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# Pharmacodynamic Evaluation of the Intracellular Activity of Antibiotics towards *Pseudomonas aeruginosa* PAO1 in a Model of THP-1 Human Monocytes

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*Pseudomonas aeruginosa* invades epithelial and phagocytic cells, which may play an important role in the persistence of infection. We have developed a 24-h model of THP-1 monocyte infection with *P. aeruginosa* PAO1 in which bacteria are seen multiplying in vacuoles by electron microscopy. The model has been used to quantitatively assess antibiotic activity against intracellular and extracellular bacteria by using a pharmacodynamic approach (concentration-dependent experiments over a wide range of extracellular concentrations to calculate bacteriostatic concentrations [ $C_s$ ] and maximal relative efficacies [ $E_{max}$ ]; Hill-Langmuir equation). Using 16 antipseudomonal antibiotics (three aminoglycosides, nine  $\beta$ -lactams, three fluoroquinolones, and colistin), dose-response curves were found to be undistinguishable for antibiotics of the same pharmacological class if data were expressed as a function of the corresponding MICs. Extracellularly, all of the antibiotics reached a bacteriostatic effect at their MIC, and their  $E_{max}$  exceeded the limit of detection ( $-4.5 \log_{10}$  CFU compared to the initial inoculum). Intracellularly,  $C_s$  values remained unchanged for  $\beta$ -lactams, fluoroquinolones, and colistin but were approximately 10 times higher for aminoglycosides, whereas  $E_{max}$  values were markedly reduced (less negative), reaching  $-3 \log_{10}$  CFU for fluoroquinolones and only  $-1$  to  $-1.5 \log_{10}$  CFU for all other antibiotics. The decrease in intracellular aminoglycoside potency (higher  $C_s$ ) can be ascribed to the acid pH to which bacteria are exposed in vacuoles. The decrease in the  $E_{max}$  may reflect a reversible alteration of bacterial responsiveness to antibiotics in the intracellular milieu. The model may prove useful for comparison of antipseudomonal antibiotics to reduce the risk of persistence or relapse of pseudomonal infections.

*Pseudomonas aeruginosa* is among the leading causes of nosocomial infections in humans, with a particular tropism for the respiratory tract (see references 1 and 2 for reviews). Beside causing acute infections, it also chronically colonizes the lungs of cystic fibrosis patients, causing protracted and relapsing infections that are a primary cause of increased morbidity and mortality (3, 4).

*P. aeruginosa* is difficult to eradicate for at least two non-mutually exclusive reasons. First, *P. aeruginosa* is not only naturally resistant to many common antibiotics (because of the poor permeability of its outer membrane) but can also express several mechanisms of resistance to most, if not all, of the drugs that have been selected and clinically developed over the years for their antipseudomonal activity (2). Second, *P. aeruginosa* can adopt specific modes of life that afford protection from host defenses and antibiotic action. The first one consists of the production of biofilm, a physical barrier to the access of antimicrobial agents that contributes to the persistence of infection with *P. aeruginosa* (5). The second one stems from the capacity of *P. aeruginosa* to invade and survive within eukaryotic cells. This has been clearly documented for both epithelial (6–14) and phagocytic (15) cells *in vitro* and also observed *in vivo* for lung epithelial cells and alveolar macrophages of infected mice (16). Thus, although it is usually considered an extracellular organism, *P. aeruginosa* may actually behave as an opportunistic intracellular organism. On the whole, one can estimate that approximately half of the clinical, laboratory, and environmental isolates obtained demonstrate measurable internalization (17). This ability to invade depends on multiple factors, among which the level of expression of two secretion systems, H2 type VI and a type III secretion system, may play a critical role, as the former favors and the latter inhibits internalization (8, 18).

Survival of bacteria in eukaryotic cells has been associated with the reduced efficacy of most antibiotics, as demonstrated for

*Staphylococcus aureus*, *Listeria monocytogenes*, *Legionella pneumophila*, and *Mycobacterium tuberculosis* (see references 19 to 21 for typical examples and references 22 and 23 for reviews). However, no data are available regarding the activity of antipseudomonal antibiotics against the intracellular forms of *P. aeruginosa*. The aim of the present study was therefore to set up a model of intracellular *P. aeruginosa* infection allowing a comparative evaluation of the activities of antipseudomonal antibiotics. We selected THP-1 human monocytes as host cells because these have proven useful in obtaining reproducible levels of intracellular infection with other bacteria and in quantitatively assessing the activities of antibiotics by a pharmacodynamic approach (19, 20, 24–26). We included in our study most of the currently used antipseudomonal agents (27) and tested them against reference strain PAO1.

## MATERIALS AND METHODS

**Bacterial strain, susceptibility testing, and time and dose-kill curve studies in extracellular medium.** *P. aeruginosa* strain ATCC PAO1 was used for all experiments. Bacteria were grown in Mueller-Hinton broth, and CFU counting was performed by plating on tryptic soy agar. MICs

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were measured by serial 2-fold microdilution according to CLSI guidelines (inoculum of approximately  $10^6$  CFU/ml, reading after 20 to 24 h of incubation) in cation-adjusted Mueller-Hinton broth (CA-MHB) (28) or in the medium used for experiments with THP-1 cells (RPMI 1640 medium supplemented with 10% fetal calf serum buffered at pH 7.4 with 10 mM phosphate buffer for MIC testing and with 2 g/liter  $\text{NaHCO}_3$  for cell cultures incubated in a 5%  $\text{CO}_2$  atmosphere). Concentration-kill curves were determined as previously described, with a starting inoculum of  $10^6$  CFU/ml (19) in the same media.

**Cells, cell culture, and intracellular infection.** Human THP-1 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum as described previously (19). Intracellular infection was performed on the basis of the protocol previously developed for *S. aureus* (19), with specific adaptations. In brief, bacteria were opsonized by 1 h of incubation with 10% human serum in RPMI 1640 medium without fetal calf serum. Phagocytosis was allowed for 2 h at a bacterium/cell ratio of 10:1, after which nonphagocytosed and adherent bacteria were eliminated by incubation for 60 min with gentamicin at 100 mg/liter. After three washes in phosphate-buffered saline (PBS) and resuspension in culture medium, the inoculum was typically  $5 \times 10^5$  to  $7 \times 10^5$  CFU/mg of cell protein (defined as time zero). Extracellular contamination at the end of the incubation period was assessed in pilot experiments by CFU counting in the culture fluid pooled with the liquids used for successive washings (see Fig. 1).

**Intracellular antibiotic activity.** Antibiotics were added to infected cells at extracellular concentrations ranging from 0.01 to 200 mg/liter to obtain a complete description of the concentration-response curve, as described in detail in a previous publication (19). After appropriate incubation times (up to 24 h), cells were collected by centrifugation, resuspended in PBS, centrifuged again to eliminate extracellular bacteria, and collected in distilled water. Complete cell lysis was achieved by sonication (10 s), and lysates were used for determination of CFU counts (after plating on agar of appropriate dilutions) and of protein content by Lowry's assay with a commercially available detection kit (Bio-Rad DC Protein Assay, Bio-Rad laboratories, Hercules, CA). The activity was expressed as the change from the initial inoculum after 24 h of incubation.

**Assessment of cell viability.** The percentage of dead cells was assessed by trypan blue exclusion test, with unstained and stained cells counted by optical microscopy after 5 min of incubation with the dye at a final concentration of 0.5% in PBS.

**Morphological studies.** Infected cells were harvested, washed with PBS, fixed with 2% glutaraldehyde (30 min, 4°C) in 0.1 M sodium cacodylate buffer (pH 7.4), washed, postfixed for 1 h with 1% osmium tetroxide in cacodylate buffer in the dark, and then stained en bloc with uranyl acetate (29). Ultrathin sections (75 nm) were counterstained with uranyl acetate and lead citrate and observed in a Philips CM12 microscope.

**Antibiotics.** Amikacin (disulfate salt; potency, 73.6%), ticarcillin (disulfate salt; potency, 84.65%), piperacillin (sodium salt; potency, 94.2%), and colistin (sulfate salt; potency, 67.50%) were purchased from Sigma-Aldrich (St. Louis, MO). The following antibiotics were obtained as microbiological standards from their manufacturers: ciprofloxacin (chlorhydrate; potency, 85%) and moxifloxacin (chlorhydrate; potency, 91%) from Bayer AG, Wuppertal, Germany; tobramycin (potency, 100%) from SMB-GalePhar, Marche-en-Famenne, Belgium; and imipenem (potency, 100%) from Merck, Darmstadt, Germany. The following antibiotics were procured as commercial products registered in Belgium or France for parenteral use from their marketing authorization holders or resellers: gentamicin (Gentalline; potency, 100%) from MSD-France (Courbevoie, France), levofloxacin (Tavanic; potency, 95%) from Aventis (Romainville, France), piperacillin-tazobactam (Tazocin; potency, 97%) from Wyeth Pharmaceuticals (Ottignies-Louvain-la-Neuve, Belgium), cefepime (Maxipime; potency, 84.5%) and aztreonam (Azactam; potency, 95.4%) from Bristol-Myers Squibb Co. (Brussels, Belgium), ceftazidime (Glazidim; potency, 88.2%) from Glaxo-SmithKline (Genval, Belgium), meropenem (Merone; potency, 74.0%) from Astra Zeneca

(Brussels, Belgium), and doripenem (Doribax; potency, 95.7%) from Janssen-Cilag (Beerse, Belgium).

**Other reagents.** Unless stated otherwise, all other reagents were of analytical grade and were purchased from Sigma-Aldrich-Fluka. Cell culture or microbiology media were from Invitrogen (Paisley, Scotland) and Difco (Sparks, MD).

**Curve fittings and statistical analyses.** Curve fittings were performed with GraphPad Prism (version 4.02) software for Windows (GraphPad Prism Software, San Diego, CA), and statistical analyses were performed with GraphPad InStat 3 version 3.10 (GraphPad Prism Software).

## RESULTS

**Susceptibility testing.** Table 1 shows the MICs of a series of antibiotics currently used for the treatment of infections with *P. aeruginosa*, together with their maximal concentrations in serum ( $C_{\text{max}}$ ) of humans (total drug) as determined in patients receiving conventional dosages. MICs were measured in CA-MHB at pHs 7.4 and 5.5 to mimic the extracellular and phagolysosomal environments, respectively, and in RPMI 1640 supplemented with 10% fetal calf serum (i.e., the medium used in experiments with THP-1 cells). Acid pH decreased the potency of aminoglycosides (MICs increased by at least 2  $\log_2$  dilutions) but not that of the other antibiotics (maximum, 1-dilution increase). When measured in RPMI 1640, MICs were similar to or only 1 dilution higher than those observed in CA-MHB.

**Validation of the intracellular model.** In a first series of experiments, we examined in parallel (i) the level of internalization of PAO1 by THP-1 cells exposed for 1 or 2 h to increasing inocula and (ii) the extent of cell death induced by bacteria under these conditions (Fig. 1, left panel). Internalization of bacteria clearly increased with the inoculum and the time of incubation but was accompanied by a commensurate toxicity for the host cells. On the basis of these data, we selected a phagocytosis time of 2 h and an initial bacterium-to-cell ratio of 10, which allowed us to obtain reproducibly an intracellular inoculum of about  $7 \times 10^5$  CFU/mg cell protein with only 15% cell death. We then monitored, under these conditions, the apparent intracellular growth of bacteria for 24 h in the presence of low concentrations of gentamicin. This antibiotic was selected on the basis of the model developed previously for intracellular infection of THP-1 cells with *S. aureus* because it was shown to prevent extracellular contamination while at the same time only minimally impairing its intracellular growth (19). As shown in Fig. 1 (right panel), the increase in the number of cell-associated CFU at 24 h was almost as large as for bacteria grown in CA-MHB as long as the gentamicin concentrations were lower than the MIC measured in RPMI 1640. However, extracellular contamination was very important (leading to an overestimation of the cell-associated counts) and associated with a marked (>20%) loss of cell viability. When the extracellular gentamicin concentration was brought to 5 mg/liter (i.e., close to its MIC in RPMI 1640 [4 mg/liter]), extracellular contamination became negligible but bacteria still grew in cells (with a gain of about 1  $\log_{10}$  CFU over the 24-h incubation period) and with no apparent increase in cell toxicity (trypan blue assay). Any further increase in the extracellular gentamicin concentration caused complete impairment of intracellular growth, denoting the development of significant activity in cells beyond what was obtained against extracellular bacteria.

**Morphological studies.** Electron microscopy was used to examine the morphology of infected cells, as well as the subcellular localization of *P. aeruginosa* (Fig. 2). At the end of the phagocyto-

TABLE 1 MICs of antibiotics in CA-MHB at pHs 7.4 and 5.5 and in RPMI 1640 against *P. aeruginosa* PAO1 compared to the  $C_{\max}$ 

Antibiotic	Abbreviation	MIC (mg/liter)			Dosage <sup>a</sup>	Human $C_{\max}$ (mg/liter) <sup>b</sup>	Reference(s)
		CA-MHB		RPMI 1640			
		pH 7.4	pH 5.5	(pH 7.4)			
<b>Aminoglycosides</b>							
Gentamicin	GEN	2	8	4	3–5 mg/kg/day i.v.	18	37
Amikacin	AMK	4	64	4	7.5 mg/kg/day i.v.	38	37
Tobramycin	TOB	1	8	1	3–5 mg/kg/day i.v.	6	37
<b><math>\beta</math>-Lactams</b>							
Ticarcillin	TIC	32	64	32	2 g/3–4 days i.v.	200	37
Piperacillin	PIP	16	16	16	4 g/3–4 days i.v.	389	37
Piperacillin-tazobactam	TZP	16	16	16	4 g/3–4 days i.v.	224	37
Cefepime	CEP	4	8	4	2 g/2–3 days i.v.	160	37
Ceftazidime	CAZ	2	4	2	2 g/2–3 days i.v.	160	37
Aztreonam	ATM	8	16	8	2 g/3–4 days i.v.	210	37
Meropenem	MEM	1	1	2	1 g/2–3 days i.v.	57	37, 38
Imipenem	IMI	1	1	1	1 g/3–4 days i.v.	91	37, 38
Doripenem	DOR	0.5	1	1	1 g/3–4 days i.v.	73	37, 38
<b>Fluoroquinolones</b>							
Moxifloxacin	MXF	1	2	1	0.4 g/day p.o.-i.v.	4.3	37
Levofloxacin	LVX	1	2	1	0.5 g/day p.o.-i.v.	6.2	37
Ciprofloxacin	CIP	0.125	0.25	0.125	0.4 g/day i.v.	4.6	37
<b>Colistin (polymyxin)</b>							
Colistin (polymyxin)	CST	1	2	2	2.5–5 mg/kg/day i.v.	5	39

<sup>a</sup> Conventional doses for the treatment of *P. aeruginosa* infections. p.o., oral; i.v., intravenous.

<sup>b</sup>  $C_{\max}$ , commonly observed maximal concentration (total drug) in serum after intravenous or oral administration of these doses.

sis period (2 h), we observed bacteria located in vacuoles limited by a double membrane (suggesting a phagosomal nature) and close to the cell surface, where they started multiplying. After 24 h of incubation (in the presence of 5 mg/liter gentamicin in the extracellular medium), we noticed the presence of larger vacuoles hosting several bacteria.

**Kinetics and influence of concentration on antibacterial effects in the MIC-to- $C_{\max}$  range.** The activity of antibiotics used for the treatment of *P. aeruginosa* infections was first examined over time and at fixed concentrations (1× the MIC in broth and at

the human  $C_{\max}$ ) against both extracellular and intracellular bacteria with a panel of antibiotics covering the aminoglycoside, fluoroquinolone, and  $\beta$ -lactam classes and colistin. These data are illustrated for one representative antibiotic in each of these pharmacological classes (gentamicin, ciprofloxacin, meropenem) and for colistin in Fig. 3. With respect to extracellular activity, gentamicin and colistin caused a transient decrease in the inoculum at 5 h when used at an extracellular concentration corresponding to the MIC, and all antibiotics were eventually bacteriostatic at 24 h, as anticipated. When used at its human  $C_{\max}$  (see Table 1 for

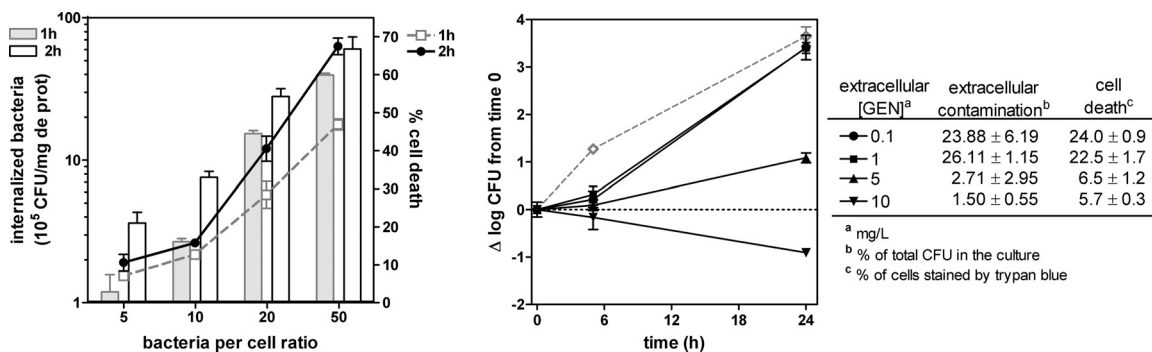


FIG 1 Setting up the intracellular model. (Left panel) Internalization of *P. aeruginosa* PAO1 after 1 (gray bars) or 2 (open bars with black border) h of phagocytosis of PAO1 at increasing bacterium-to-cell ratios (left axis) and percent mortality of THP-1 cells as assessed at the end of the phagocytosis period (right axis). All data are means ± standard deviations ( $n = 3$ ). (Right panel) Apparent intracellular growth of *P. aeruginosa* PAO1 in THP-1 cells incubated for 24 h with increasing extracellular concentrations of gentamicin compared to the extracellular growth (dotted gray line) measured in RPMI culture medium supplemented with 10% fetal calf serum in the absence of antibiotic. The graph shows the change in the number of CFU ( $\Delta \log$  CFU), from the initial, postphagocytosis inoculum achieved after 2 h of phagocytosis for an initial inoculum of 10 CFU/cell (approximately  $7 \times 10^5$  CFU/mg of protein). The table shows the percentages of contamination of the culture medium by *P. aeruginosa* and of cell death at the end of the experiment. GEN, gentamicin. All data are means ± standard deviations ( $n = 3$ ).

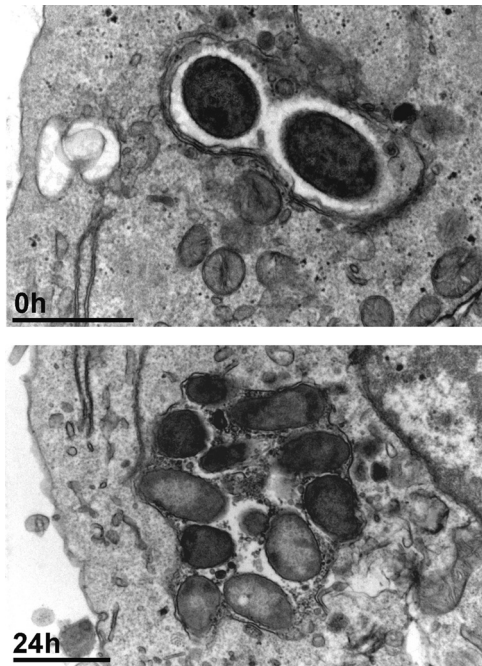


FIG 2 Electron microscopic appearance of THP-1 cells infected with *P. aeruginosa* PAO1. Infected cells were observed after 2 h of phagocytosis (corresponding to the postphagocytosis conditions) and after 24 h of incubation in the presence of gentamicin at an extracellular concentration of 5 mg/liter. Bars, 1  $\mu$ m.

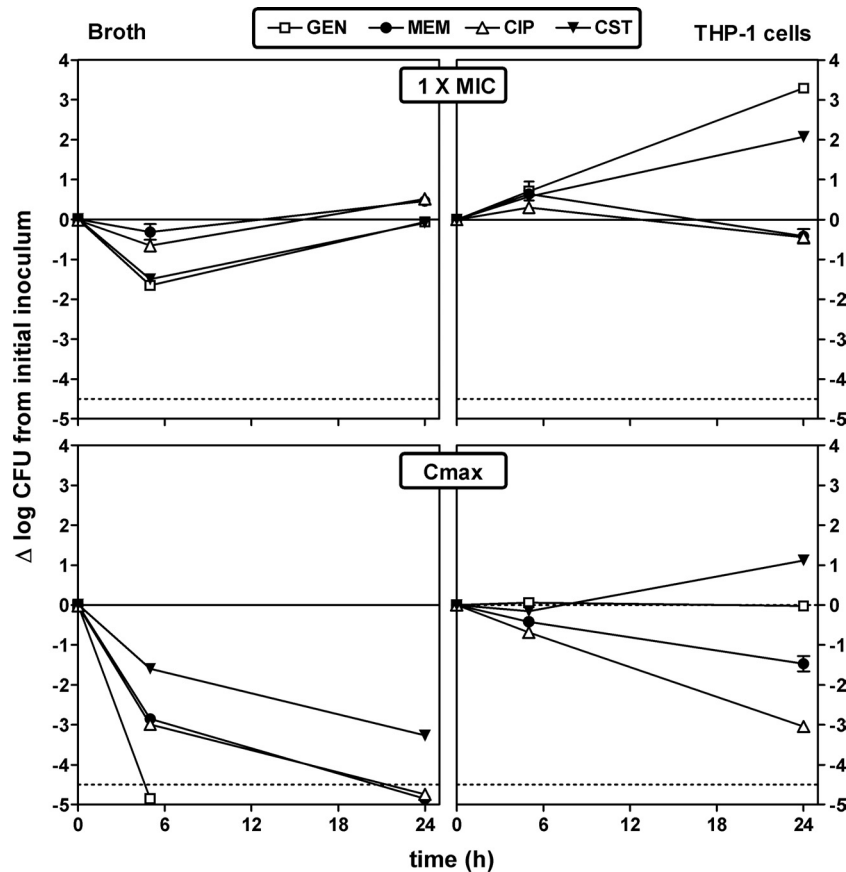
values), gentamicin was rapidly bactericidal toward extracellular bacteria, yielding a decrease in the CFU count corresponding to the limit of detection at 5 h. Ciprofloxacin and meropenem were also bactericidal against extracellular bacteria at 5 h (3  $\log_{10}$  CFU decrease), but colistin required 24 h of incubation to reach this threshold. With respect to intracellular activity, gentamicin and colistin did not prevent bacterial growth when used at extracellular concentrations corresponding to their MICs, while meropenem and ciprofloxacin reached a bacteriostatic effect after 24 h of incubation. At the human  $C_{max}$ , colistin still did not completely impair the intracellular growth of *P. aeruginosa*, gentamicin was bacteriostatic, and meropenem and ciprofloxacin caused 1.5- and 3- $\log_{10}$  CFU decreases at 24 h, respectively. Full data for the other antibiotics tested (amikacin, tobramycin, moxifloxacin, levofloxacin, piperacillin, piperacillin-tazobactam, ticarcillin, ceftazidime, cefepime, imipenem, doripenem, and aztreonam) are shown in Fig. S1 in the supplemental material together with those of the four antibiotics described above. Globally, all of the antibiotics were bacteriostatic at their MICs at 24 h against extracellular bacteria and bactericidal at their  $C_{max}$ , with the exception of cefepime. Together with gentamicin, amikacin was the only antibiotic capable of sterilizing the culture in 5 h. Against intracellular bacteria and at 24 h, aminoglycosides did not prevent intracellular growth when added to the culture medium at concentrations corresponding to their MICs in RPMI 1640 medium, whereas all  $\beta$ -lactams were bacteriostatic and fluoroquinolones showed variable effects. At extracellular concentrations corresponding to their  $C_{max}$ , aminoglycosides were almost bacteriostatic at 24 h,  $\beta$ -lactams reduced the inoculum by approximately 1  $\log_{10}$  CFU, but fluoroquinolones reduced the intracellular inoculum by close or equal to 3  $\log_{10}$  CFU compared to the postphagocytosis value.

**Concentration-effect relationships (pharmacological comparisons).** Because only minimal decreases in intracellular CFU counts were observed at 5 h, full pharmacological concentration-response studies (aimed at determining and comparing the apparent bacteriostatic concentrations [ $C_s$ ] and maximal relative efficacies [ $E_{max}$ ] of antibiotics against extracellular and intracellular bacteria) were made only after 24 h of incubation. Typical data are illustrated in Fig. 4 for the same four representative antibiotics. In all cases, monophasic sigmoidal functions (with a slope factor of 1) could be fitted to the experimental values and these were used for interpretation of the data. Against extracellular bacteria, an apparent bacteriostatic effect was observed for each antibiotic at concentrations close to the MIC and the  $E_{max}$  was always below the actual limit of detection (4.5- $\log_{10}$  decrease compared to the original inoculum).  $E_{max}$  values were considerably lower (less negative) against intracellular bacteria than against extracellular bacteria for all four drugs, reaching only a 1- to 1.5- $\log_{10}$  CFU decrease from the original, postphagocytosis inoculum for colistin, gentamicin, and meropenem and a 2.6- $\log_{10}$  CFU decrease for ciprofloxacin. The  $C_s$  of colistin, meropenem, and ciprofloxacin were close to the MICs (measured at pH 7.4), while that of gentamicin was about 10-fold higher. These studies were then extended to 12 other antibiotics, and the corresponding data (grouped by drugs of the same pharmacological class) are illustrated in Fig. 5 (for matching pharmacodynamic parameters for each individual antibiotic, see Table S1 in the supplemental material). Quite interestingly, all of the antibiotics within a pharmacological class showed similar activity profiles when the extracellular concentrations were expressed in multiples of the respective MICs (at pH 7.4). A single curve could thus be fitted to the whole set of data obtained with different antibiotics within that class (Table 2 contains the pharmacodynamic parameters calculated from these single-fit curves; see Tables S1 to S3 in supplemental material for the pharmacodynamic parameters of individual curves and detailed statistical analyses); only the aztreonam and amikacin intracellular  $E_{max}$  values were significantly different from those of their respective classes, and only the tobramycin  $EC_{50}$  (concentration yielding a response halfway between the  $E_{min}$  and  $E_{max}$  responses in concentration-dependent experiments) was significantly different from that of the other aminoglycosides. Two major observations could be made from these studies. First, the  $C_s$  values were similar for intracellular and extracellular bacteria with  $\beta$ -lactams and fluoroquinolones but were globally 10 times higher (lower potency) for aminoglycosides. Second, the  $E_{max}$  values for intracellular *P. aeruginosa* were lower (less negative) than those of their extracellular counterparts but to a variable extent. Thus, the  $E_{max}$  still reached about  $-2.9 \log_{10}$  CFU for fluoroquinolones but only about  $-1.2 \log_{10}$  CFU for all other antibiotics. The MICs for bacteria collected at the end of the experiment were measured and found to be unchanged from those for the infecting strain, indicating that we were not dealing with the mere selection of stable resistant subpopulations (data not shown).

## DISCUSSION

The present study has allowed the development of a well-defined 24-h model of intracellular infection of phagocytic cells with *P. aeruginosa* that could be used to document the response of intracellular *P. aeruginosa* to antibiotics on a pharmacodynamic basis.

Considering the model, optimization of the bacterium-to-cell ratio and the time of phagocytosis was critical in obtaining a re-



**FIG 3** Influence of time on the rate and extent of activity of antibiotics against extracellular (broth; left panels) and intracellular (THP-1 cells; right panels) *P. aeruginosa* PAO1 upon incubation at fixed extracellular concentrations corresponding to their MICs in broth (top) and to concentrations (total drug) corresponding to their  $C_{max}$  observed in humans after the administration of conventional doses (bottom). The ordinate shows the change in the number of CFU (log scale) per milliliter for extracellular bacteria or per milligram of cell protein for intracellular bacteria. The solid horizontal line corresponds to a bacteriostatic effect (no change from the initial inoculum), and the dotted horizontal line shows the limit of detection ( $-4.5$ -log CFU decrease). Values are means  $\pm$  standard deviations ( $n = 3$ ); when not visible, error bars are smaller than the symbols. GEN, gentamicin; MEM, meropenem; CIP, ciprofloxacin; CST, colistin.

producibile infection while at the same time avoiding extensive killing of the host cells. This is important for *P. aeruginosa* because it produces a variety of virulence factors that can affect host cell viability. In strain PAO1, pili, flagella, a type III secretion system, exotoxin A, and lipopolysaccharide are all known to contribute to virulence (30, 31). Because the expression of these factors is variable between strains and critically depends on their growth status, preliminary investigations such as those performed here will probably be necessary when expanding the model to other laboratory or clinical strains. Selection of the appropriate extracellular concentration of gentamicin to prevent extracellular contamination without impairing the intracellular growth of *P. aeruginosa* also proved to be critical. Our data suggest that a concentration close to the MIC for the strain (as determined in cell culture medium at pH 7.4) is the most appropriate, as previously established in a model of *S. aureus*-infected THP-1 cells (19), but we also see that both lower and higher concentrations may give rise to spurious results. Lastly, we show that *P. aeruginosa* is able to grow slowly within THP-1 monocytes, with a net population increase of about  $1 \log_{10}$  CFU over 24 h. However, it must be emphasized that intracellular growth seems to start only after a 5-h period following phagocytosis. In this context, few studies have monitored the long-term intracellular fate of *P. aeruginosa*, which makes com-

parisons of other models with ours quite difficult. However, prolonged intracellular persistence of *P. aeruginosa* has been demonstrated in airway epithelial cells (14) or in HeLa cervical cancer cells (8) and transient growth has been demonstrated in corneal epithelial cells (9). A switch from apparent intracellular persistence to a net increase in the number of intracellular bacteria is probably related to an equilibrium among intrinsic bacterial growth properties, expression of virulence factors, and the level of host defenses. The latter are notoriously poor in THP-1 cells (32). Electron microscopy suggests that intracellular *P. aeruginosa* remains confined in vacuoles in THP-1 cells for at least 24 h and can actively replicate therein. A vacuolar localization of *P. aeruginosa* has also been generally observed in epithelial cells (7, 13, 33), but the nature of the infected vacuoles has not yet been determined (31).

Moving now to the use of the model for the study of antibiotics, we show here that their general pharmacodynamic profile is globally comparable to that observed in the same host cells for other bacteria such as *S. aureus*, *L. monocytogenes*, and *L. pneumophila* (19, 20, 24–26). Thus, (i) all antibiotics clearly showed concentration-dependent effects following the general model described for drugs in interaction with their targets and described by the Hill-Langmuir sigmoid function, (ii) the relative intracellular  $E_{max}$  is

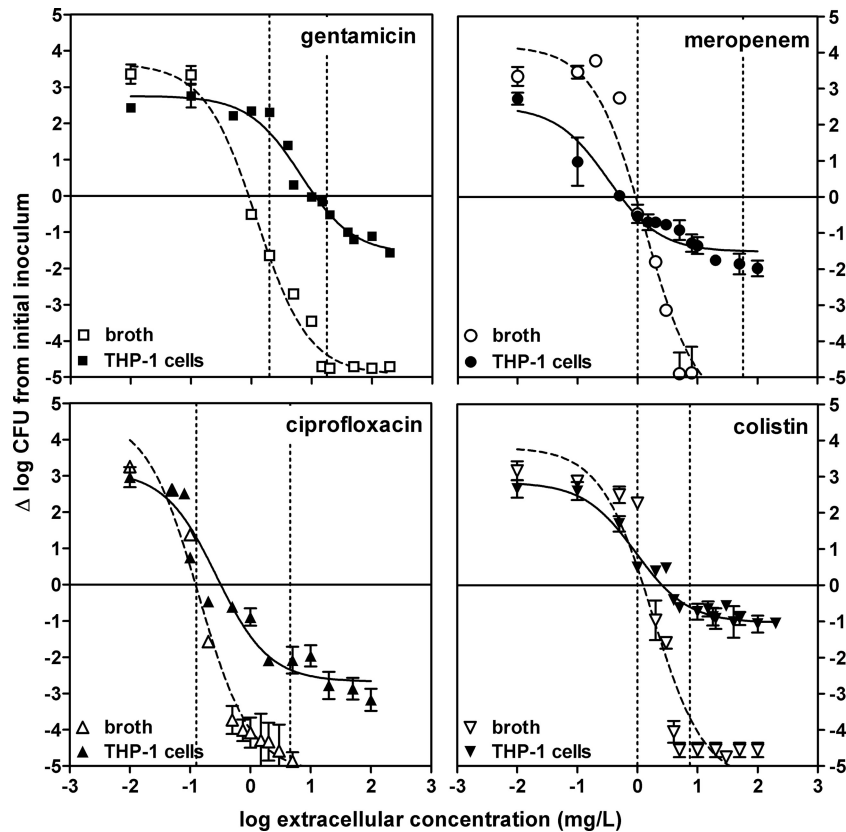


FIG 4 Concentration-response curves of selected antibiotics against extracellular and intracellular *P. aeruginosa*. The graphs show the changes in the number of CFU ( $\Delta \log \text{CFU}$  from the initial inoculum) per milliliter of broth (extracellular, open symbols, dotted lines) or per milligram of cell protein (intracellular, closed symbols, solid lines) in THP-1 cells after 24 h of incubation at increasing extracellular concentrations (expressed in milligrams per liter [total drug]). The solid horizontal line corresponds to a bacteriostatic effect (no change from the initial inoculum); the vertical dotted lines show the MIC-to- $C_{\text{max}}$  range limits. Data are means  $\pm$  standard deviations ( $n = 3$ ); when not visible, error bars are smaller than the symbols.

always considerably lower than that observed in broth, and (iii) the apparent  $C_s$  for intracellular bacteria is similar to the value for bacteria in broth, except that of aminoglycosides (which is markedly increased, denoting a corresponding loss of potency). Remarkably, all of the drugs within a given pharmacological class behave similarly when the bacterial response is expressed as a function of multiples of the respective MICs. This suggests that the intracellular activity of antibiotics depends on the intrinsic activity shown in broth (MIC), which will determine the  $C_s$  on the one hand, and on their pharmacological class, which will determine their relative  $E_{\text{max}}$  on the other hand. The latter parameter reflects the maximal killing capacity of a given antibiotic in a specific environment. It therefore takes into account not only the way the drug can express its activity in the infected compartment but also the bacterial responsiveness to that antibiotic and the cooperation with cell defense mechanisms to achieve killing (22). The marked reduction in maximal relative intracellular efficacy (compared to what is seen in broth) must therefore be interpreted as clear evidence that a significant part of the intracellular inoculum apparently becomes insensitive to the action of the antibiotic. This could be explained by the slow growth of bacteria when in the intracellular milieu or the ability of intracellular *P. aeruginosa* to switch to a less susceptible phenotype. While slow growth has been documented here, we know that *P. aeruginosa* may adopt a biofilm-like phenotype inside cells that makes it poorly responsive to

antibiotics (14). Both changes, however, must be reversible because no difference in the MIC was noticed when testing bacteria collected from cells (after phagocytosis and exposure of these cells to antibiotics for 24 h) in comparison with bacteria maintained in broth.

Our previous studies using the THP-1 monocyte model allow comparison of the pharmacodynamic properties of antibiotics against bacteria located in distinct subcellular compartments of the same host. Against an organism that thrives in vacuoles, such as *S. aureus*, the  $E_{\text{max}}$  values of aminoglycosides and  $\beta$ -lactams are of the same order of magnitude as those seen here (about  $-1$  to  $-1.5 \log_{10} \text{CFU}$ ). The values of fluoroquinolones seem species specific, varying from  $-2 \log_{10} \text{CFU}$  for *S. aureus* (25, 34, 35) to  $-2$  to  $-3 \log_{10} \text{CFU}$  for *L. pneumophila* (25) and  $-3 \log_{10} \text{CFU}$  for *P. aeruginosa*. Against an organism multiplying in the cytosol, such as *L. monocytogenes*, the  $E_{\text{max}}$  values of both  $\beta$ -lactams ( $-2 \log_{10} \text{CFU}$ ) and fluoroquinolones ( $-3$  to  $-4 \log_{10} \text{CFU}$ ) are higher (more negative) (24–26) than those we observed here and aminoglycosides are totally ineffective (36). Available data (reviewed in references 22 and 23) show that  $\beta$ -lactams and fluoroquinolones, although they are recovered mainly in the cytosol after cell fractionation studies, are probably capable of moving freely throughout all cell compartments. Conversely, aminoglycosides are always found associated with, and probably restricted to, the endocytic and lysosomal compartment of the cells. Thus, while

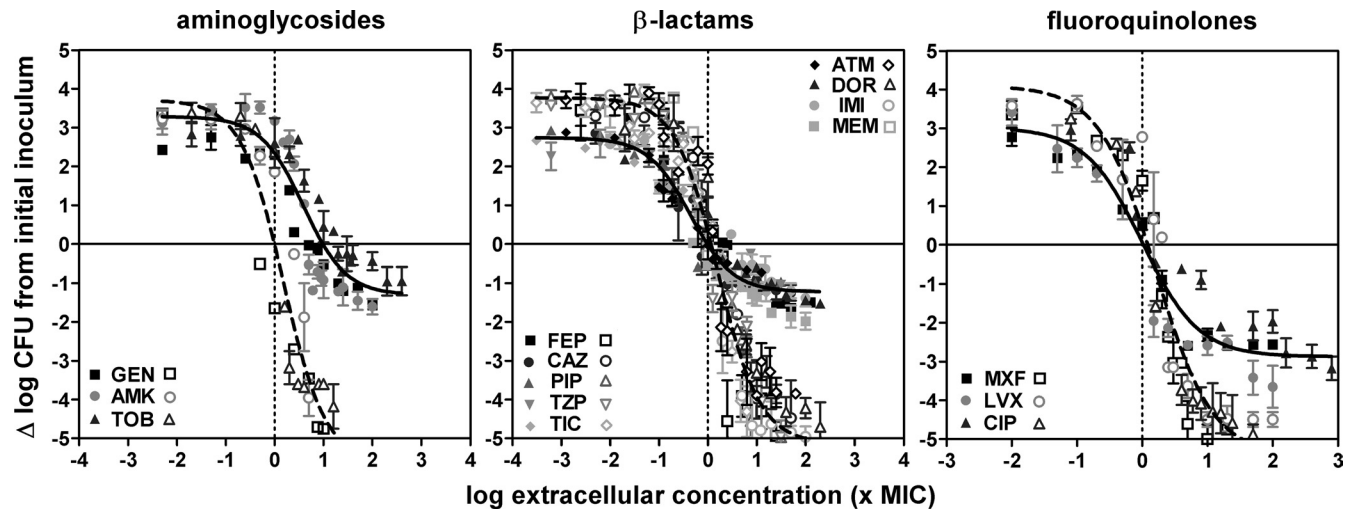


FIG 5 Concentration-response curves of antibiotics against extracellular and intracellular *P. aeruginosa*. The graphs show changes in the number of CFU ( $\Delta \log$  CFU from the initial inoculum) per milliliter of broth (extracellular; open symbols and dotted line) or per milligram of cell protein (intracellular; closed symbols and solid line) in THP-1 cells after 24 h of incubation at increasing extracellular concentrations (expressed in multiples of the MIC). The solid horizontal line corresponds to a bacteriostatic effect (no change from the initial inoculum); the vertical dotted line shows the MIC. Data are means  $\pm$  standard deviations ( $n = 3$ ); when not visible, error bars are smaller than the symbols. For definitions of drug name abbreviations, see Table 1.

$\beta$ -lactams, fluoroquinolones, and aminoglycosides can be effective against intravacuolar *P. aeruginosa* and *S. aureus* if their extracellular concentration is high enough (which is what the  $E_{\max}$  will document), only  $\beta$ -lactams and fluoroquinolones might be able to reach and act on cytosolic *L. monocytogenes*. Access to target only, however, does not explain why fluoroquinolones are more effective against intracellular *P. aeruginosa* than against intracellular *S. aureus* (when comparing these two bacteria in the same cell host) and more effective than  $\beta$ -lactams against *P. aeruginosa* in the model studied here. This suggests a specific role of bacterial responsiveness to fluoroquinolones that needs to be investigated further.

Previous studies with other bacteria showed that the relative potencies of drugs rely on their MICs on the one hand (intrinsic potency) and on their cellular concentrations on the other (for a review, see reference 23). We have selected the  $C_s$  as a more useful parameter than the  $EC_{50}$  commonly used to describe relative antibiotic potencies in pharmacological studies. This is because  $EC_{50}$

are critically dependent on the amplitude of the concentration-response curve, which in our case is entirely related to the  $E_{\max}$  (the  $E_{\min}$ , which describes bacterial growth in the absence of an antibiotic, is essentially similar for all of the groups studied here). Thus, a given  $EC_{50}$  may actually correspond to very different bacterial response levels (growth, an apparent bacteriostatic effect, or a decrease in the CFU count), depending on how much lower the  $E_{\max}$  value is than that of the initial inoculum, which makes it poorly informative in terms of an antibacterial effect. Conversely, the  $C_s$  for intracellular bacteria tends to be constant when comparing antibiotics (even if the  $E_{\max}$  values are different) and actually close to the MIC measured in broth or in RPMI 1640 medium for all antibiotics except aminoglycosides. While  $\beta$ -lactams reach intracellular concentrations close to the extracellular ones, fluoroquinolones accumulate to much higher concentrations in cells (22). The most plausible explanation for the fact that fluoroquinolones are not more potent than  $\beta$ -lactams against intracellular bacteria is that only a fraction of an accumulated fluoroquinolone

TABLE 2 Pertinent regression parameters of dose-response curves for extracellular (broth) and intracellular (THP-1 cells) activities of antibiotics against strain PAO1<sup>a</sup>

Antibiotic(s)	Mean extracellular activity (95% confidence interval)					Mean intracellular activity (95% confidence interval)				
	$E_{\min}^b$	$E_{\max}^c$	$EC_{50}^d$	$C_s^e$	$R^2$	$E_{\min}^b$	$E_{\max}^c$	$EC_{50}^d$	$C_s^e$	$R^2$
Aminoglycosides	3.80 (3.37–4.23)aA <sup>f</sup>	>–4.5A	1.77 (1.31–2.37)aA	1.18	0.90	3.34 (3.04–3.64)aA	–1.31 (–1.62 to –1.00)aB	4.22 (3.01–5.93)aA	10.77	0.83
$\beta$ -Lactams	3.77 (3.57–3.98)aA	>–4.5A	1.58 (1.37–1.83)aA	1.17	0.93	2.74 (2.60–2.88)bB	–1.23 (–1.34 to –1.11)aB	0.44 (0.36–0.53)bA	0.98	0.90
Fluoroquinolones	4.10 (3.57–4.63)aA	>–4.5A	1.61 (1.29–2.00)aA	1.21	0.94	3.02 (2.65–3.39)aB	–2.88 (–3.13 to –2.63)bB	1.00 (0.73–1.36)bA	1.04	0.91
Colistin	3.81 (2.75–4.87)aA	>–4.5A	1.78 (1.08–2.95)aA	1.25	0.92	2.84 (2.50–3.18)bB	–1.04 (–1.22 to –0.86)aB	0.94 (0.63–1.40)bA	2.52	0.92

<sup>a</sup> Cells were incubated for 24 h with extracellular drug concentrations ranging from 0.01 to 200 mg/liter. A single curve was fitted to the data obtained for different antipseudomonal antibiotics within a single class. The corresponding data are shown in Fig. 5; for the parameters for the individual fits, see Table S1 in the supplemental material.

<sup>b</sup> CFU count increase (in  $\log_{10}$  units) at 24 h from the corresponding initial inoculum as extrapolated for an infinitely low antibiotic concentration.

<sup>c</sup> CFU count decrease (in  $\log_{10}$  units) at 24 h from the corresponding initial inoculum as extrapolated from an infinitely high antibiotic concentration.

<sup>d</sup> Extracellular concentration (total drug; in multiples of the MIC) causing a reduction halfway between  $E_{\min}$  and  $E_{\max}$ , as calculated from the Hill equation of the concentration-response curve (slope factor of 1).

<sup>e</sup>  $C_s$ , i.e., the extracellular concentration (total drug; in multiples of the MIC) resulting in no apparent bacterial growth (number of CFU identical to the initial inoculum), as calculated from the Hill equation of the concentration-response curve.

<sup>f</sup> Statistical analysis per column was done by one-way analysis of variance with Tukey's test for multiple comparisons of each parameter for all classes. Values followed by different lowercase letters are significantly different from each other ( $P < 0.05$ ). Statistical analysis per row was done by unpaired, two-tailed  $t$  test between corresponding parameters of extracellular and intracellular activities. Values followed by different uppercase letters are significantly different from each other ( $P < 0.05$ ).



expresses its activity at the site of action, as already concluded from studies assessing the susceptibility of intracellular *S. aureus* and *L. monocytogenes* to fluoroquinolones with distinct levels of accumulation (see reference 26 and the references cited therein). Colistin accumulation and subcellular distribution are still unknown, making any discussion premature in this context. For aminoglycosides, the marked difference in  $C_s$  for intracellular bacteria from the MIC measured in broth at neutral pH can be understood if considering that both the drug and the target could be located in an acidic environment (lysosomes and phagolysosomes on the basis of the data from our electron microscopic studies). The  $C_s$  of aminoglycosides for intracellular bacteria are actually close to their MICs if measured at pH 5.5. Data in the literature, however, suggest that the vacuoles in which *P. aeruginosa* survives and multiplies may be distinct from lysosomes (and therefore not as acidic), at least in epithelial cells (33). Further studies aimed at identifying the real nature of the vacuoles identified here and at determining their actual pH would be useful in this context.

The present study used only one reference strain of *P. aeruginosa* (ATCC PAO1), which may be considered a major limitation to the application of our conclusions to clinical situations. The choice of a unique, well-characterized strain was actually essential for the development and validation of the model, as well as for the correct comparative assessment of the intrinsic intracellular activity of each antibiotic class. Many resistance mechanisms and virulence factors vary in their level of expression in clinical strains, depending on the environment. This would have created much uncertainty in what is primarily a pharmacological study. Focusing on this aspect, our data show a clear superiority of fluoroquinolones over  $\beta$ -lactams and aminoglycosides when dealing with intracellular forms of *P. aeruginosa*. They also highlight the poor efficacy of colistin against these forms. Within the limitations inherent to all *in vitro* experiments, including those that are specific to the model used and that are discussed in details in our previous publications (19, 20, 24), our studies may represent a first step in the rationalization of current antibiotic choices and in the better assessment of new molecules. Further studies examining key clinical strains and other host cells but based on a similar pharmacodynamic approach may eventually further contribute to limitation of the risk of therapeutic failures related to persistence and/or recurrence of pseudomonal infections.

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**Table S1. Pertinent regression parameters of dose-response curves for intracellular and extracellular activity of antibiotics against PAO1 strain <sup>a</sup>.**

Antibiotic	Extracellular activity <sup>c</sup>					Intracellular activity <sup>a</sup>				
	$E_{min}^d$	$E_{max}^d$	$EC_{50}^e$	$C_s^f$	$R^2$	$E_{min}^d$	$E_{max}^d$	$EC_{50}^e$	$C_s^f$	$R^2$
GEN	3.66 (3.20 to 4.11)	>-4.5	0.63 (0.47 to 0.84)	0.46	0.98	2.76 (2.44 to 3.08)	-1.59 (-1.96 to -1.23)	3.30 (2.18 to 4.99)	5.70	0.96
AMK	3.70 (3.01 to 4.39)	>-4.5	1.20 (0.79 to 1.82)	0.69	0.94	3.78 (3.33 to 4.22)	-2.17 (-2.79 to -1.55)	2.04 (1.32 to 3.14)	3.53	0.89
TOB	4.28 (2.76 to 5.81)	>-4.5	0.69 (0.42 to 1.14)	0.55	0.90	2.99 (2.63 to 3.36)	-1.02 (-1.38 to -0.65)	3.64 (2.26 to 5.86)	10.69	0.88
Aminoglycosides <sup>g</sup>	3.83 (3.37 to 4.28)	>-4.5	0.74 (0.57 to 0.98)	0.56	0.93	3.34 (3.04 to 3.64)	-1.31 (-1.62 to -1.00)	2.12 (1.51 to 2.98)	5.40	0.84
MXF	4.04 (3.21 to 4.87)	>-4.5	1.97 (1.40 to 2.77)	1.28	0.94	2.75 (2.49 to 3.01)	-2.87 (-3.11 to -2.64)	1.10 (0.85 to 1.42)	1.05	0.98
LVX	3.94 (3.00 to 4.88)	>-4.5	1.82 (1.11 to 2.98)	1.37	0.90	3.45 (2.86 to 4.04)	-3.22 (-3.58 to -2.88)	0.49 (0.30 to 0.79)	0.48	0.95
CIP	4.66 (3.49 to 5.82)	>-4.5	1.09 (0.74 to 1.60)	0.98	0.93	3.15 (2.39 to 3.90)	-2.66 (-3.02 to -2.29)	2.15 (1.22 to 3.77)	2.55	0.92
Fluoroquinolones <sup>g</sup>	4.10 (3.57 to 4.63)	>-4.5	1.61 (1.29 to 2.00)	1.21	0.94	3.02 (2.65 to 3.39)	-2.88 (-3.13 to -2.63)	1.00 (0.73 to 1.36)	1.04	0.91
TIC	3.24 (3.01 to 3.47)	>-4.5	0.54 (0.33 to 0.75)	1.27	0.97	2.53 (2.33 to 2.73)	-1.44 (-1.71 to -1.17)	0.59 (0.43 to 0.82)	1.04	0.96
PIP	3.87 (3.46 to 4.27)	>-4.5	1.94 (1.37 to 2.76)	1.29	0.97	2.96 (2.64 to 3.28)	-1.13 (-1.41 to -0.85)	0.26 (0.17 to 0.40)	0.68	0.96
TZP	3.69 (3.22 to 4.15)	>-4.5	1.85 (1.22 to 2.86)	1.36	0.94	2.50 (2.27 to 2.74)	-0.91 (-1.22 to -0.61)	0.56 (0.33 to 0.95)	1.54	0.95
FEP	3.97 (3.15 to 4.79)	>-4.5	1.29 (0.81 to 2.04)	0.93	0.92	3.14 (2.62 to 3.66)	-1.52 (-1.93 to -1.11)	0.75 (0.42 to 1.34)	1.56	0.93
CAZ	3.51 (3.01 to 4.00)	>-4.5	1.97 (1.40 to 2.78)	1.45	0.96	3.13 (2.56 to 3.69)	-1.33 (-1.67 to -1.00)	0.31 (0.19 to 0.53)	0.74	0.85
ATM	3.87 (3.43 to 4.31)	-4.36 (-5.02 to -3.69)	1.67 (1.18 to 2.36)	1.48	0.92	2.92 (2.62 to 3.22)	-0.75 (-0.99 to -0.52)	0.20 (0.14 to 0.30)	0.79	0.96
MER	4.62 (3.17 to 6.06)	>-4.5	1.02 (0.55 to 1.89)	0.74	0.92	2.65 (2.17 to 3.13)	-1.57 (-1.80 to -1.34)	0.36 (0.23 to 0.57)	0.61	0.94
IMI	4.23 (3.62 to 4.85)	>-4.5	0.77 (0.47 to 1.25)	0.63	0.97	2.80 (2.42 to 3.19)	-1.07 (-1.33 to -0.81)	0.74 (0.47 to 1.17)	1.95	0.92
DOR	3.51 (2.77 to 4.26)	>-4.5	1.98 (1.34 to 2.94)	1.51	0.93	2.11 (1.72 to 2.51)	-1.33 (-1.60 to -1.08)	1.21 (0.74 to 1.98)	1.91	0.95
$\beta$ -lactams <sup>g</sup>	3.77 (3.57 to 3.98)	>-4.5	1.58 (1.37 to 1.83)	1.16	0.93	2.74 (2.60 to 2.88)	-1.23 (-1.34 to -1.11)	0.44 (0.36 to 0.53)	0.98	0.90
CST	3.81 (2.75 to 4.87)	> -4.5	1.78 (1.08 to 2.95)	1.25	0.92	2.84 (2.50 to 3.18)	-1.04 (-1.22 to -0.86)	0.94 (0.63 to 1.40)	2.52	0.92

<sup>a</sup> Data are based on a 24-h post-phagocytosis exposure. Regression parameters were calculated using all data from antibiotic concentrations of  $10^{-2}$  to  $10^3$  to times the MIC. The parameters described in the table were derived from analysis of the curve shown in Fig 5. See Table S2 and 3 for statistical analyses

<sup>b</sup> Determined in THP-1 macrophages with an initial phagocytosed inoculum of about  $10^6$  CFU/mg of protein.

<sup>d</sup> Log CFU decrease at 24 h from the corresponding original inoculum, as extrapolated for an infinitely large antibiotic concentration. Samples yielding less than  $-5$  log CFU were considered below the detection level.

<sup>e</sup> extracellular concentrations (total drug; in multiples of the MIC) causing a reduction of the inoculum halfway between the initial ( $E_0$ ) and the maximal ( $E_{max}$ ) values, as obtained from the Hill equation.

<sup>f</sup> Static concentration, i.e., the extracellular concentration (total drug; in multiples of the MIC) resulting in no apparent bacterial growth (number of CFU identical to the initial inoculum), as calculated from the Hill equation of the concentration-response curve.

<sup>g</sup> reproduced from Table 2; data from all antibiotics within the class were used to fit a single curve

**Table S2: statistical analyses of EC<sub>50</sub> presented in Table S1**

Black and dark gray cells compare parameters for extracellular and intracellular activity of individual antibiotics (black) or classes as a whole (dark grey) (\*\*\*,  $p < 0.01$ ; NS, not significant).

Colored cells compare antibiotics within a same class (orange: aminoglycosides; green:  $\beta$ -lactams; blue: fluoroquinolones) or classes as whole among them (yellow). Light and dark colors correspond to statistical analysis for extracellular and intracellular activities, respectively (\*:  $p < 0.05$ ; NS: not significant).

EC <sub>50</sub>		Antibiotics																		
		AMIN.	GEN	AMK	TOB	Blact	TIC	PIP	TZP	FEP	CAZ	ATM	MEM	IMI	DOR	FQ	LVX	MOX	CIP	CST
Antibiotics	AMIN.	***	NS	NS	*	*										*				*
	GEN	NS	***	NS	NS															
	AMK	NS	*	NS	NS															
	TOB	NS	NS	*	***															
	Blact	NS				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS				NS
	TIC					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
	PIP					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
	TZP					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
	FEP					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
	CAZ					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
	ATM					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
	MEM					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
	IMI					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
	DOR					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS					
	FQ	NS				NS										NS	NS	NS	NS	NS
	LVX															NS	NS	NS	NS	
	MOX															NS	NS	NS	NS	
	CIP															NS	NS	NS	NS	
	CST	NS				NS										NS				NS

**Table S3: statistical analyses of E<sub>max</sub> presented in Table S1**

Black and dark gray cells compare parameters for extracellular and intracellular activity of individual antibiotics (black) or classes as a whole (dark grey) (\*\*\*, p< 0.01; NS, not significant).

Colored cells compare antibiotics within a same class (orange: aminoglycosides; green: β-lactams; blue: fluoroquinolones) or classes as whole among them (yellow). Light and dark colors correspond to statistical analysis for extracellular and intracellular activities, respectively (\*: p<0.05; NS: not significant; NA: not applicable [because E<sub>max</sub> > detection limit]).

E <sub>max</sub>		Antibiotics																			
		AMIN.	GEN	AMK	TOB	Blact	TIC	PIP	TZP	FEP	CAZ	ATM	MEM	IMI	DOR	FQ	LVX	MOX	CIP	CST	
Antibiotics	AMIN.	***	NS	*	NS	NS										*				NS	
	GEN	NA	***	NS	*																
	AMK	NA	NA	***	NS																
	TOB	NA	NA	NA	***																
	Blact	NA				***	NS	NS	NS	NS	NS	*	NS	NS	NS	*					NS
	TIC					NA	***	NS	*	NS	NS	*	NS	NS	NS						
	PIP					NA	NA	***	NS	NS	NS	*	NS	NS							
	TZP					NA	NA	NA	***	*	*	NS	*	NS	*						
	FEP					NA	NA	NA	NA	***	NS	*	NS	*	NS						
	CAZ					NA	NA	NA	NA	NA	***	*	NS	NS	NS						
	ATM					NA	NA	NA	NA	NA	NA	***	*	NS	*						
	MEM					NA	NA	NA	NA	NA	NA	NA	***	NS	NS						
	IMI					NA	NA	NA	NA	NA	NA	NA	NA	***	NS						
	DOR					NA	NA	NA	NA	NA	NA	NA	NA	NA	***						
	FQ	NA				NA										***	NS	NS	NS	*	
	LVX															NA	***	NS	*		
	MOX															NA	NA	***	NS		
	CIP															NA	NA	NA	***		
	CST	NA				NA										NA					***

**Figure S1.** Influence of the time on the rate and extent of activity of antibiotics against extracellular and intracellular *P. aeruginosa* PAO1 upon incubation at a fixed extracellular concentration corresponding to their MIC in broth (open symbols and dotted lines) and to their maximal concentration (total drug) observed in humans after administration of conventional doses ( $C_{max}$ ; closed symbols and plain lines). The ordinate shows the change in the number of CFU (log scale) per mL for extracellular bacteria or per mg of cell protein for intracellular bacteria. The plain horizontal line corresponds to a bacteriostatic effect (no change from initial inoculum) and the dotted horizontal line shows the limit of detection ( $-4.5 \log_{10}$  CFU decrease). Values are means  $\pm$  standard deviations ( $n = 3$ ); when not visible, error bars are smaller than the symbols.

