well predictable by the MIC alone based on the model.

Introduction

The increasing problem of antibiotic resistance due to overuse and misuse of antibiotics is threatening to return us to a pre-antibiotic era.[1–3] In combating existing resistance and limiting the enrichment of resistant bacteria during treatment, it is essential to understand the relationship between common resistance mutations and the time profile of bacterial growth and death when exposed to antibiotics.[4] Relevant information to predict optimal dosing strategies for antibiotics can be obtained from pharmacokinetic/pharmacodynamic (PK/PD) *in silico* models based on *in vitro* time–kill experiments.[5–8] By predicting the bacterial killing following different doses and dosing intervals from such models, drug development could be guided in the selection between candidate drugs and current dosing schemes could be optimized, not only to increase efficacy but also to limit resistance development. For the last decade, model-based drug development has been recommended by the regulatory agencies to strengthen the scientific evidence to base key decisions on[9,10] and PK/PD models have lately gained increased attention from the pharmaceutical industry for this purpose. A framework, aiming to be able to make reasonable predictions based on a limited number of future experiments, is of high value for making drug development more efficient.

In silico PK/PD models have been developed for different bacterial strains and antibiotics.[4,6,11–17] The aim of the models may differ, however. For example, some models focus on evaluating mechanism-based theory on the data and some models focus more on making drug development more efficient, e.g. for forecasting unstudied outcomes based on limited new information or predictions of different dosing regimens in the presence of less susceptible bacteria. From a mechanism-based PK/PD model, we can explore different dosing regimens in the presence of resistant mutants to investigate how resistance selection can be reduced or overcome. For a model to be applicable for drug development, a general basis for the model structure is desirable. Nielsen *et al.*[5] have previously developed a mechanism-based *in silico* model describing the *in vitro* time–kill profiles for *Streptococcus pyogenes* exposed to antibiotics of different classes. The model fitted data well from both static and dynamic time–kill experiments[18]

and predicted the *in vivo* determined categories and magnitudes of PK/PD indices.[16] Further, the Nielsen model has been shown to provide a useful base structure that allows for extension with structural elements that describe adaptive resistance, e.g. as observed in experiments of *Escherichia coli* exposed to gentamicin[11] and *Pseudomonas aeruginosa* exposed to colistin.[12]

In this study, we have developed the Nielsen model further for the study of *E. coli* exposed to a fluoroquinolone. Fluoroquinolones are an important class of antibiotics that are commonly used against Gram-negative infections. Resistance to fluoroquinolones, however, has been reported as increasing worldwide over the last decade.[19,20] Ciprofloxacin is a fluoroquinolone that acts by inhibiting DNA gyrase and topoisomerase IV, which leads to inhibition of cell division and bacterial death.[21,22] Common mutations in *E. coli* leading to ciprofloxacin resistance include mutations in *gyrA1*, *gyrA2* (DNA gyrase), *parC* (topoisomerase IV) and *marR* (repressor of efflux system AcrAB). These mutations are typically associated with higher MIC values compared with WT[23] and may also reduce bacterial fitness.[24] How these mutations affect the parameters describing the PK/PD relationships and hence the time course of bacterial killing after drug exposure is of interest, since such information can provide valuable information for the design of future studies and in development of dosing regimens.

The aim was to develop a mechanism-based *in silico* model that can describe *in vitro* time–kill experiments of *E. coli* MG1655 WT and six isogenic mutants exposed to ciprofloxacin and to identify relationships that could be used to simplify future characterizations in a similar setting. The effects of specific mutations on model parameters describing the time course of bacterial killing for different ciprofloxacin concentrations were investigated and a common PK/PD model structure was developed.

Material and methods

In vitro experiments

E. coli strains and antibiotic used

The strains were isogenic, only differing in the mutation(s) outlined in Table 1. Their MICs (Etest) and fitness values (as determined by growth competition assays in tubes) have been reported earlier.[24] Ciprofloxacin from Sigma–Aldrich (17850-5G-F) was used in all experiments. The antibiotic was dissolved in 0.1 M HCl as recommended by the supplier, either freshly dissolved or from a 10 mg/mL stock solution not older than 4 weeks (stored at +4°C). Further dilutions were made in Mueller–Hinton II (MHII) broth (BD BBL™).

MIC determination

The previously reported MIC values were determined using Etest (Table 1).[24] In our study, the MICs were determined by macrodilution in MHII broth in order to reflect the MIC in the time–kill experiments. The macrodilution MICs (reported as modal MICs) for all strains were evaluated for a minimum of 6 times on different occasions and in different labs (Table 1). The experiments were performed with 2 mL of MHII and a starting inoculum of 2×10^5 cfu/mL. A 2-fold half dilution scheme was used and the results were read at 24 h. To investigate if resistant bacteria were selected during the experiment, the MIC was determined in some 0.5–1× MIC experiments for WT bacteria and for one of the mutants.

Time–kill experiments

Time–kill experiments were performed in at least triplicate for all seven strains. At least one replicate for each strain was tested at three different laboratories working in a joint consortium to increase the robustness of the results by showing reproducibility of the experiments. There was no obvious trend for systematic differences between laboratories. The high variability at concentrations between 0.25× and 1× MIC is likely due to biological random variability (e.g. influencing the starting inocula) in combination with small experimental variations (e.g. temperature fluctuations). Interlaboratory variation was minimized by strictly following a detailed standard protocol. All incubations were made at 37°C (liquid cultures in a rocking water bath at 150 rpm) and dilutions were made into pre-warmed MHII broth. From single colonies, overnight cultures (15–17 h) were prepared in 2 mL of MHII and then diluted 1:100 in 2 mL of MHII broth. A second dilution (1:100 in 2 mL of MHII broth) was made after 1.5 h when the culture had reached log phase ($OD_{600}=0.1-0.3$, $\sim10^7-10^8$ cfu/mL) to achieve a starting inoculum of $\sim10^6$ cfu/mL. After an initial bacterial sample was extracted, antibiotic was added to obtain a concentration of 0×, 0.0625×, 0.125×, 0.25×, 0.5×, 1×, 2×, 4× and 8× MIC for the strain (MICs from macrodilution were used, see Table 1). Additional samples were removed at 1, 2, 4, 6, 9, 12 and 24 h after the addition of the antibiotic. Different dilutions were prepared in sterile 0.9% NaCl and plated for each sampling timepoint.

The samples were spread by glass beads (five beads, Hecht 1401/6) onto MHII agar plates. Colonies were counted manually after ~20 h. The limit of detection (LOD) for viable counts was 10 cfu/mL. Antibiotic

Table 1. Mutations, relative fitness compared with LM347 and measured MICs for the different *E. coli* MG1655 strains used in the experiments

						MIC (mg/L)	
Strain	GyrA1	GyrA2	MarR	ParC	Relative fitness[a]	Etest[b]	macrodilution[c]
LM347 (WT)	—	—	—	—	1.0	0.016	0.023
LM202	—	—	Δ	—	0.83	0.032	0.047
LM378	S83L	—	—	—	1.01	0.38	0.38
LM534	—	D87N	—	—	0.99	0.25	0.25
LM625	S83L	D87N	—	—	0.97	0.38	0.50
LM693	S83L	D87N	—	S80I	1.01	32	32
LM707	S83L	D87N	Δ	S80I	0.89	32	48

[a]Mean fitness per generation, relative to WT from Marcusson *et al.*[24]
[b]The MIC was determined by Etest on Mueller–Hinton II agar plates incubated for 16–18 h at 37°C as described by Marcusson *et al.*[24] and is summarized as modal MIC.
[c]The MIC was determined by macrodilution in Mueller–Hinton II broth incubated for 24 h at 37°C in this study and is summarized as modal MIC.

carry-over was handled by adding the sample to one side of the plate, allowing the antibiotic to sink into the agar before spreading out the bacteria. When counting colonies, a visual inspection of the distribution of colonies on the plate was conducted and if a clear zone was present on the plate, the plate was divided into two and only the zone that the sample was not added to was counted, with the count being doubled to represent the whole plate.

Particle size in culture

To determine the degree of filamentation in our time–kill assay, flow cytometry was used to study the bacteria particle size over time. A GFP gene-labelled analogue of LM347 that can be measured with flow cytometry was used. This strain has previously been constructed and studied by Gullberg *et al.*[25] and we used an identical analogue. Overnight cultures were grown in 2 mL of MHII broth. The next day, 20 μL of the culture was transferred to 2 mL of fresh pre-warmed MHII broth using the same procedure as in the time–kill experiments. Ciprofloxacin was added to each tube to final concentrations of 0×, 0.5× and 1× MIC. Samples were taken at 1, 2, 4, 6, 9 and 24 h after the antibiotic was added. From the tube without antibiotic, 5 μL of culture was sampled at each timepoint and diluted with 500 μL of sterile-filtered 0.9% NaCl. From the tubes with ciprofloxacin, between 5 μL and 1 mL of sample was taken at each timepoint. To obtain an appropriate concentration of ∼10^6 cfu/mL, cells were either diluted in 0.9% NaCl or concentrated by spinning at 5000 rpm for 6 min and resuspended in 0.9% NaCl. For each sample, 10^5 cells were analysed. After removal of the supernatant, sufficient volumes of PBS were added to resuspend the cell pellet. The samples were vortexed for 5 s before being analysed by flow cytometry (BD FACS Aria Cell Sorter). The fluorescence (Blue 488 nm laser) was detected by FITC-filter (502 nm Long pass filter, 530/30 nm band pass). We first gated away noise and debris based on side-scatter light height (SSC-H) and only analysed cells with yellow fluorescence. There was a clear separation between fluorescent cells and non-fluorescent cells and debris. We gated only fluorescent cells. At least 100 000 events were recorded for each sample.

oCelloScope experiments

Cell morphology at 0.5× MIC was monitored over time using an oCelloScope™ (Unisensor, Allerod, Denmark). Bacterial inocula were prepared as above in pre-warmed MHII broth, except that the second dilution was done 1:50 to make a further 1:2 dilution into the microtitre plate (96-well microtitre plate with lid; Thermo Scientific Nunc, cat. no. 10378937) already containing 100 μL of MHII with ciprofloxacin at different concentrations. Representative images of filamentation were chosen by zooming into the best-focused section and exporting to bitmap.

In silico modelling

Mechanism-based PK/PD model

The general mechanism-based PK/PD model previously developed for *S. pyogenes* by Nielsen *et al.*[5] was used as the starting point for model development. During development of the ciprofloxacin–*E. coli* model, different structural models were tested, including the characterization of pre-existing subpopulations with different drug susceptibility, subpopulations of non-colony-forming bacteria and adaptive resistance mechanisms.[11,12] A parsimonious model with a common structure for all seven strains was searched for.

Ciprofloxacin has been reported to be stable in culture media[26,27] and was shown to be stable over 24 h at 37°C in pilot studies; hence, the concentration was assumed to be constant during the 24 h time–kill experiments. The time during which some of the bacteria in the system were transferred into the non-colony-forming state was estimated by application of the MTIME function in NONMEM.

Data analysis

The typical model parameters and the residual errors were estimated simultaneously based on all data in NONMEM 7;[28] thus, all strains were fitted using the same model. The Laplacian estimation method and ADVAN13 were used. When selecting between nested models, the difference in objective function value (OFV) was used. In NONMEM, the OFV is −2 times the log of the likelihood. The more complex model was selected when the reduction in OFV was ≥10.83 corresponding to a P value of <0.001 for 1 degree of freedom. Baseline variability in the starting inocula was handled using the B2 method,[29] where the observed starting inoculum was treated as a covariate associated with a residual error having the same estimated residual variability as the other bacterial counts. All detectable bacterial counts (n=3438) from all experiments were included in the analysis and there were generally between one and three observations (dilutions) per sample timepoint and experiment. All bacterial counts were transformed into natural logarithms prior to data analysis. Typical parameter values and residual errors were estimated. No between-experiment variability was estimated. When tested, inclusion of interexperimental variability resulted in misspecification of the baseline value and overestimated the variability. The residual error was additive on the logarithmic scale. To avoid bias due to correlations between replicate samples, the residual error was estimated as one residual common for all replicates of the same timepoint in an experiment and one residual that was replicate specific.[5]

Model performance was evaluated with visual predictive check (VPC) plots by simulating 1000 replicates of the dataset from the developed model and its parameter estimates (without uncertainty) and constructing an 80% prediction interval based on the simulated data.[30–32] The VPC was stratified by bacterial strain and drug concentration. The model predictions and calculations to produce the VPCs were performed using PsN,[33] visualized using Xpose version 4.4.2.2[34] and R 2.10 (www.R-project.org). For samples taken at the same timepoint, where all dilutions were below the LOD, the M3 method[35,36] was used to estimate the probability for the observation to be below the LOD. It has been shown that acknowledging the presence of LOD data reduces the bias in parameter estimates.[28,35]

Results

Time–kill profiles

The time–kill curves for WT and mutant bacteria had similar shapes when normalizing the ciprofloxacin concentration to the MIC for each strain (Figure 1). For all strains, most variability between experiments was observed at concentrations of 0.25–1× MIC. The triple (LM693) and quadruple mutants (LM707) had the highest MICs (32 and 48 mg/L, respectively) and also the highest experimental variability at concentrations around the MIC.

It was observed that at 3–6 h after initial killing, regrowth occurred in all experiments with a ciprofloxacin concentration of 0.5× MIC (Figure 1). For higher ciprofloxacin concentrations, regrowth at the end of the experiment was observed in some, but not all experiments. For the same multiple of MIC, regrowth was more frequently observed in experiments with WT bacteria compared with any of the mutants. An increase in the MIC at later timepoints was noted in some of the investigated experiments with regrowth. Due to technical difficulties with ciprofloxacin concentrations >48 mg/L, no data were generated for >1× MIC for LM693 (*gyrA* S83L, *gyrA* D87N and *parC* S80I) or LM707 (*gyrA* S83L, *gyrA* D87N, *parC* S80I and Δ*marR*).

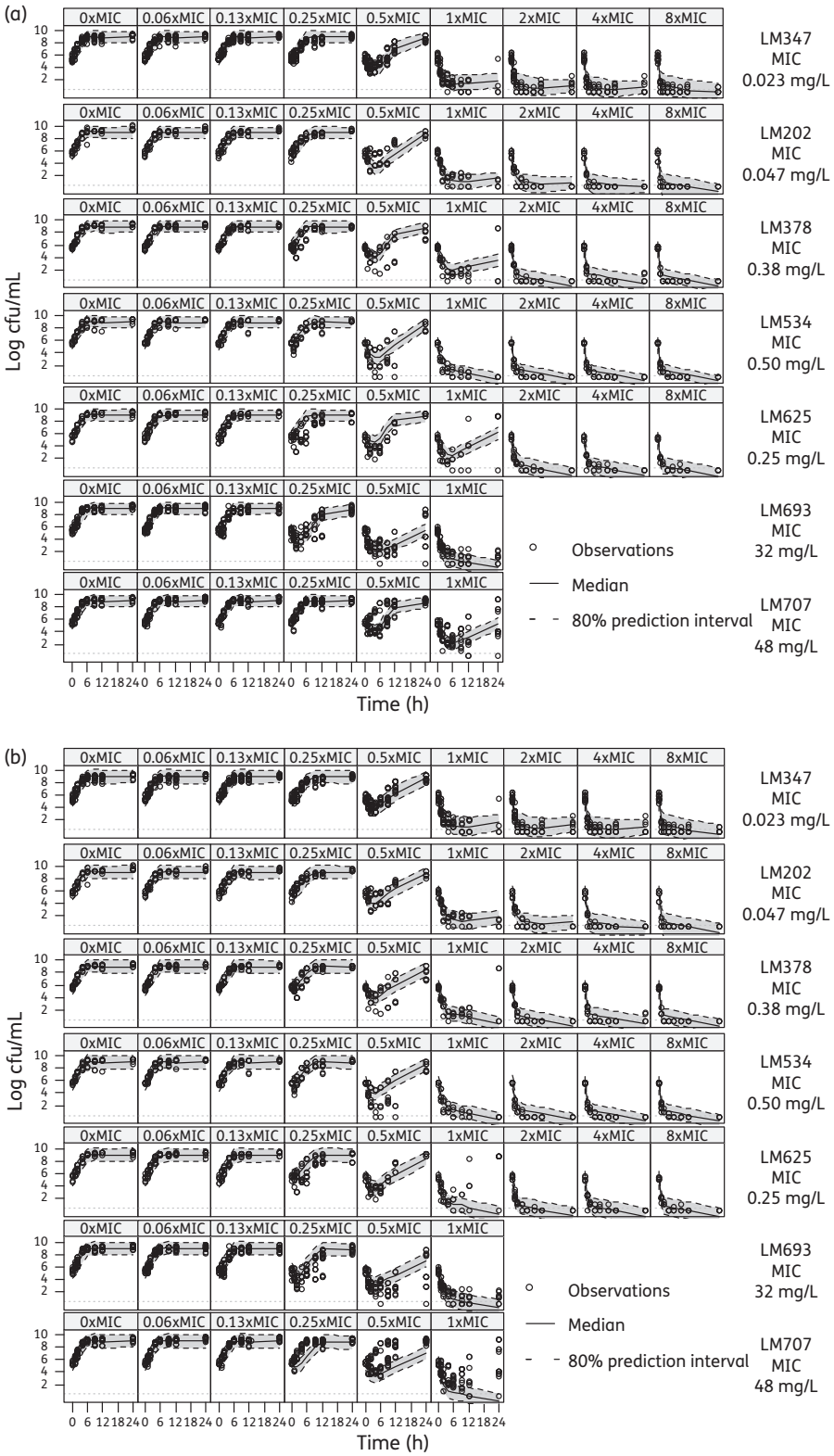

Figure 1. VPCs for *E. coli* MG1655 WT and six well-characterized mutants thereof. (a) Model fit for full model and (b) model fit for the EC_{50}/MIC correlation model. Each panel includes all observations (o), i.e. at least three different experiments with all dilutions of detectable bacteria, the black continuous line represents the simulated median. Observations below the dashed grey line are observations below the LOD and are plotted as 0.15×LOD (1.5). One thousand simulations from the final model were performed to construct 80% prediction intervals, representing estimated and thereby expected experimental variability.

PK/PD modelling

Model structure

The mechanism-based model by Nielsen *et al.*,[5] in its original version, could satisfactorily characterize the initial killing rate at high drug concentrations and the maximum bacterial load in the system, but did not describe the initial killing and regrowth observed at concentrations of ~0.5× MIC, nor the slow regrowth at later timepoints mainly seen for WT bacteria. Therefore, the model structure was further developed: the final model structure includes compartments for drug-susceptible growing bacteria (S), resting non-growing bacteria (R) and non-colony-forming drug-susceptible bacteria (Nc) that are rendered non-growing under the influence of ciprofloxacin and unable to grow on agar plates (Figure 2). Bacteria were also divided into two subpopulations, Subpopulation 1 (S1, R1 and Nc1) and Subpopulation 2 (S2, R2 and Nc2). Subpopulation 2 is representing less susceptible pre-existing resistant bacteria (Figure 2) describing the slow regrowth seen in some experiments. The inclusion of compartments of non-colony-forming bacteria satisfactorily described the initial killing and regrowth at ~0.5× MIC. The final model successfully fitted the data from all experiments as shown in the VPCs (Figure 1). The model performed better at lower and higher drug concentrations, while the observed bacterial counts at concentrations of ~0.25–1× MIC varied more within and between experiments and were consequently not as well predicted.

The structure and assumptions that created the best model are as follows. At the start of each experiment, all bacteria were assigned to be drug susceptible (S1 or S2). The change in bacterial load for susceptible bacteria (equation 1) was affected by rate constants describing bacterial replication (k_{growth}), natural kill (k_{death}), drug effect (k_{DRUG}) and transfer (k_{death}) from S to R as well as transformation into (k_{SNc}) and from (k_{NcS}), the non-colony-forming state:

$$\frac{dS}{dt} = k_{growth} \times S - k_{death} \times S - k_{DRUG} \times S - k_{SR} \times S - k_{SNc} \times S + k_{NcS} \times Nc \quad (1)$$

The change in bacterial load for the non-susceptible non-growing bacteria (equation 2) was affected by the transfer from S to R (k_{SR}) as well as the natural kill rate (k_{death}):

$$\frac{dR}{dt} = k_{SR} \times S - k_{death} \times R \quad (2)$$

The rate constant of bacterial transfer from the S state to the R state (k_{SR}) was dependent on an estimated proportionality constant (PC) and the total amount of bacteria in the system (equation 3), i.e. the more bacteria in the culture, the higher the rate of transfer from S to R. This structure has previously been shown to describe stationary-phase data well.[5,11,12] The transfer from the non-growing phase (R) back to the growing phase (S) was in these experiments assumed to be negligible as there is no information about this transformation in our data, thus making R1 and R2 identical. However, R1 and R2 may in reality be able to transfer back to S1 and S2 if ciprofloxacin is removed or if stationary-phase bacteria are diluted in new media. When only the S and Nc states were assumed to affect k_{SR}, the model fit was worse (the OFV increased by 30 U).

$$k_{SR} = PC \times (S1 + R1 + Nc1 + S2 + R2 + Nc2) \quad (3)$$

In the model, susceptible bacteria can transfer into the non-colony-forming state. Non-colony-forming bacteria are also affected by drug effect, but can recover and transfer back to the susceptible growing state. Rates of change of the non-colony-forming bacteria were described by equation 4:

$$\frac{dNc}{dt} = k_{SNc} \times S - k_{NcS} \times Nc - k_{death} \times Nc - k_{DRUG} \times Nc \quad (4)$$

Within strains, different EC_{50} (antibiotic concentration that results in 50% of the maximum kill rate) values for susceptible bacteria ($EC_{50,1}$) were allowed to be estimated (Table 2).

The rate constant for transformation from susceptible to non-colony-forming bacteria was described by a function that approaches a maximum rate ($k_{SNc,max}$) at high ciprofloxacin concentrations (C) (equation 5). The driver for the transformation was a ratio of the antibiotic concentration divided by the estimated EC_{50} for the drug effect for each mutant. tr_{50} is the sensitivity parameter (resulting in 50% of the maximum rate) and γ_{Nc} is the estimated Hill factor for this function. This allowed the same parameters to be used for all mutants. It was observed that γ_{Nc} was estimated by the model to a very high value with high uncertainty. Therefore, it was fixed to the value of 20, which was the lowest value not resulting in a significantly reduced fit of the data. A high value of γ_{Nc} predicts that k_{SNc} rapidly changes from a value of 0 to $k_{SNc,max}$ around tr_{50} (estimated at ~0.25× EC_{50}).

$$k_{SNc} = \frac{k_{SNc,max} \times (C/EC_{50})^{\gamma_{Nc}}}{tr_{50}^{\gamma_{Nc}} + (C/EC_{50})^{\gamma_{Nc}}} \quad (5)$$

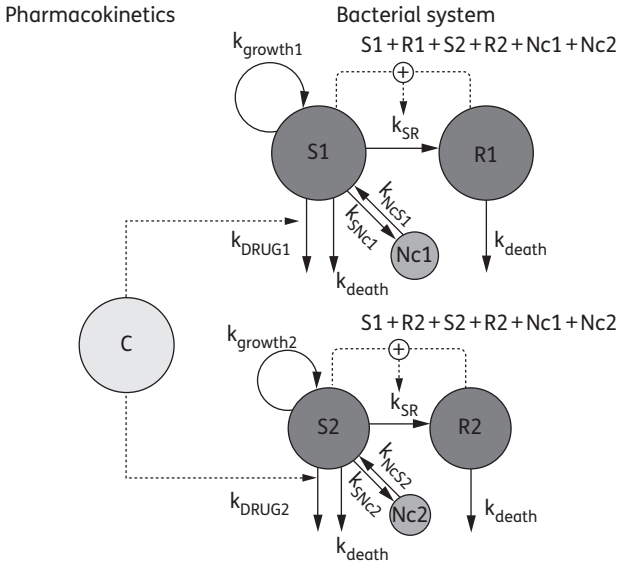

Figure 2. Mechanism-based PK/PD model developed for ciprofloxacin and *E. coli* MG1655 WT and six well-characterized mutants exposed to ciprofloxacin. S1, susceptible bacteria; R1, resting/non-susceptible, non-growing bacteria; Nc1, non-colony-forming bacteria for Subpopulation 1. Subpopulation 2 represents pre-existing resistant bacteria (S2, R2 and Nc2).

Table 2. Final model parameter estimates with relative standard error (RSE%) for the full model and the EC_{50}/MIC correlation model

Parameter	Full model	EC_{50}/MIC correlation model
$k_{growth1}$ (h^{-1})	1.70 (2.1%)	1.73 (2.5%)
$EC_{50,1\ LM347}$ (mg/L)	0.0368 (3.7%)	0.030[a]
$EC_{50,1\ LM202}$ (mg/L)	0.0573 (5.1%)	0.061[a]
$EC_{50,1\ LM378}$ (mg/L)	0.654 (3.4%)	0.474[a]
$EC_{50,1\ LM534}$ (mg/L)	0.295 (4.1%)	0.314[a]
$EC_{50,1\ LM625}$ (mg/L)	1.00 (3.3%)	0.619[a]
$EC_{50,1\ LM693}$ (mg/L)	31.0 (3.3%)	36.2[a]
$EC_{50,1\ LM707}$ (mg/L)	91.6 (3.3%)	53.8[a]
E_{max} (h^{-1})	5.24 (5.8%)	5.84 (7.2%)
γ	1.98 (4.4%)	1.77 (4.6%)
S2 starting conc. per 10^6 cfu/mL	0.819 (14%)	0.703 (16%)
$k_{growth2}$ (h^{-1})	0.344 (4.5%)	0.384 (3.5%)
$EC_{50,2}$ (mg/L)	1.25 (12%)	1.15 (9.9%)
$k_{SNc,max}$ (h^{-1})	5.83 (7.5%)	4.85 (9.8%)
sf_{NcS} (h^{-1})	0.174 (13%)	0.163 (22%)
tr_{50}	0.240 (4.8%)	0.214 (6.9%)
PC (h^{-1}/cfu/mL)	1.86×10^{-9} (7.5%)	1.72×10^{-9} (9.5%)
t_{Nc} (h)	5.34 (2.8%)	3.37 (4.1%)

[a]EC_{50} for the EC_{50}/MIC correlation model was estimated by two parameters using MIC as covariate ($1.22\times MIC^{0.978}$) (RSE 4.5% and 0.2%, respectively).

The transfer back to S from Nc was also dependent on the scaled ciprofloxacin concentration (equation 6) multiplied by an estimated scale factor sf_{NcS}. The inverse relationship to C indicates that the higher the drug concentration, the lower is the rate of recovery back to the susceptible state from the non-colony-forming state. C was set to a very low value for control experiments to avoid division by 0.

$$k_{NcS} = sf_{NcS} \times \left(\frac{EC_{50}}{C}\right) \quad (6)$$

The time during which some of the bacteria in the system were transferred into the non-colony-forming state (t_{Nc}) was estimated to be 5.3 h after the start of drug exposure. At this estimated timepoint, the transfer rate constant from the S to the Nc compartment (k_{SNc}) was switched off and set to 0. By adding Nc compartments, the initial decline and subsequent regrowth observed for ciprofloxacin concentrations at ~0.5× MIC were adequately described and improved the model fit by resulting in a decrease in OFV of 650 U. When a third subpopulation was evaluated to describe the transient decline, the OFV was 564 U higher compared with the final model.

The natural death rate constant (k_{death}) of the bacteria was fixed to a value of 0.179 h^{-1} as previously reported by Nielsen *et al.*[5] as there was little information in the current data for this parameter. All bacteria, including the resting and non-colony-forming bacteria, were assumed be affected by the natural death rate. Fixing k_{death} to 0.1 and 0.3 h^{-1} resulted in an increase in OFV of 28 and 5 U, respectively.

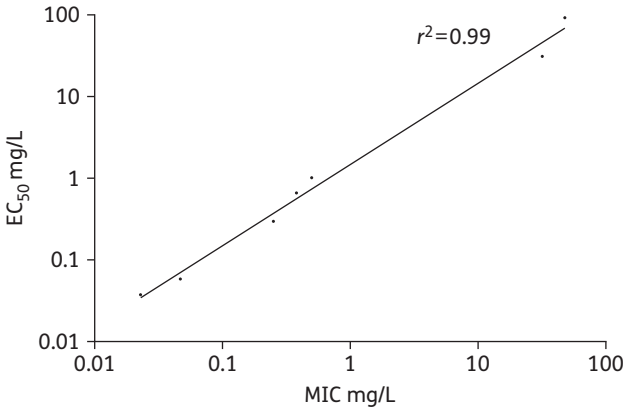

Figure 3. EC_{50} as estimated by the full model versus experimentally determined MICs by macrodilution.

The rate constants determining the drug-induced bacterial killing (k_{DRUG}) of S and Nc were described with E_{max} models (equation 7):

$$k_{DRUG} = \frac{E_{max} \times C^{\gamma}}{EC_{50}{}^{\gamma} + C^{\gamma}} \quad (7)$$

As anticipated, the different strains had different susceptibility to ciprofloxacin and consequently it was significant to estimate different EC_{50} values for each strain (Table 2). When the bacterial growth parameter ($k_{growth1}$) was allowed to be strain dependent, there was no significant improvement in the model fit. $k_{growth1}$ was hence a shared parameter for all strains.

The fraction of pre-existing less susceptible bacteria in the culture (S2) at the start of the experiment was estimated to be 0.82 cfu/mL per 10^6 cfu/mL in the starting inoculum as a common parameter for all experiments. Excluding pre-existing resistant bacteria from the model structure generated an increased OFV of 42 U with three fewer model parameters. The inclusion of pre-existing resistant bacteria described the slow regrowth seen mainly in the experiments with WT bacteria.

The pre-existing resistant subpopulations were assumed to have different replication rates ($k_{growth2}$) and EC_{50} ($EC_{50,2}$) compared with the susceptible populations; the $EC_{50,2}$ was assumed to be the same for all seven studied strains. The replication rate constant for the pre-existing resistant bacteria ($k_{growth2}$) was estimated to be only one-fifth of the replication rate constant in Subpopulation 1 ($k_{growth1}$), while $EC_{50,2}$ was higher than $EC_{50,1}$ for five of the seven strains. There was limited information in the data on Subpopulation 2 and drug effect parameters characterizing the non-colony-forming bacteria were therefore assumed to be the same as for the corresponding susceptible state (S1 to Nc1 and S2 to Nc2).

EC_{50}/MIC correlation model

The estimated EC_{50} values for the different strains were highly correlated to the experimentally measured MICs with $r^2 = 0.99$ for the final model with shared E_{max} and k_{growth} parameters (Figure 3). Also, for a model where E_{max} and k_{growth} parameters were allowed to be strain specific, the EC_{50}/MIC correlation was 0.99. A reduced, alternative model where the EC_{50} values for all

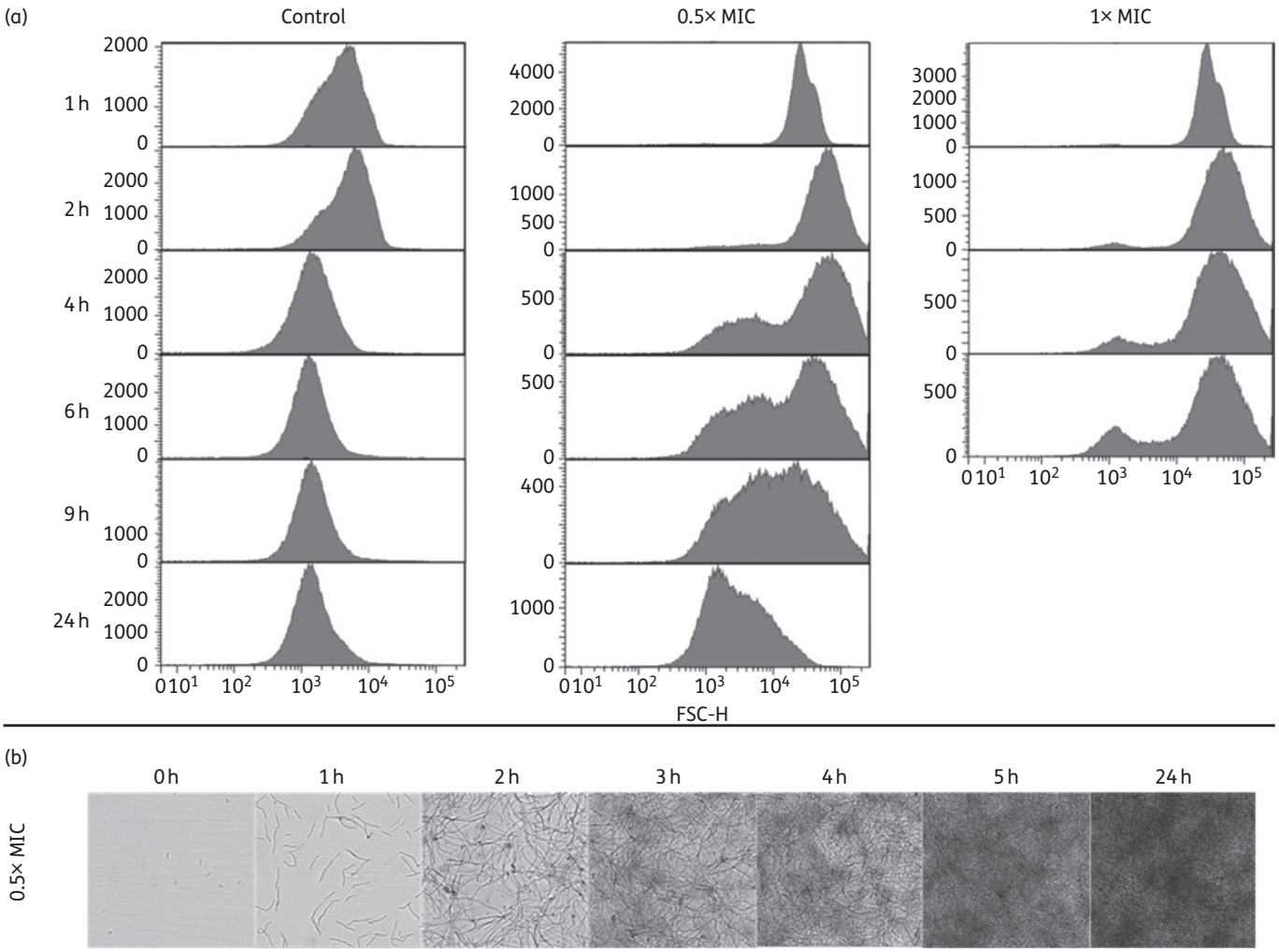

Figure 4. (a) Fluorescence-activated cell sorting results for strain LM347, with relative bacterial density on the *y*-axis and forward-scatter light height (FSC-H) corresponding to size of the particles (bacteria) on the *x*-axis. (b) oCelloScope images showing cell morphology for strain LM347 over 24 h at 0.5× MIC of ciprofloxacin.

strains were forced to be related to their experimentally determined MIC was therefore evaluated and resulted in equation 8:

$$EC_{50} = 1.22 \times MIC^{0.978} \quad (8)$$

In this reduced model, only two parameters to describe EC_{50} for all seven strains were hence estimated. The other parameters for the reduced model were similar to the full model, e.g. $k_{growth1}$ changed from 1.70 to 1.73 h^{-1} and E_{max} changed from 5.24 to 5.84 h^{-1} (Table 2). The reduced model satisfactorily described the data, as depicted in the VPCs (Figure 1b). The full model was, however, significantly better than the reduced model, with a 432 U lower OFV.

Flow cytometry and cell morphology

Flow cytometry was used to study the bacteria particle size in the culture over time for the control (no drug) and ciprofloxacin concentrations of 0.5× and 1× MIC. The results show that particle sizes at 0.5× and 1× MIC were increased between 1 and 4 h compared with control. At 0.5× MIC, particle sizes clearly decreased again after 4 h, while at 1× MIC the particle size decrease after 4 h was less pronounced (Figure 4a).

Cell morphology at 0.5× MIC was studied over 24 h using oCelloScope and longer chains of *E. coli* indicating filamentation were observed (Figure 4b).

Discussion

In this study, we developed a mechanism-based PK/PD model for ciprofloxacin and *E. coli*. The developed model successfully characterized the time–kill curves for both WT and six *E. coli* mutants as depicted in the VPCs (Figure 1). The model structure includes susceptible growing bacteria, resting non-growing bacteria and non-colony-forming non-growing bacteria, divided into two subpopulations. Subpopulation 1 represents susceptible bacteria and Subpopulation 2 represents pre-existing resistant bacteria (Figure 2). These elements were needed to describe the data, but to describe the full mechanism would be the scope of

other studies aiming to elucidate these relationships. Here, similarities of model parameters were observed across strains and thus some parameters were shared between strains (Table 2).

In the time–kill data, an initial decline in bacterial load and a fast rebound between 2 and 6 h was observed, primarily for concentrations of ∼0.5× MIC. This was in the model described by inclusion of the non-colony-forming compartment, which significantly improved the description of the data. There may be additional variables affecting this, but a non-colony-forming compartment satisfactorily describes the outcome and is experimentally supported. After an estimated time of 5.3 h, the transfer of bacteria from the susceptible to the non-colony-forming compartment was switched off; this resulted in model predictions better describing the observed time–kill profiles. It is well known that filamentation occurs when *E. coli* is exposed to ciprofloxacin.[21,37] The oCelloScope™ results indicating filaments (Figure 4b) and the flow cytometry results (Figure 4a) on particle sizes over time show that the proportion of bacteria with a smaller cell size increases again after 4–6 h, which strengthens the hypothesis that non-colony-forming bacteria and filaments are highly associated.

It has been reported that bacteria undergoing filamentation can either die or recover and become growing 'plateable' cells.[21] The flow cytometry results indicate that at 0.5× MIC, filaments form between 1 and 4 h and then cells recover and start to replicate again because the ciprofloxacin concentration is not high enough to kill the filaments (Figure 4a). For concentrations at the MIC level, an increase in particle size similar to the increase at 0.5× MIC was observed; however, recovery was less pronounced. This indicates that more bacteria are killed around the MIC and the filaments are less prone to recover and start growing.

Our mechanistic hypothesis is that bacteria become affected by ciprofloxacin and form filaments but do not die. These bacteria are not fully able to proliferate because they form filaments and if the drug concentration is not sufficiently high to kill the filamented bacteria, they may recover and revert back to susceptible growing bacteria in the model. It should, however, be acknowledged that these experiments were not designed to fully characterize the filamentation, but inclusion of the non-colony-forming compartment significantly improved the description of the PK/PD relationship for ciprofloxacin.

The mechanism-based model includes two subpopulations with the second subpopulation hypothesized to represent a subpopulation of pre-existing resistant bacteria. In some experiments, but not all, regrowth was observed at the end of the experiments. The presence of subpopulations with lower susceptibility to ciprofloxacin was experimentally supported by MIC measurements in the case of regrowth. Because of limited information in the data about these pre-existing resistant bacteria (the experiments were not designed to characterize these bacteria), all strains were in the final model assumed to share all the parameter estimates specific to this bacterial population, including the fraction of this population in the starting inocula, $k_{growth2}$, and $EC_{50,2}$. Because $EC_{50,2}$ was set to be the same for all strains, S2 was most important for bacteria with low EC_{50} (i.e. the WT) and of less importance for mutant strains with higher EC_{50}. In addition, there was no significant difference in E_{max} for the two subpopulations. If the starting inoculum is high compared with the strains' normal mutation rate, there is a high probability of having a less susceptible subpopulation in the system. Here, we estimated a starting concentration of 0.82 cfu/mL per 10^6 cfu/mL in the starting inocula to be less susceptible. A pre-existing resistant subpopulation model structure has previously been used for modelling of ciprofloxacin.[14] Bulitta *et al.*[13] estimated a similar fraction, 0.03–0.7 cfu/mL per 10^6 cfu/mL, for the least susceptible population in different *P. aeruginosa* strains treated with colistin. The estimated replication rate of the pre-existing resistant bacteria was much lower than the normal replication rate, indicating a high fitness cost associated with resistance. However, since our experiments were not designed to characterize this subpopulation and the estimates are based on a few experiments and timepoints, they should be treated with caution.

The concept of relating PK/PD model parameters to the MIC has been discussed before on a theoretical level.[38–40] In our work, the estimated model parameters for EC_{50} correlated well with the experimentally determined MICs by macrodilution ($r^2 = 0.99$) (Figure 3). The reduced EC_{50}/MIC correlation model uses MIC as a covariate for EC_{50} and therefore it may be possible to predict the *in vitro* time–kill profile for a different *E. coli* strain by measuring only the MIC experimentally and then applying the here-developed model structure and model parameters for the reduced EC_{50}/MIC correlation model (Table 2). Yano *et al.*[15] have previously described an EC_{50}/MIC correlation for meropenem, imipenem, cefpirome and ceftazidime in *E. coli*, *P. aeruginosa* and *Staphylococcus aureus*. Further, Katsube *et al.*[41] applied that correlation to estimate the EC_{50} using the MIC as a covariate for *P. aeruginosa* treated with meropenem, doripenem and imipenem. For predictions of new *E. coli* strains, the EC_{50}/MIC correlation model would hence be used inserting the MIC as a covariate when performing simulations/predictions. When predicting bacterial killing under new conditions for the here-studied strains, such as higher starting inocula or from a dynamic drug concentration system, the full model is however anticipated to give the best prediction. If the replication rate is indicated to be lower than for the here-studied strains, also a lower k_{growth} may be considered to fully describe the data.

Several of the EC_{50} values for the full model were highly (>95%) correlated to each other with the highest correlation between EC_{50} for LM693 and EC_{50} for LM707 (98%). This is indeed expected when all data are modelled simultaneously and other parameters are shared between the strains. Other parameters were less correlated and estimated with reasonable precision.

A strength of our study is that we examined different well-characterized strains with known single and multiple resistance mutations. Our results show that all six *E. coli* mutants have similar replication rates as the WT bacteria as no significant differences in k_{growth} could be estimated from the data in this study, as was expected from the results of Marcusson *et al.*[24] that has previously shown that resistance mutations for *E. coli* MG1655 often only give a low or no fitness cost. When strain-specific replication rate constants were allowed, the range of $k_{growth1}$ values was between 1.58 and 1.71 h^{-1} with LM707 having the lowest $k_{growth1}$ value, but the difference did not reach statistical significance.

Our study also has limitations. Firstly, we did not study data outside traditional static *in vitro* time–kill conditions, such as different starting inocula or dynamic ciprofloxacin concentrations. Secondly, the studied strains are isogenic except for the resistance mutations and no clinical isolates of *E. coli* were included. The developed mechanism-based model's predictive ability thus needs to be evaluated for different *E. coli* strains including clinical

strains and for other experimental conditions to better understand its value for extrapolations and usefulness in drug development. Thirdly, the experiments were not designed to fully characterize the pre-existing resistant subpopulation or the non-colony-forming bacteria. Finally, for strains LM693 and LM707, we studied only concentrations up to 1× MIC due to technical difficulty in producing these data. Initial test experiments indicated that there may be a paradoxical drug effect in these experiments with higher concentrations of drug resulting in lower drug effects (i.e. bacterial killing),[42,43] possibly due to solubility issues at concentrations >67 mg/L.[44] Since the C_{max} of ciprofloxacin for a 500 mg oral dose is ∼2 mg/L,[45] no further evaluation of concentrations >48 mg/L was made.

In summary, we have shown that our model structure can be applied to describe the time–kill profiles of seven different *E. coli* MG1655 strains. The similarities in model parameters, including same growth for all studied strains, indicate that the here-investigated strains mainly differ in EC_{50} which is correlated to the MIC. After further validation,[46–48] e.g. with clinical strains, dynamic concentration experiments and different starting inocula, this model may be a valuable tool in predicting improved dosing strategies in a patient population to effectively treat infections caused by WT and resistant *E. coli*. This experimental and modelling framework can then be used in the search for more effective dosing regimens.

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Transparency declarations

None to declare.

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