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Intra- and Extracellular Activities of Dicloxacillin against *Staphylococcus aureus In Vivo* and *In Vitro*▽

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Antibiotic treatment of *Staphylococcus aureus* infections is often problematic due to the slow response and recurrences. The intracellular persistence of the staphylococci offers a plausible explanation for the treatment difficulties because of the impaired intracellular efficacies of the antibiotics. The intra- and extracellular time- and concentration-kill relationships were examined *in vitro* with THP-1 cells and *in vivo* by use of a mouse peritonitis model. The *in vivo* model was further used to estimate the most predictive pharmacokinetic/pharmacodynamic (PK/PD) indices (the ratio of the maximum concentration of drug in plasma/MIC, the ratio of the area under the concentration-time curve/MIC, or the cumulative percentage of a 24-h period that the free [*f*] drug concentration exceeded the MIC under steady-state pharmacokinetic conditions [fT_{MIC}]) for dicloxacillin (DCX) intra- and extracellularly. In general, DCX was found to have similar intracellular activities, regardless of the model used. Both models showed (i) the relative maximal efficacy (1-log-unit reduction in the numbers of CFU) of DCX intracellularly and (ii) the equal relative potency of DCX intra- and extracellularly, with the MIC being a good indicator of the overall response in both situations. Discordant results, based on data obtained different times after dosing, were obtained from the two models when the extracellular activity of DCX was measured, in which the *in vitro* model showed a considerable reduction in the number of CFU from that in the original inoculum (3-log-unit decrease in the number of CFU after 24 h), whereas the extracellular CFU reduction achieved *in vivo* after 4 h did not exceed 1 log unit. Multiple dosing of DCX *in vivo* revealed increased extra- and intracellular efficacies (2.5 log and 2 log units of reduction in the numbers of CFU after 24 h, respectively), confirming that DCX is a highly active antistaphylococcal antibiotic. PK/PD analysis revealed that fT_{MIC} is the index that is the most predictive of the outcome of infection both intra- and extracellularly.

Staphylococcus aureus is a major cause of both community- and hospital-acquired infections (28, 30), which range from simple and uncomplicated skin and wound infections (2, 24) to more serious and life-threatening situations such as pneumonia (15, 36), endocarditis (16, 37), osteomyelitis (13, 25), and meningitis (34). *S. aureus* infections often show poor and slow responses to therapy, with recurrences and ensuing mortality (8, 9, 27, 37, 38, 46). These responses could be caused by the ability of the bacteria to invade and survive inside cells (5, 10, 21, 22, 31, 32). Intracellular antimicrobial activity depends on both drug- and bacterium-related factors (penetration, accumulation, subcellular bioavailability, expression of activity in the local environment, and the state of responsiveness of the organisms [42, 44]). In general, intracellular antimicrobial activity is markedly impaired compared to the activity seen in broth or the extracellular milieu (3, 39, 45), although we know about situations in which the opposite is true (7). Thus, the direct assessment of antibiotic activity in the pertinent models is warranted. Several *in vitro* models with either human or animal cells have been developed to study the intracellular activities of antibiotics (3, 6, 14, 21, 35, 41), and a corresponding *in vivo* model (a modified version of a murine peritonitis model) has recently been described (39). We have now combined these models and report here our results obtained by using dicloxacillin (DCX) as a prototype of antistaphylococcal β-lactam antibiotics. Isoxazolyl penicillins have usually been preferred for the treatment of methicillin-susceptible *S. aureus* (MSSA) infections (2, 20, 26, 30). DCX has been the main choice in Denmark and many other countries due to its stability against penicillinases, low level of toxicity, and availability for both oral and intravenous administration (19). We examined the intra- and extracellular time- and concentration-kill relationships for two MSSA strains *in vitro* using macrophages and performed corresponding intra- and extracellular dose-kill studies with the murine peritonitis model. In combination with pharmacokinetic (PK) analysis and measurement of the amount of free drug (*f*) versus protein-bound drug, this allowed us to estimate which PK/pharmacodynamic (PD) index best predicts the efficacy of DCX intra- and extracellularly.

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MATERIALS AND METHODS

Bacterial strains, antimicrobial agents, and sources of other products. Strains ATCC 25923 (American Type Culture Collection, Manassas, VA) and E19977 (a clinical MSSA strain; Statens Serum Institut) were used throughout the study. DCX was procured as Diclocil (the parenteral form of DCX; Bristol-Myers

TABLE 1. Dosing regimens applied for the PK/PD study in the mouse peritonitis model

Dosing regimen no.	Dose (mg/kg)	Dosing interval[a]	Cumulative total dose (mg/24 kg · h)	Result for the following PK/PD index (free drug):		
				% fT_{MIC}	$fAUC_{24}$/MIC (h)	fC_{max}/MIC
1	76	q2h	912	100.00	227.43	28.18
2	30	q2h	360	45.83	39.32	6.27
3	80	q3h	640	66.67	168.69	30.64
4	120	q3h	960	75.56	350.73	61.60
5	160	q3h	1,280	96.11	556.40	81.58
6	200	q4h	1,200	86.25	616.07	101.92
7	80	q8h	240	25.00	63.49	30.64
8	240	q6h	960	65.28	572.83	136.18
9	200	q6h	800	57.50	411.61	101.92
10	120	q6h	480	37.78	175.82	61.60
11	400	q12h	800	35.97	644.84	291.53
12	300	q12h	600	35.28	439.15	196.76
13	340	q24h	340	17.78	259.37	233.35
14	120	q24h	120	9.44	43.95	61.60

[a] q2h, q3h, q4h, q6h, q8h, q12h, and q24h, dosing every 2, 3, 4, 6, 8, 12, and 24 h, respectively.

Squibb Company, New York, NY) and was used in compliance with the provisions of the European Pharmacopeia. *Sarcina lutea* (ATCC 9391) was used for the microbiological bioassay. Unless stated otherwise, cell culture media were from Invitrogen (Carlsbad, CA), microbiological media were from BD (Franklin Lakes, NJ), and other products were from Sigma-Aldrich, St. Louis, MO).

***In vitro* susceptibility studies.** MICs were determined at pH 5.4 and 7.4 by microdilution, as described previously (41), and for all other aspects, the recommendations of the Clinical and Laboratory Standard Institute (11) were followed.

***In vitro* extracellular and intracellular time- and concentration-kill studies.** Extracellular time-kill and concentration-kill studies were performed as described previously (41). In brief, bacteria at a density of 10^6 CFU/ml were exposed to DCX concentrations that varied over a wide range (to obtain a full description of the pharmacological response), and quantification of the numbers of CFU was performed after 5 and 24 h of incubation. Intracellular time- and concentration-kill studies were performed with phagocytized bacteria, as described previously (3, 29, 40, 41). In brief, opsonized *S. aureus* was added to the cell culture medium at a 4:1 bacteria/macrophage ratio for 45 min. Adherent, nonphagocytized bacteria were eliminated by washing and a short incubation in phosphate-buffered saline (PBS) containing gentamicin (50 mg/liter), and thereafter, the cells were incubated with DCX for up to 24 h over a wide range of extracellular concentrations. The numbers of cell-associated CFU and the amount of cell protein were measured at time zero and the end of the incubation. The large sample dilutions involved in the process of bacteria and cell collection ensured the absence of a carryover effect (see references 3 and 33 for details). Cell viability was checked by the trypan blue exclusion test for concentrations of DCX up to 500 mg/liter maintained for 24 h.

Pharmacokinetic studies of DCX in NMRI mice. Single-dose plasma pharmacokinetic studies were performed with NMRI mice given subcutaneous doses of DCX. Plasma DCX concentrations in blood samples collected and placed in tubes containing EDTA were measured by a microbiological bioassay with *S. lutea* (ATCC 9391) as the test organism (limit of detection, 1.5 mg/liter; linearity up to 100 mg/liter; coefficient of variation, ~5%; absence of interference by sample matrix checked by using the exact same matrix composition for the standard curve).

Pharmacokinetic indices (terminal half-life, area under the concentration-time curve [AUC], maximum concentration of drug in plasma [C_{max}]) were determined by noncompartmental modeling with the initESTIM program in the PKPDsim software package (http://www.ssi.dk/pkpdsim). The terminal half-life was estimated from the last four experimental time points by fitting of a monoexponential expression (least-squares regression). Exponential expression was also used to extrapolate the time-concentration curve data from the last experimental concentration data collection point out to 24 h for each dose tested. The AUC at 24 h (AUC_{24}) was therefore calculated partly from the experimental mean concentration points (trapezoidal rule) and partly from the fitted exponential curve (exact integration). The time-concentration curves from these single-dose PK studies were used to model a series of dosage regimens for the predictive PK/PD index estimations.

Protein binding of DCX in NMRI mouse plasma. Protein binding in plasma was measured by ultrafiltration of blood samples from both healthy and infected NMRI mice collected in EDTA-coated tubes. The samples were spiked with DCX at concentrations ranging from 10 to 400 mg/liter (no pH changes were observed). Controls were made with PBS spiked with similar concentrations of DCX. The samples were incubated for 1 h at 37°C and were then subjected to centrifugation through filter devices (at 3,000 × *g* for 8 min at 37°C; Centricon YM-30 filter; Millipore, Bedford, MA). The DCX concentrations in the original samples and filtrates were measured by the microbiological bioassay mentioned above. Two mathematical expressions, a simple square root expression as well as a theoretical expression based on binding kinetics, were fitted to the free (unbound) fraction of DCX as a function of the total DCX concentration in plasma by using the BinKin program in the PKPDsim software package (see the Appendix). The expression with the best fit was chosen on the basis of the least sum-of-squares criterion to model the variable free fraction of the drug.

Mouse peritonitis model. The mouse peritonitis model used has been described earlier (18, 39) (the protocol was approved by the Danish Animal Experimentation Inspectorate [license no. 2004/561-835]). In short, outbred female NMRI mice (Harland Netherlands, Horst, Netherlands) were inoculated by intraperitoneal injection of $10^{7.4}$ CFU in 0.5 ml. When they were sampled, the mice were euthanized and subjected to a peritoneal flush (2.0 ml Hanks balanced salt solution) to collect murine cells and bacteria.

(i) Intra- and extracellular dose-kill studies. Single doses of DCX (0.25 ml) were administrated subcutaneously 2 h after inoculation, and samples were obtained after 4 h of drug exposure.

TABLE 2. *In vitro* intra- and extracellular regression data for the activity of DCX against *S. aureus* in concentration-kill studies (24-h drug exposure protocol)

S. aureus strain	MIC (mg/liter)		Extracellular data[a] (95% confidence interval)			
	pH 7.4	pH 5.4	E_{max}[c] (Δlog CFU)	EC_{50}[d]		C_{static}[e]
				mg/liter	Multiple of MIC	mg/liter
ATCC 25923	0.125	0.031	−3.19 (−4.25 to −2.14)	0.06 (0.04 to 0.15)	0.49 (0.19 to 1.26)	0.07 (0.04 to 0.13)
E19977	0.5	0.125	−3.81 (−4.50 to −3.13)	0.50 (0.23 to 1.08)	1.00 (0.46 to 2.16)	0.43 (0.25 to 0.78)

[a] The initial (postphagocytosis) inocula (at time zero) were (1.78 ± 0.34) × 10^6 CFU/ml for strain ATCC 25923 and (1.23 ± 0.25) × 10^6 CFU/ml for strain E19977 (n = 3).

[b] The initial (postphagocytosis) inocula (at time zero) were (7.14 ± 2.59) × 10^6 CFU/mg protein for strain ATCC 25923 and (9.69 ± 1.23) × 10^6 CFU/mg protein for strain E19977 (n = 3).

[c] E_{max}, the decrease in the colony count (in $\log_{10}$ CFU) at time 24 h compared to the initial colony count extrapolated for an infinitely large antibiotic concentration, as determined by the Hill equation.

[d] EC_{50}, the concentration causing a change in the inoculum halfway between the changes in the numbers of CFU extrapolated for an infinitely low (E_{min}) and an infinitely high (E_{max}) dose, as obtained from the Hill equation.

[e] $C_{stastic}$, the concentration resulting in no apparent bacterial growth (the number of CFU is identical to that in the initial inoculum), as determined by graphical interpolation.

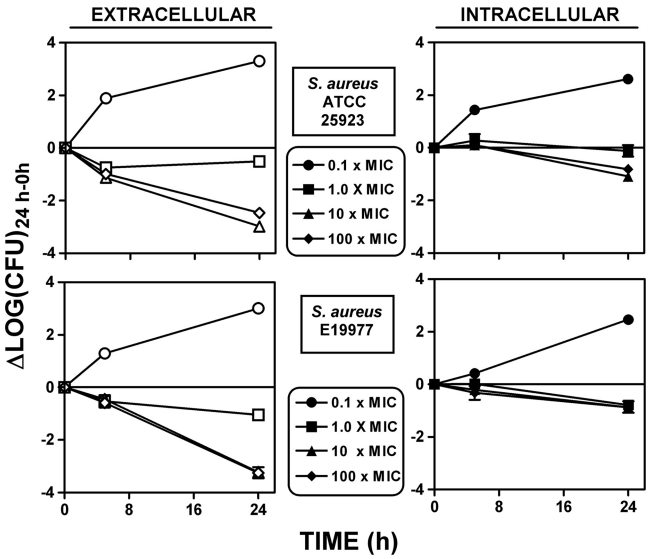

FIG. 1. *In vitro* time-kill studies of DCX against *S. aureus* at different fixed extracellular concentrations. (Left panels) Activity against the extracellular form of the bacteria measured in broth; (right panels) activity against the intracellular form of the bacteria measured in THP-1 macrophages. The activity was measured against two different MSSA strains (upper panels, ATCC 25923 [MIC = 0.125 mg/liter]; lower panels, E19977 [MIC = 0.5 mg/liter]). The graphs show the activity as changes in the numbers of CFU (Δ log CFU; means ± standard deviations; $n = 3$). The change in the numbers of CFU is measured as the numbers of CFU/ml in the extracellular studies and the numbers of CFU/mg cell protein in the intracellular studies.

(ii) Predictive PK/PD index studies. On the basis of the time-concentration curves obtained from the single-dose PK studies and the mathematical expression for the variable free fraction of the drug with the best fit, a model for a series of dosage regimens was made. This was carried out by linear scaling and interpolation, followed by truncation and repetition of the curves, according to the dose size and dosage interval, by using the studyDESIGN program in the PKPDsim software package. A subset of 14 regimens was chosen (on the basis of the distribution of the cumulative percentage of a 24-h period that the drug concentration exceeds the MIC under steady-state pharmacokinetic conditions [fT_{MIC}], $f\text{AUC}_{24}$/MIC, and fC_{max}/MIC (1) (Table 1). A control group was included for the first 6 h of infection. At this point, the mice met the clinical signs of irreversible sickness and were euthanized. The predictive PK/PD index was determined by using the data obtained with strain E19977.

(iii) Separation of intra- and extracellular *S. aureus* in peritoneal fluid. An assay for the separation of intra- and extracellular *S. aureus* in peritoneal fluid was performed by use of a modified version of a procedure described previously (39). The peritoneal fluid collected from each mouse was diluted 1:1 with Hanks balanced salt solution and divided into two equal fractions (fractions A and B). The supernatant from fraction A was used for extracellular CFU quantification (by centrifugation at 300 × *g* and room temperature for 10 min). Lysostaphin was added to fraction B (15 mg/liter), and the mixture was incubated (15 min, room temperature). The lysostaphin was removed by centrifugation (300 × *g*) and the fraction was prepared for intracellular CFU quantification, as described previously (39).

Data analysis. The data from all concentration- and dose-kill studies were analyzed by fitting a sigmoidal dose-response equation (by use of a slope factor of 1) which allowed calculation of the minimal relative efficacy (E_{min}; defined as the increase in the $\log_{10}$ numbers of CFU for an infinitely low antibiotic concentration), the maximal relative efficacy (E_{max}; defined as the decrease in the $\log_{10}$ numbers of CFU for an infinitely large antibiotic concentration), the relative potency (the 50% effective concentration [EC_{50}], measured as the concentration that caused a change in the $\log_{10}$ numbers of CFU halfway between E_{min} and E_{max}), the concentration that caused no apparent change in the numbers of CFU compared to the numbers of CFU in the original inoculum (C_{static}), and goodness of fit (R^2). The best-fit values for E_{max} and EC_{50} were compared between the intra- and the extracellular data and between strains by using the extra sum-of-squares F test. A P value of <0.05 was considered significant. The correlation between the PK/PD index and the infection outcome were calculated by using nonlinear regression and least-squares fit. All curve fittings and statistical analyses were performed by using Prism software (version 5.0; GraphPad Prism Software, San Diego, CA).

RESULTS

Susceptibility studies. The MICs of DCX at pHs 7.4 and 5.4 (to mimic the pH values of the extracellular and phagolysosomal environments, respectively) are shown in Table 2. The MICs for both strains (ATCC 25923 and E19977) dropped 4-fold when the pH was decreased from 7.4 to 5.4.

***In vitro* intra- and extracellular time- and concentration-kill studies.** DCX did not cause an increase in the percentage of THP-1 macrophages stained by trypan blue up to a concentration of 500 mg/liter (<1% of the macrophages were dead after 24 h of incubation). Figure 1 shows the results of the intra- and extracellular time-kill studies performed with DCX and strains ATCC 25923 and E19977. Considering the extracellular activity first, we observed a less than 1-$\log_{10}$-unit decrease in the counts of both strains after 5 h at all concentrations equal to or greater than the MIC. After 24 h, a 3-$\log_{10}$-unit decrease in the bacterial counts was observed at concentrations above the MIC. A static effect was observed at between 5 and 24 h of drug exposure at concentrations equal to the MIC value. Bacterial growth occurred at the MICs. When the intracellular activity is considered, an overall impairment of the activity of DCX was seen. Thus, only a static effect was observed at 5 h at concentrations equal to or greater than the MIC value. After 24 h, a less than 1-$\log_{10}$-unit decrease in the bacterial counts was observed at concentrations greater than the MIC value for both strains. At concentrations equal to the MIC value, a static effect was observed for ATCC 25923 after 24 h of drug exposure and a 1-$\log_{10}$-unit decrease was observed for E19977. Figure 2 shows the full pharmacological response of extracellular and intracellular isolates of strains ATCC 25923 and E19977 to DCX at 24 h when they were exposed to a wide

TABLE 2—*Continued*

Extracellular data[a] (95% confidence interval)		Intracellular data[b] (95% confidence interval)					
C_{static}[e]	R^2	E_{max} (Δlog CFU)	EC_{50}		C_{static}		R^2
Multiple of MIC			mg/liter	Multiple of MIC	mg/liter	Multiple of MIC	
0.59 (0.30 to 1.08)	0.95	−1.19 (−1.85 to −0.52)	0.04 (0.01 to 0.14)	0.33 (0.10 to 1.12)	0.11 (0.05 to 0.22)	0.86 (0.36 to 1.75)	0.87
0.87 (0.49 to 1.56)	0.87	−1.01 (−1.65 to −0.37)	0.31 (0.07 to 1.37)	0.62 (0.14 to 2.75)	0.89 (0.28 to 2.45)	1.78 (0.56 to 4.90)	0.90

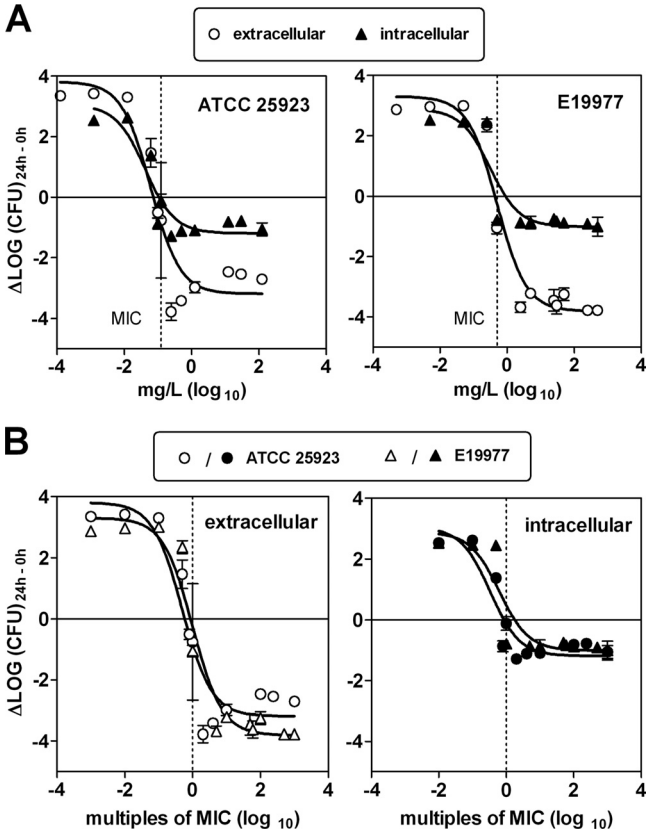

FIG. 2. *In vitro* dose-response curves of DCX against extracellular (measured in broth) and intracellular (measured in THP-1 cells) *S. aureus* (strains ATCC 25923 and E19977). The graphs show the activity as changes in the numbers of CFU (Δ log CFU; means ± standard deviations; $n = 3$). The change in the numbers of CFU is measured as the numbers of CFU per ml broth in the extracellular studies and as the numbers of CFU per mg cell protein in the intracellular studies. The initial inocula for the extracellular study (time zero) were $(1.78 \pm 0.34) \times 10^6$ CFU/ml for strain ATCC 25923 and $(1.23 \pm 0.25) \times 10^6$ CFU/ml for strain E19977 ($n = 3$). The initial (postphagocytosis) inocula for the intracellular study (time zero) were $(7.14 \pm 2.59) \times 10^6$ CFU/mg protein for strain ATCC 25923 and $(9.69 \pm 1.23) \times 10^6$ CFU/mg protein for strain E19977 ($n = 3$). The sigmoidal function was used for fitting (see Table 2 for goodness-of-fit and regression parameters). (A) Comparison of the intra- and extracellular activities against each strain versus the extracellular concentration (mg/liter); (B) comparison of the extracellular and intracellular activities against the two strains versus equipotent extracellular concentrations (multiples of the MICs).

range of extracellular concentrations. The pertinent regression parameters are listed in Table 2.

The E_{max} values for the intracellular concentration-kill curve were significantly lower than the extracellular E_{max} values ($P < 0.001$ for strain E19977 and $P = 0.0015$ for strain ATCC 25923). Despite the impairment of its intracellular antibiotic activity, DCX still showed a maximal relative efficacy of a 1-$\log_{10}$-unit decrease. When the relative potencies (EC_{50}s) of DCX intra- and extracellularly are compared, no significant difference was observed ($P = 0.4539$ for strain E19977 and $P = 0.5473$ for strain ATCC 25923). The C_{static} values also seemed to be equal intra- and extracellularly and were close to the MIC measured at pH 7.4. When the results of these studies were expressed as multiples of the MIC, no significant difference between the responses of ATCC 25923 and those of E19977 was seen, whether the responses were measured intra- or extracellularly.

TABLE 3. Protein binding of DCX to NMRI mouse plasma

Concn (mg/liter)	% protein binding in plasma from:	
	Healthy mice	Infected mice[a]
400	83.7	
270	84.9	80.7
180	87.2	
90	88.7	85.6
60	89.5	
30	91.5	91.9
10	93.9	93.6

[a] Plasma from NMRI mice infected intraperitoneally (6 h) with *S. aureus* E19977.

Protein binding and pharmacokinetic studies. Table 3 shows that the binding of DCX to proteins was concentration dependent but was saturable. The level of binding in uninfected mice was lower than that in infected animals, but only for the high concentrations. As can be seen from Fig. 3, the data from the binding study were best fit by a simple square root expression when the free fraction was described as a function of the total concentration. The pharmacokinetics of DCX in NMRI mouse plasma obtained from four primary PK studies are shown in Fig. 4a. Peak levels were observed after 9 to 24 min. The half-life was very short and varied from 19 to 30 min. DCX exhibited nonlinear pharmacokinetics with respect to the dose size, as clearly illustrated in Fig. 4b; with full linearity, the four curves would have been identical (within the limits of uncertainty).

***In vivo* intra- and extracellular single dose-kill studies (4 h).** Figure 5 shows the results of the intra- and extracellular dose-kill studies observed 4 h after the administration of single doses of DCX, and the pertinent regression parameters listed in Table 4. The E_{max} values for the intracellular dose-kill curve were not significantly different from the extracellular E_{max} value ($P = 0.1152$ for strain E19977 and $P = 0.3878$ for strain ATCC 25923). The DCX intracellular activity *in vivo* was a 1-$\log_{10}$-unit decrease in the numbers of CFU compared to the

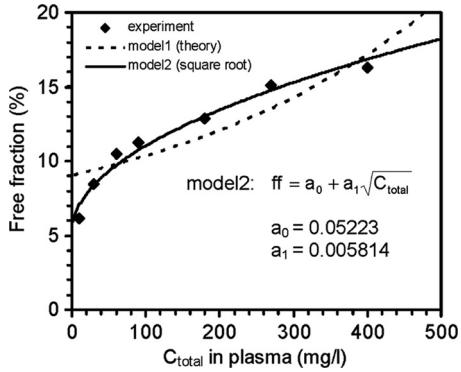

FIG. 3. Protein binding of DCX in plasma from healthy mice upon an increase in the drug concentrations. The graph shows the fitting of two mathematical expressions to the experimental data. See the Appendix for a detailed description of the theoretical model.

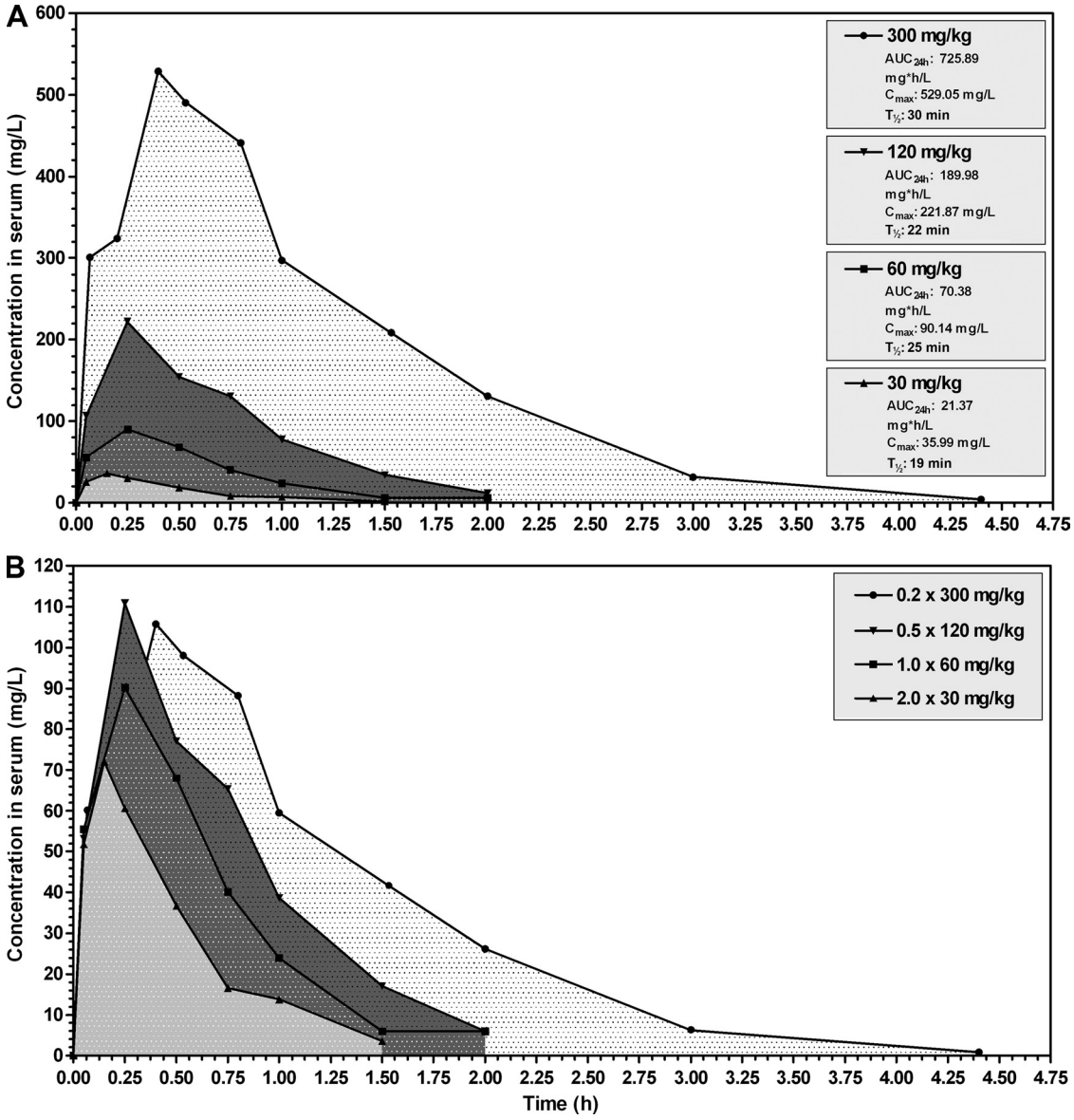

FIG. 4. Plasma DCX concentrations in NMRI mice following the subcutaneous administration of single doses of 300, 120, 60, and 30 mg/kg of body weight. (A) Measured total concentrations; (B) linearly scaled total concentrations simulating the response of a 60-mg/kg dose for all four curves.

initial inoculum. The EC_{50}s for the intra- and extracellular activities were not significantly different for either strain used ($P = 0.0717$ for strain E19977 and $P = 0.9080$ for strain ATCC 25923). The C_{static} values were slightly higher intracellularly than extracellularly for strain E19977 and almost equal for strain ATCC 25923. The *f*AUC values (mg · h/liter) for each static dose (C_{static}) were calculated and divided by the drug exposure time (4 h), resulting in the average concentration (mg/liter) achieved through the 4 h of treatment. The mean concentrations were 0.095 and 0.120 mg/liter extracellularly and 0.09 and 0.137 mg/liter intracellularly for strains ATCC 25923 and E19977, respectively. These concentrations were close to the static concentrations and the MIC values measured *in vitro* (Table 2).

Predictive intra- and extracellular PK/PD index determinations (24 h). The impacts of the three well-accepted PK/PD indices (*f*AUC/MIC ratio, the fC_{max}/MIC ratio, and fT_{MIC}) on the infection outcome were determined by correlating the number of bacteria in the peritoneum after 24 h of therapy (both as the total bacterial count and as the extra- and intracellular bacterial counts) to the calculated values for each of the dosing regimens tested (Table 1). No or very little interrelationship (less than 27%) was recorded between the PK/PD index values of the selected dosing regimens. The fitted mathematical expression for the free variable fraction of the drug (model 2 in Fig. 3) was used for the calculations (it was critical to take into account the saturable character of DCX protein binding, as this markedly affects $fAUC_{24}$/MIC and fC_{max}/MIC,

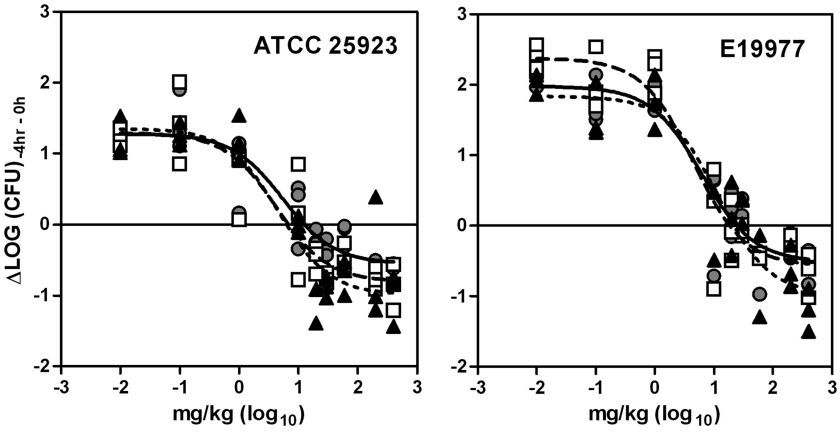

FIG. 5. *In vivo* dose-response curves after single doses of dicloxacillin against *S. aureus* (strains ATCC 25923 and E19977) measured in a mouse peritonitis model (4-h drug exposure). The graphs displays (i) the total activity (gray circles and solid line), (ii) the activity considered to be extracellular (open squares and dashed line), and (iii) the activity considered to be intracellular (closed triangles and dotted line). The activity is displayed as changes in the numbers of CFU (Δ log CFU), with each datum point corresponding to one animal. The initial total inocula were $(9.80 \pm 4.85) \times 10^5$ CFU/ml for strain ATCC 25923 and $(6.26 \pm 2.87) \times 10^5$ CFU/ml for strain E19977 ($n = 6$). The initial extracellular inocula were $(4.26 \pm 3.03) \times 10^4$ CFU/ml for strain ATCC 25923 and $(3.03 \pm 0.85) \times 10^4$ CFU/ml for strain E19977 ($n = 6$). The initial intracellular inocula were $(6.36 \pm 4.41) \times 10^5$ CFU/ml for strain ATCC 25923 and $(2.88 \pm 1.72) \times 10^5$ CFU/ml for strain E19977 ($n = 6$). The sigmoidal function was used for fitting (see Table 4 for the goodness-of-fit and regression parameters).

while it leaves fT_{MIC} almost unchanged). The results of these calculations are shown in Fig. 6. The average colony count for the untreated control group, sampled 4 h after treatment initiation, was included in the regression analysis but displayed as a small value for the PK/PD index. At this time point, the inocula were $(1.70 \pm 0.11) \times 10^7$ CFU/ml in total and $(1.56 \pm 1.05) \times 10^6$ CFU/ml extracellularly, while the intracellular inoculum was $(4.51 \pm 3.94) \times 10^6$ CFU/ml ($n = 8$).

The best correlation to the infection outcome in total, extracellularly, and intracellularly was observed for the fT_{MIC} index ($R^2 \geq 78\%$). A modest significant correlation was observed, however, for the $f\text{AUC}_{24}$/MIC index (R^2, between 40 and 52%). A poor or no correlation was observed for the fC_{max}/MIC index. As also illustrated in Fig. 6, high correlations were observed between the infection outcome and the cumulative total 24-h dose (Table 1) of DCX ($R^2 \geq 71\%$).

DISCUSSION

In the studies described here, we quantified and compared the activities of DCX against extracellular and intracellular *S. aureus* in cell culture and animal models, and these represent some of the first attempts to systematically compare these two approaches.

In the cell culture model (THP-1 macrophages), the antibiotic concentration and the number of cells remain fairly constant, which allows exploration of the effect of antibiotics over a wide range of known concentrations. THP-1 cells also display poor intrinsic defenses against intracellular infection (29). This model therefore offers valuable information concerning the specific intra- and extracellular capacities of the drug by excluding other parameters that could affect the antimicrobial activity. This approach revealed that the maximal relative intracellular activity (E_{max}) of DCX is considerably lower than its extracellular activity (measured in broth). This complements previous findings for β-lactams in the same model used in the present study as well as in other *in vitro* cell models (4, 29). The relative potency and the C_{static} of DCX, however, were not different for extracellular and intracellular bacteria (with, for C_{static}, a value close to the DCX MIC being measured at pH 7.4). This indicates that the loss of intracellular activity mainly affects the extent of eradication that can be obtained, with the clear persistence of a significantly larger inoculum intracellu-

TABLE 4. *In vivo* intra- and extracellular regression data for dose-kill studies of activity of DCX against *S. aureus* (4-h treatment protocol)

S. aureus strain	Total data[a] (95% confidence interval)				Extracellular data[b] (95% confidence interval)	
	E_{max}[d] (Δlog CFU)	EC_{50}[e] (mg/kg)	C_{static}[f] (mg/kg)	R^2	E_{max} (Δlog CFU)	EC_{50} (mg/kg)
ATCC 25923	−0.55 (−0.79 to −0.32)	5.49 (2.30 to 13.11)	12.71	0.87	−0.80 (−1.10 to −0.51)	4.27 (1.48 to 12.28)
E19977	−0.51 (−0.83 to −0.19)	6.28 (2.84 to 13.88)	24.21 (13.24–43.24)	0.88	−0.57 (−0.89 to −0.26)	4.49 (2.07 to 9.73)

[a] The initial total inocula (at time zero) were $(9.80 \pm 4.85) \times 10^5$ CFU/ml for strain ATCC 25923 and $(6.26 \pm 2.87) \times 10^5$ CFU/ml for strain E19977 ($n = 6$).
[b] The initial extracellular inocula (at time zero) were $(4.26 \pm 3.03) \times 10^4$ CFU/ml for strain ATCC 25923 and $(3.03 \pm 0.85) \times 10^4$ CFU/ml for strain E19977 ($n = 6$).
[c] The initial intracellular inocula (at time zero) were $(6.36 \pm 4.41) \times 10^5$ CFU/ml for strain ATCC 25923 and $(2.88 \pm 1.72) \times 10^5$ CFU/ml for strain E19977 ($n = 6$).
[d] E_{max}, the decrease in the colony count (in $\log_{10}$ CFU) from the corresponding initial inoculum and at time 24 h, as extrapolated for an infinitely large dose and by use of the Hill equation.
[e] EC_{50}, the dose causing a reduction of the inoculum halfway between the changes in the number of CFU extrapolated for an infinitely low (E_{min}) and an infinitely high (E_{max}) dose, as obtained from the Hill equation.
[f] C_{static}, the dose resulting in no apparent bacterial growth (the number of CFU is identical to that in the initial inoculum), as determined by graphical interpolation.

larly than in broth being noted. The data also show that the killing of bacteria by DCX largely becomes concentration independent both intracellularly and extracellularly, once its concentration exceeds the MIC. Furthermore, the activity was found to be mainly time dependent, with a long incubation time (24 h) being required to cause a significant decrease in the numbers of CFU. This matches previous observations for β-lactams (12, 17) and helps to reconcile what could appear to be contradictory statements concerning the concentration and time dependence of the activities of these antibiotics when they are examined in different models.

Similar to the cell culture studies, we found no significant differences *in vivo* between the intra- and extracellular potencies (EC_{50}s) and C_{static} values. Interestingly, the free mean concentrations of DCX during treatment were closely related to the MIC, suggesting that, as in the cell culture model, MIC values are good indicators of the potency of DCX both intracellularly and extracellularly. The cell culture and the *in vivo* models, however, differ markedly with respect to the E_{max}s observed for intra- and extracellular bacteria. Thus, *in vivo*, the E_{max} values of DCX are similar but weak (only a 1-$\log_{10}$-unit decrease in the numbers of CFU) in both situations. For β-lactams, the rate of bacterial killing is highly dependent upon the generation time (43), and this can at least partly explain why the measurements of the extracellular activity are so different between the two models. In the cell culture model, the extracellular killing was measured in broth without cells, which offers optimal growth conditions for the bacteria, and activity spans over almost 8 $\log_{10}$ units between values for infinitely low and infinitely high antibiotic concentrations (E_{min} and E_{max}, respectively). In the *in vivo* model, the extracellular compartment is hostile to the bacteria and a reduced growth rate is expected, leading to the reduced bactericidal activity of DCX. The antimicrobial activity is further expected to be reduced *in vivo* due to the protein binding of the drug and general elimination of the drug from the infection site. The cell culture model may therefore overestimate the *in vivo* extracellular capacity of DCX. It is interesting to note, in this context, that the E_{min}-E_{max} span in the *in vivo* model roughly approximates what is seen for intracellular bacteria in the *in vitro* model, suggesting that the expression of the activity of DCX is impaired to the same extent in both situations.

The *in vivo* model can also be differentiated from the cell culture model in terms of the point of the cell life cycle. Cell turnover is expected at the infection site *in vivo* (39) because of the constant exchange of bacterial cells that are either becoming extracellular due to cell lysis or becoming intracellular by phagocytosis from newly migrated granulocytes. This could also contribute to the impaired extracellular activity seen *in vivo* compared to that seen *in vitro*.

An important question in the type of study presented here is whether what is observed is specific to one strain of bacteria. We show here that the reference MSSA strain, ATCC 25923, and a clinical MSSA strain, E19977, essentially behave alike and that the responses in the cell culture model as well as *in vivo* are essentially determined by their *in vitro* susceptibility to DCX. This suggests that MIC determination measures the true intrinsic potency of the drug and can be used to categorize isolates with respect to both extracellular and intracellular susceptibilities.

Knowledge of the intra- and extracellular activities is important when the relevance of using DCX for the treatment of intracellular *S. aureus* infections is considered. Although we have shown that the relative efficacy (E_{max}) of DCX is reduced intracellularly, we found that DCX is able to control the intracellular bacterial count and even decrease this load, provided that enough time (24 h) is allowed. When the concentration is on strategies for the treatment of intracellular staphylococcal infections, the next logical question to be asked is, what is the optimal dosing regimen for the eradication of both intra- and extracellular organisms? We found in the present study that the fT_{MIC} index is best correlated to the infection outcome both intracellularly and extracellularly. Whereas a value of approximately 10% is sufficient for a static effect, a value of 100% is needed to obtain a maximal effect both intracellularly and extracellularly. The determination that T_{MIC} is the driving force for the activities of the β-lactams is not new (12, 17, 26). What the present study does, however, is show that this index is predictive of activity against both the intracellular and the extracellular forms of *S. aureus*, which, to our knowledge, has not been detected in previous studies. We must, however, emphasize that this is because of (i) the low MICs of DCX toward the organisms used and (ii) the fact that the drug dosages used made the concentration to exceed the MIC for a significant proportion of the exposure time, thus showing a pattern of time dependence of DCX in our *in vivo* model. Given this caveat, the data clearly support the use of frequent doses or even the constant infusion of DCX (at concentrations above the MIC) for prolonged periods of time to optimize the *in vivo* treatment efficacy both intracellularly and extracellularly. These conclusions concur with the clinical findings. Thus, Hughes et al. (23) found that a continuous infusion of oxacillin improved the rates of microbiological cure in patients with endocarditis compared to those achieved with intermittent infusion. Furthermore, Jensen et al. (26) found that a dosage of dicloxacillin of 1 g four times daily or 2 g three times daily (which provided a T_{MIC} value of 100%) was superior to a dosage of 1 g three times daily (which provided a T_{MIC} value of 78%) in terms of both the rates of mortality and recurrence of infection in patients with *S. aureus* bacteremia. The rate of mortality was also significantly higher for patients treated for less than 14 days (23%) than for patients treated for 14 days or more (4%).

TABLE 4—*Continued*

Extracellular data[b] (95% confidence interval)		Intracellular data[c] (95% confidence interval)			
C_{static} (mg/kg)	R^2	E_{max} (Δlog CFU)	EC_{50} (mg/kg)	C_{static} (mg/kg)	R^2
6.84	0.83	−0.99 (−1.3 to −0.67)	4.65 (1.71 to 12.62)	6.34	0.84
18.58 (10.05 to 31.48)	0.90	−0.98 (−1.4 to −0.56)	11.67 (5.63 to 24.19)	21.83 (13.18 to 33.65)	0.86

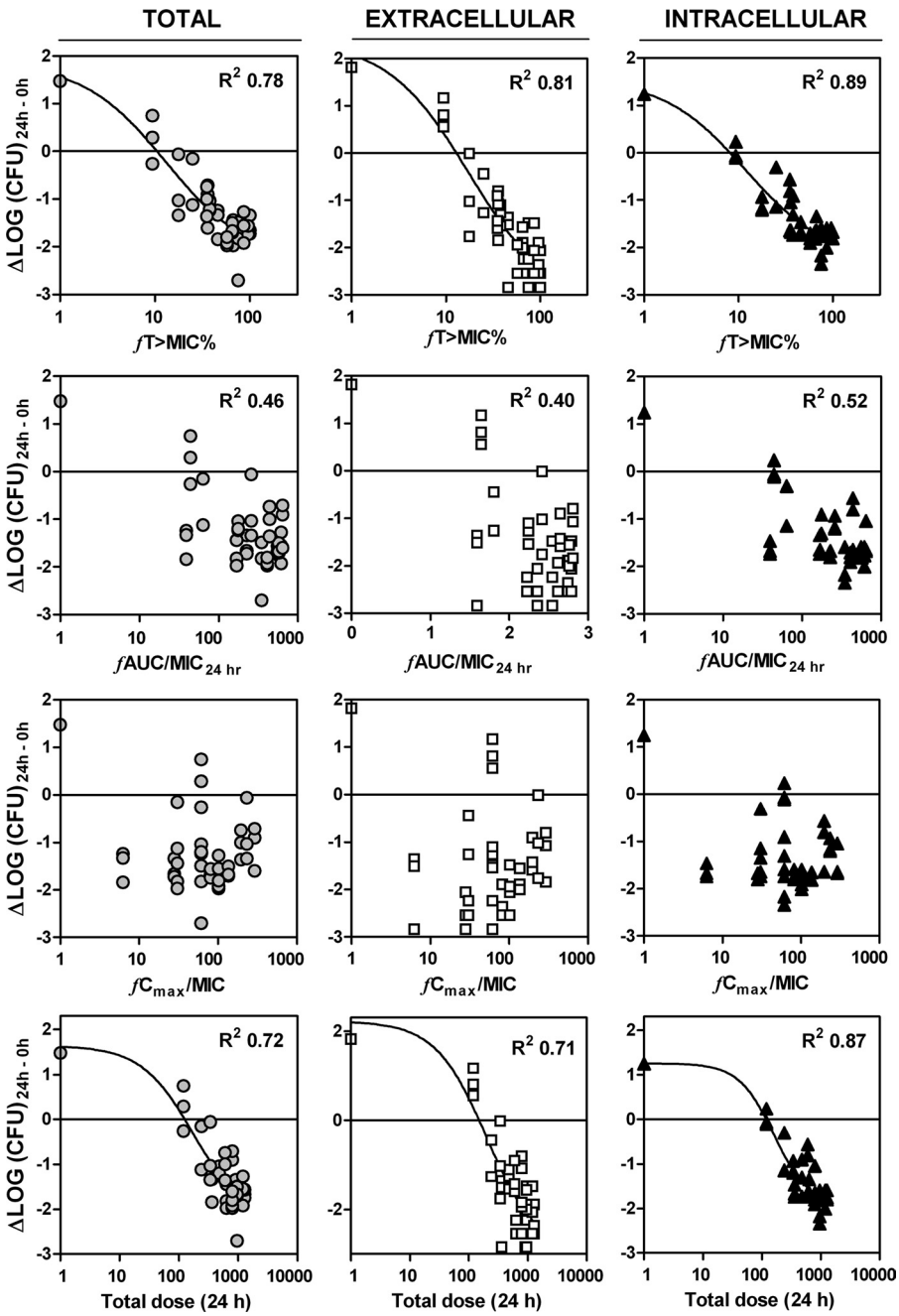

FIG. 6. Relationship between fT_{MIC}, fAUC/MIC, fC_{max}/MIC, and the total 24-h dose for the 24-h accumulated amount of free drug and the efficacy of DCX against MSSA strain E19977, as measured in the mouse peritonitis model (24 h of drug exposure). See Table 1 for the dosing regimens applied. The graphs display (i) the total activity (left panels, gray circles), (ii) the activity considered to be against extracellular bacteria (middle panels, open squares), and (iii) the activity considered to be against intracellular bacteria (right panels, closed triangles). The activity is displayed as changes in the numbers of CFU (Δ log CFU), with each datum point corresponding to one animal. The initial total, extracellular, and intracellular inocula were $(5.61 \pm 0.15) \times 10^5$, $(2.12 \pm 1.51) \times 10^4$, $(2.86 \pm 2.81) \times 10^5$ CFU/ml, respectively ($n = 8$). The sigmoidal function was used, and R^2 represents the goodness of fit.

Measurement of the intra- and extracellular activities of DCX in both a cell culture model and in an vivo model offers a unique opportunity to compare the findings obtained with and discuss the use of the two models. As seen from the data presented above, similar conclusions concerning the intracellular activity of DCX were, in general, reached, regardless of the model used. The *in vitro* model is suitable as a tool for the first screening of intracellular activity and has the advantage of being able to study a specific constellation of drug and cell types. The *in vivo* model, however, should be used when extended knowledge of drug efficacy and PK/PD relationships is needed. The two models therefore complement each other

very well and may be used to obtain important knowledge of the intra- and extracellular activities of both potentially new antibiotics and well-established antibiotics such as DCX.

APPENDIX

With no influx or removal of antibiotics from the plasma compartment, a general equation for the reversible binding mechanism can be written as

$$\frac{dC_b}{dt} = -\frac{dC_f}{dt} = k_b C_b (C_{b\,\max} - C_b) - \frac{C_b}{T_b} \quad (1)$$

where C_b and C_f denote the concentration of protein-bound and free (unbound) antibiotics, respectively; t is time; $C_{b\max}$ is the binding capacity; k_b is the binding affinity; and T_b is the mean time in the bound state.

By setting the right-hand side in equation 1 equal to 0, we obtain the following relationship between C_b and C_f at equilibrium:

$$C_f = \frac{C_b}{k_b T_b (C_{b\,\max} - C_b)} \quad (2)$$

Equation 2 can be rewritten to give the free fraction (*ff*) as a function of the total concentration and the binding parameters:

$$\frac{C_f}{C_{\text{total}}} = ff(C_{\text{total}}, k_b T_b, C_{b\,\max}) \quad (3)$$

where $C_{\text{total}} = C_b + C_f$.

Fitting of equation 3 to the experimental binding data for dicloxacillin in Table 3 gives the following parameter values: $C_{b\max}$ equals 637 mg/liter and $k_b T_b$ equals 0.0158 liter/mg. Figure 3 shows the fitted curve as model 1 (theory).

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