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Modulation of the Cellular Accumulation and Intracellular Activity of Daptomycin towards Phagocytized *Staphylococcus aureus* by the P-Glycoprotein (MDR1) Efflux Transporter in Human THP-1 Macrophages and Madin-Darby Canine Kidney Cells[▽]

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P-glycoprotein (P-gp; MDR1), a major efflux transporter, recognizes various antibiotics and is present in macrophages. We have examined its effect on the modulation of the intracellular accumulation and activity of daptomycin towards phagocytized *Staphylococcus aureus* (ATCC 25923) in human THP-1 macrophages, in comparison with MDCK epithelial cells (wild type and MDCK-MDR1 overexpressing P-gp; the bulk of the protein was immunodetected at the surface of all three cell types). Daptomycin displayed concentration-dependent intracellular activity (Hill equation pattern) in THP-1 and MDCK cells with (i) 50% effective drug extracellular concentration (EC_{50}; relative potency) and static concentrations at 9 to 10 times the MIC and (ii) maximal efficacy (E_{max}; CFU decrease at infinite extracellular drug concentration) at 1.6 to 2 log compared to that of the postphagocytosis inoculum. Verapamil (100 μM) and elacridar (GF 120918; 0.5 μM), two known inhibitors of P-gp, decreased daptomycin EC_{50} (about threefold) in THP-1 and MDCK cells without affecting E_{max}. Daptomycin EC_{50} was about three- to fourfold higher and accumulation in MDCK-MDR1 commensurately lower than in wild-type cells. In THP-1 macrophages, (i) verapamil and ATP depletion increased, and ouabain (an inducer of *mdr1* [the gene encoding P-gp] expression) decreased the accumulation of daptomycin in parallel with that of $DiOC_2$ (a known substrate of P-gp); (ii) silencing *mdr1* with duplex human *mdr1* siRNAs reduced the cell content in immunoreactive P-gp to 15 to 30% of controls and caused an eight- to 13-fold increase in daptomycin accumulation. We conclude that daptomycin is subject to efflux from THP-1 macrophages and MDCK cells by P-gp, which reduces its intracellular activity against phagocytized *S. aureus*.

Active efflux of drugs from eukaryotic cells is now recognized as a major determinant in the modulation of their pharmacokinetic properties in relation to absorption, distribution, and excretion, and needs, therefore, to be fully taken into account in the assessment of their activity and toxicity (20, 27, 40, 49, 66). In this context, P-glycoprotein (P-gp), one of the main members of the superfamily of the ATP-binding cassette (ABC) efflux proteins, has been shown to recognize antibiotics (59, 61), and could therefore modulate their intracellular activity. Thus, P-gp-mediated efflux was found to partially defeat the cellular accumulation and to reduce the activity of azithromycin towards *Staphylococcus aureus* phagocytized by murine J774 macrophages (46, 47).

In the present study, we have examined and assessed the role of P-gp in the modulation of the intracellular accumulation and activity of daptomycin in human THP-1 macrophages. To better ascertain the specificity of the effects seen to the activity of P-gp, we also used Madin-Darby canine kidney (MDCK) cells, for which stable lines overexpressing P-gp are available (12). Originally described in the mid-1980s as LY 146032 (11), daptomycin has become the lead member of the new class of the so-called acidic lipopeptide antibiotics. These are characterized by a membrane-related mode(s) of action (53), which results in a marked bactericidal activity against multiresistant gram-positive organisms, including methicillin-resistant *S. aureus* (14, 23). Intracellular survival of *S. aureus* is often considered as an important determinant in the relapsing and recurrent character of staphylococcal infections (see references 13 and 31 for a discussion). Yet, the intracellular uptake and activity of daptomycin have been little explored so far (63). We show here that daptomycin exerts concentration-dependent activity against intracellular *S. aureus* in the two types of cells studied but that its accumulation, and thereby its activity, is partially defeated by efflux through P-gp.

MATERIALS AND METHODS

Materials. Daptomycin was obtained as laboratory samples for microbiological evaluation from Novartis Pharma AG, Basel, Switzerland. Verapamil, ouabain, gemfibrozil, and 3-ethyl-2-[5-(3-ethyl-2(3H)-benzoxazolylidene)-1,3-pentadienyl]-iodide ($DiOC_2$) were from Sigma-Aldrich (St. Louis, MO). Elacridar (GF120918) was the generous gift of GlaxoWellcome Research and Development (Laboratoire Glaxo Wellcome, Les Ulis, France). Unless stated otherwise, cell culture media and sera were from Invitrogen, antibodies from Sigma-Aldrich, and other products from Merck KGaA (Darmstadt, Germany).

Bacterial strain and susceptibility testing. *S. aureus* ATCC 25923 (methicillin sensitive) was used throughout our experiments. Daptomycin MIC, as determined by microdilution in Mueller-Hinton broth supplemented with 50 mg/liter $CaCl_2$ (15), was 0.125 mg/liter (determinations made in triplicate; value checked for consistency throughout our experiments).

Cell cultures and assessment of cell viability. Experiments were performed with (i) THP-1 cells (ATCC TIB-202), a human myelomonocytic cell line displaying macrophage-like activity (57) and obtained from the American Tissue Collection (LGC Promochem Ltd., Teddington, United Kingdom), and (ii) MDCK wild-type cells (29) and MDCK cells transfected with human *mdr1*

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(MDCK-MDR1) (12), both obtained from P. Borst (The Netherlands Cancer Institute, Division of Molecular Biology and Centre of Biomedical Genetics, Amsterdam, The Netherlands). Maintenance of cell viability in the presence of daptomycin and P-gp inhibitors was assessed by trypan blue exclusion test (validated against the accepted method of measuring lactate dehydrogenase release used in our previous studies) (4) with CSA-13, a membrane-damaging antibiotic (25). Verapamil was dissolved in water, and gemfibrozil and elacridar were dissolved in dimethyl sulfoxide (DMSO; we checked that the concentrations of DMSO brought in the final culture medium [0.5%] did not interfere with the cell accumulation of daptomycin).

Cell infection and assessment of intracellular activities of daptomycin. Infection and assessment of intracellular activity in THP-1 cells were performed exactly as previously described (26). Infection of MDCK and MDCK-MDR1 cells was performed following the protocol previously described for J774 macrophages (45), except that (i) phagocytosis was allowed to take place for 2 h with an inoculum of 10^7 bacteria/ml, and (ii) nonphagocytized bacteria were removed by extensive washing only (to avoid using gentamicin, which caused a marked loss of cell viability if used immediately after phagocytosis). For both cell types, the postphagocytosis inoculum comprised between 1.8 and 2.7×10^6 CFU per mg of cell protein, a value close to what was obtained in our previous studies (3, 26). Intracellular activity was assessed as previously described for J774 cells.

Assay of cell-associated daptomycin and $DiOC_2$ and calculation of apparent cellular-to-extracellular concentration ratios. Daptomycin and $DiOC_2$ were assayed by fluorimetry, according to previously described procedures (51, 54), with the following modifications and performance characteristics. For daptomycin, excitation and emission wavelengths were set at 380 and 425 nm, and linearity was obtained between 1 and 250 mg/liter ($R^2 > 0.99$). For $DiOC_2$, excitation and emission wavelengths were set at 485 nm and 620 nm, and linearity was obtained between 0.05 and 10 mg/liter ($R^2 > 0.99$). We checked in pilot experiments that verapamil, gemfibrozil, and ouabain did not interfere with this assay. In contrast, elacridar intrinsic fluorescence made it impossible to assay daptomycin in its presence. Cell protein was assayed in parallel using the Folin-Ciocalteu/biuret method (30). The cell-associated contents in daptomycin and $DiOC_2$ were expressed by reference to the total cell protein content and converted into apparent concentrations using a conversion factor of 5 μl per mg of cell protein as commonly used for cultured cells. The level of accumulation of each compound was then expressed as the ratio of this apparent cell concentration to the corresponding extracellular concentration.

Measurements of free cell Ca^{2+} concentrations. Calcium-sensor dye Fluo-3 (19, 36), under its acetoxymethyl ester form (Fluo-3-AM; Invitrogen), was dissolved in DMSO with the nonionic detergent Pluronic F-127. THP-1 macrophages were harvested, suspended in Ca^{2+}-free Hanks balanced salt solution supplemented with 5 g/liter bovine serum albumin, and exposed for 30 min at 25°C to 5 μM Fluo-3-AM in the same medium supplemented with 1 mM probenecid (to reduce cell efflux of Fluo-3 after its intracellular deesterification). Cells were thereafter washed in Ca^{2+}-free Hanks balanced salt solution-bovine serum albumin-probenecid medium, incubated for 30 min at 25°C in the same medium, and thereafter incubated without (controls) or with either 100 μM verapamil or 250 μM gemfibrozil or ionomycin (50 μM; a Ca^{2+} ionophore [6]). Whole-cell fluorescence was then read continuously for up to 60 min at room temperature (λ_{exc}, 485 nm; λ_{em}, 530 nm) using a Packard FluoroCount microplate reader instrument (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA).

Modulation of P-gp expression by siRNA (gene silencing). THP-1 macrophages (10^7 cells) were transfected with 0.75 μg of duplex human *mdr1* small interfering RNA (siRNA) (Santa-Cruz Biotechnology, Heidelberg, Germany). We used a Nucleofector II electroporation apparatus (Amaxa Biosystems, Cologne, Germany), following the general procedure described for THP-1 cells (program V-001), with cells suspended in Royal Park Memorial Institute (RPMI) 1640 culture medium supplemented with 10% fetal bovine serum and either (i) 1.25% DMSO (medium a) (32) or (ii) 25 mM HEPES, pH 7.4 (medium b) (43). We checked that both media allowed for an efficient penetration of the nonpermeant dye trypan blue in cells when added to the electroporation buffer (83.4 ± 2.6 of cells stained versus 1.1 ± 0.1 for controls [no electroporation] with medium a; 87.7 ± 0.2 versus 3.4 ± 1.32 with medium b). Cells returned to culture medium and reincubated for 1 h after electroporation before being again exposed to trypan blue excluded the dye for >80%, indicating a satisfactory cell membrane resealing and maintenance of viability.

Western blotting. For analysis of P-gp expression, cells (~10^7) were washed three times with ice-cold phosphate-buffered saline (pH 7.4) and suspended in 500 μl of lysis buffer (64). The samples were then subjected to three successive cycles of freezing-thawing (−80°C for 5 min and 37°C for 5 min), sonicated (for three cycles of 5 s with 30-s intervals at 4°C), and finally centrifuged for 10 min at 14,000 rpm and at 4°C in an Eppendorf 5415C microcentrifuge (Eppendorf AG, Hamburg, Germany) equipped with the standard rotor F-45-24-11. Protein concentration of the supernatant was determined by a modified Bradford's procedure (39) using the Quick Start Bradford protein assay kit 3 from Bio-Rad Laboratories (Hercules, CA). Western blotting was performed following the procedure described previously (34) with a mouse monoclonal anti-P-gp (MDR) antibody (1/500) as primary antibody (Sigma-Aldrich catalog no. P7965; according to its supplier, this antibody detects specifically human MDR1 P-gp but not MDR3) and polyclonal horseradish peroxidase-labeled anti-mouse (1/1,000) as secondary antibody (equiloading of the gels was controlled with monoclonal mouse anti-β-actin antibody [Sigma-Aldrich catalog no. A2228; 1/2,000]). Bands were revealed with the SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL). Films were scanned and subjected to a densitometric analysis using Image J software (version 1.3.1; available from the Research Service branch of the National Institute of Mental Health at http://rsb.info.nih.gov/ij).

Confocal microscopy. P-gp was detected on formalin-fixed and saponin-permeabilized cells using the same mouse monoclonal anti-P-gp as for Western blotting and fluorescein isothiocyanate-labeled goat anti-mouse antibodies (Santa Cruz Biotechnology), with counterstaining for actin with 5 units of rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA), following the general techniques previously described for J774 macrophages (48, 58) with minor adaptations. Observations were made with MRC1024 confocal scanning equipment (Bio-Rad, Richmond, CA) mounted on an Axiovert confocal microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analyses. Curve-fitting analyses were made using GraphPad Prism version 4.02 for Windows (GraphPad Prism Software, San Diego, CA). Analysis of variance was made with GraphPad Instat version 3.06 (GraphPad Prism Software), and analysis of covariance with XLStat version 7.5.2 (Addinsoft SARL, Paris, France).

RESULTS

Intracellular activity of daptomycin in THP-1 macrophages and modulation by efflux transporter inhibitors. In the absence of published detailed analysis of the intracellular activity of daptomycin in macrophages, we first conducted 24-h dose-response studies using a wide range of extracellular concentrations to obtain information on key pharmacological descriptors (50% effective concentration [EC_{50}]; static concentration [C_{static}]; maximal efficacy [E_{max}]). We also used our previous studies (4, 26), which were necessary for the correct interpretation of the present data. Results are shown in Fig. 1A, with the pertinent regression parameters presented in Table 1. Daptomycin displayed clear-cut concentration-dependent activity, with EC_{50} and C_{static} values close to each other at about 9 to 10 times the MIC and an E_{max} value at about −1.6 log CFU. We then selected a daptomycin extracellular concentration of 1 mg/liter (i.e., close to the EC_{50} and C_{static} values) to test for the influence on daptomycin activity of two well-known P-gp inhibitors, namely, the Ca^{2+} channel antagonist verapamil, which is largely nonspecific, and elacridar, a much more specific inhibitor (16). Results of experiments conducted at fixed concentrations (verapamil, 100 μM; elacridar, 0.5 μM) over 5 and 24 h are shown in Fig. 1B. Both inhibitors markedly enhanced the intraphagocytic activity of daptomycin, with an effect already detectable at 5 h only. This was not due to a direct action of verapamil or elacridar on bacteria, since the presence of these inhibitors alone did not markedly affect the intraphagocytic growth of *S. aureus* (they did not affect the MIC of daptomycin, either [data not shown]). The influence of verapamil and elacridar on the activity of daptomycin was then studied in more detail by running complete dose-response studies over a 24-h period. In the first series of experiments, verapamil or elacridar was added at a fixed concentration (100

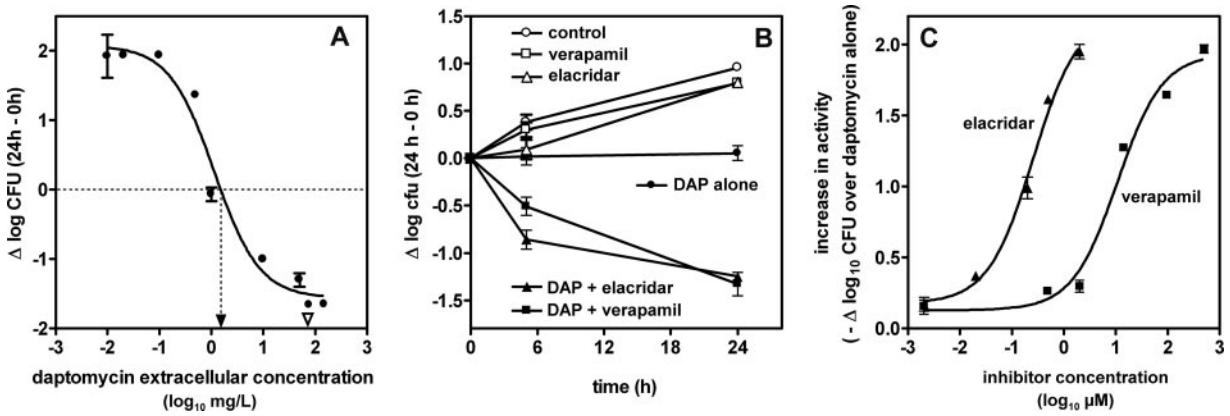

FIG. 1. Intracellular activity of daptomycin towards *S. aureus* ATCC 25923 in THP-1 macrophages. (A) Dose-response curves over a wide range of extracellular concentrations. The ordinate shows the change in the number of CFU (Δ log CFU) per milligram of cell protein at 24 h compared to the postphagocytosis inoculum. A sigmoidal (slope factor, 1) function was used for regression (see Table 1 for goodness-of-fit and regression parameters). The dotted horizontal line indicates a static effect, which was reached for the extracellular concentration shown by the vertical dotted line. For reference, the open triangle on the abscissa indicates the serum C_{max} (total drug) observed in volunteers receiving the clinically recommended dose of 4 mg/kg of body weight daptomycin (77 mg/liter) (67). (B) Influence of time and of the presence of efflux transporter inhibitors on the rate and the extent of the activity of daptomycin at a fixed extracellular concentration. The ordinate is as in panel A. Control, no treatment; DAP, daptomycin (1 mg/liter); verapamil (100 μM); elacridar (GF 120918; 0.5 μM). (C) Influence of the concentration of verapamil or elacridar on the activity of daptomycin (1 mg/liter) measured at 24 h. The ordinate shows the increase in activity defined as the difference between the change in CFU observed in the presence of the inhibitors minus what is observed with daptomycin alone (the graph shows the negative value of this difference to avoid describing increases in activity by decrements in the ordinate). All values are means ± standard deviations (n = 3; when not visible, the standard deviation bars are smaller than the symbols).

or 0.5 μM, respectively) to media containing increasing concentrations of daptomycin (over the same range as that used to generate the data of Fig. 1A). Results, shown in a synoptic fashion in Table 1, demonstrate that both inhibitors significantly increased the relative potency of daptomycin (thus causing a reduction of the EC_{50}) without affecting its E_{max} (no significant change in E_{max} value). In the second series of experiments, daptomycin was used at a fixed concentration of 1 mg/liter (shown to cause an almost static effect), and verapamil or elacridar was added at increasing concentrations (from 2 nM to 10 μM [elacridar] and 500 μM [verapamil]). Results presented in Fig. 1C show that both inhibitors acted in a concentration-dependent fashion and were able to bring the intracellular activity of daptomycin (present in the extracellular medium at a concentration of 1 mg/liter only) to the value observed for a daptomycin concentration of 100 mg/liter in cells incubated in the absence of inhibitors (and corresponding to its E_{max} [Table 1]). To check for the specificity of the changes in daptomycin activity observed with verapamil and elacridar with respect to P-gp, all experiments were repeated with gemfibrozil, an inhibitor of MRPs, the second major family of ABC transporters capable of transporting antibiotics in macrophages (34), but no significant effect was observed.

Intracellular activity of daptomycin in wild-type MDCK cells versus MDCK cells overexpressing P-gp (MDCK-MDR1) and modulation by efflux transporter inhibitors. In the first

TABLE 1. Pertinent regression parameters and statistical analysis of the dose-response curves of daptomycin alone (see Fig. 1A) or daptomycin plus transporter inhibitors (actual data not shown) towards *S. aureus* ATCC 25923 phagocytized by THP-1 macrophages or MDCK cells[a]

Condition	EC_{50} ± SD (mg/liter)[b,c]	C_{static} (mg/liter)[d]	E_{max} ± SD (log_{10} U)[b,e]	R^2
THP-1 cells				
Daptomycin alone	1.14 ± 0.14 A	1.3	−1.60 ± 0.16 A	0.977
Daptomycin + verapamil (100 μM)	0.31 ± 0.13 B	0.4	−1.82 ± 0.16 A	0.978
Daptomycin + elacridar (0.5 μM)	0.27 ± 0.17 B	0.4	−1.67 ± 0.23 A	0.964
MDCK cells				
Wild type, daptomycin alone	0.62 ± 0.09 C	0.6	−1.70 ± 0.07 A	0.993
Wild type, daptomycin + verapamil (100 μM)	0.18 ± 0.06 B	0.2	−2.01 ± 0.06 B	0.996
MDCK-MDR1, daptomycin alone	2.67 ± 0.07 D	2.1	−1.77 ± 0.06 A	0.999
MDCK-MDR1, daptomycin + verapamil (100 μM)	0.70 ± 0.15 E	0.6	−1.78 ± 0.02 A	0.989

[a] Regression parameters obtained by nonlinear regression using the Hill equation with a slope factor of 1.
[b] For statistical analysis (per column; one-way analysis of variance by the Tukey's test for multiple comparisons between each parameter for all conditions), values with different letters are significantly different from each other ($P < 0.05$).
[c] Concentrations causing a reduction of the inoculum halfway between the extrapolated initial (E_0) and maximal (E_{max}) values.
[d] Concentration resulting in no apparent bacterial growth (no change in CFU compared to the postphagocytosis inoculum; determined by graphical intrapolation).
[e] CFU decrease at 24 h from the corresponding postphagocytosis inoculum, as extrapolated for a daptomycin concentration at infinity; all CFU counts were much above the minimal detection level.

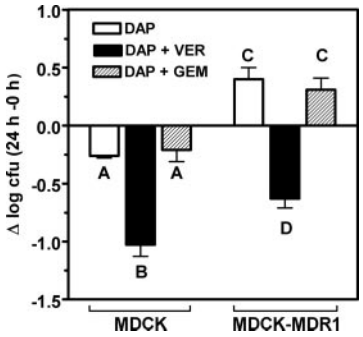

FIG. 2. Activity of daptomycin at a fixed extracellular concentration (1 mg/liter) towards *S. aureus* ATCC 25923 in MDCK (wild-type) and MDCK-MDR1 (overexpressing P-gp) cells in 24 h. The ordinate shows the change in the number of CFU (Δ log CFU) per mg of cell protein. DAP, daptomycin alone; DAP + VER, daptomycin plus verapamil (100 μM); DAP + GEM, daptomycin plus gemfibrozil (250 μM). All values are means ± standard deviations ($n = 3$). Statistical analysis: values of bars with different letters are significantly different from each other (analysis of variance; $P < 0.05$).

series of experiments, the activity of daptomycin in MDCK and MDCK-MDR1 cells infected with *S. aureus* was examined using a fixed extracellular concentration of 1 mg/liter in the absence and in the presence of verapamil (100 μM) or gemfibrozil (250 μM). Results are shown in Fig. 2. Concentrating first on wild-type cells, we see that daptomycin, which at the concentration used was almost bacteriostatic against intracellular *S. aureus*, had its activity markedly enhanced by verapamil, as in THP-1 macrophages. Moving now to MDCK-MDR1 cells, we see that daptomycin, at the same concentration as that used for wild-type cells, was unable to control the intracellular bacterial growth. Yet, the addition of verapamil allowed for the appearance of significant activity. Interestingly enough, the increase in activity obtained with verapamil was similar in MDCK and MDCK-MDR1 cells (about a 1.3-log decrease in CFU), although the basal levels were different. Finally, gemfibrozil was without significant effect on daptomycin activity with either cell line. In the second series of experiments, we ran full daptomycin dose-response curves in infected wild-type MDCK and MDCK-MDR1 cells in the presence and in the absence of verapamil (100 μM). Results shown in a synoptic fashion in Table 1 demonstrate that verapamil increased the relative potency of daptomycin about 3.5-fold (in both cell lines), as in THP-1 macrophages. There was also a small increase in E_{max} in wild-type MDCK cells.

Modulation of the cellular accumulation of daptomycin and $DiOC_2$. Based on the results presented so far, we hypothesized that verapamil increased the intracellular antibacterial effect of daptomycin in THP-1 macrophages and MDCK cells by enhancing its cellular accumulation (through impairment of a P-gp-mediated efflux mechanism). The cellular accumulation of daptomycin was therefore examined under conditions known to either impair (addition of verapamil or ATP depletion) or to enhance (addition of ouabain) (7) the activity of P-gp. These experiments had to use a large extracellular concentration of daptomycin (250 mg/liter) because of the low sensitivity of our assay method (and the unavailability of radiolabeled compound). Results shown in Table 2 demonstrate that (i) MDCK-MDR1 cells accumulated significantly less daptomycin than wild-type MDCK cells, and (ii) verapamil and ATP depletion (tested for THP-1 macrophages only) enhanced the accumulation of daptomycin (and, interestingly enough, to the same extent [about 3.6-fold in all conditions]), whereas ouabain markedly decreased it. As in all other experiments, gemfibrozil had no effect.

Since the large concentrations of daptomycin used could have caused saturation of the putative P-gp efflux transporter, we also examined the influence of verapamil, ouabain, and gemfibrozil on the accumulation of a known and specific substrate of P-gp, namely, $DiOC_2$ (24, 35), which could be used at a much lower concentration (1 mg/liter). In pilot experiments, we observed that the effect of verapamil on $DiOC_2$ accumulation was not immediate but increased over time. We therefore measured in parallel the accumulation of $DiOC_2$ (at the extracellular concentration of 1 mg/liter) and that of daptomycin (at the extracellular concentration of 250 mg/liter) over a period of 1 and 5 h in control cells versus cells coincubated with verapamil, ouabain, or gemfibrozil. Results presented in Fig. 3 show that verapamil caused an increase, and ouabain a decrease, of the accumulation of $DiOC_2$, which was highly correlated with that of daptomycin. In contrast, the effect of gemfibrozil was minimal.

Influence of verapamil concentration on restoration of daptomycin activity and accumulation in MDCK-MDR1 cells. The data of Tables 1 and 2 show that the addition of 100 μM verapamil to MDCK-MDR1 cells did not bring the daptomycin EC_{50} value and accumulation level to the same values as for

TABLE 2. Modulation of the cellular accumulation of daptomycin in THP-1 macrophages and MDCK cells[a]

Condition	Apparent cellular-to-extracellular concn ratio ± SD (mg/liter)[d]		
	THP-1 cells	MDCK cells[c]	
		Wild type	MDCK-MDR1
Daptomycin alone	0.21 ± 0.05 A	0.23 ± 0.13 A;a	0.08 ± 0.11 A;a
Daptomycin + verapamil (100 μM)	0.75 ± 0.05 B	0.83 ± 0.20 B;a	0.30 ± 0.05 B;b
ATP depletion[b]	0.82 ± 0.07 B		
Daptomycin + ouabain (1 μM)	0.12 ± 0.04 C	0.04 ± 0.01 C;a	0.03 ± 0.01 C;a
Daptomycin + gemfibrozil (250 μM)	0.18 ± 0.06 A;C	0.18 ± 0.06 A;a	0.07 ± 0.06 A;a

[a] Cells incubated for 24 h with 250 mg/liter daptomycin.
[b] Cells incubated for 1 h with 5 mM NaN_3 and 60 mM 2-deoxyglucose before being exposed to daptomycin in the presence of the same inhibitors.
[c] Empty cells, exposure of MDCK and MDCK-MDR1 cells to the inhibitors used for ATP depletion in THP-1 cells caused massive cell detachment.
[d] For statistical analysis, one-way analysis of variance by the Tukey's test for multiple comparisons between conditions (analysis per column; capital letters) or between cells (analysis per row for MDCK cells; small letters) was conducted; values with different letters are significantly different from each other ($P < 0.05$).

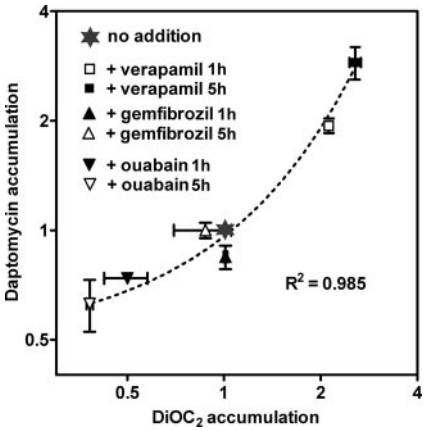

FIG. 3. Comparative modulation of the accumulation of daptomycin and $DiOC_2$ in THP-1 macrophages by verapamil (100 μM), gemfibrozil (250 μM), or ouabain (1 μM). Cells were incubated with daptomycin (250 mg/liter) or $DiOC_2$ (1 mg/liter) alone or in the presence of the modulators for 1 or 5 h. The graph shows the changes in accumulation of daptomycin (ordinate) and of $DiOC_2$ (abscissa) expressed as the fraction of the respective values observed in the absence of the modulator. All values are means ± standard deviations ($n = 3$; when not visible, the standard deviation bars are smaller than the symbols). The dotted line shows the regression curve obtained by using an exponential growth function.

wild-type MDCK cells. This could indicate either an incomplete P-gp inhibition at this verapamil concentration or the presence of another efflux mechanism in MDCK-MDR1 cells. *S. aureus*-infected MDCK-MDR1 cells were, therefore, incubated with 1 mg/liter daptomycin in the presence of increasing concentrations of verapamil (0 to 500 μM). While a verapamil concentration of 100 μM yielded only a modest effect, increasing its concentration to 500 μM yielded a ≈1.5-log CFU decrease (similar to what was seen with 100 μM verapamil with control MDCK cells).

Immunohistodetection of P-gp in THP-1 macrophages and MDCK cells. The previous experiments strongly suggested the presence of an efflux transporter for daptomycin in THP-1 cells that could be potentially identified as P-gp. With only limited data being available as to the presence of this protein in THP-1 macrophages (17), we undertook documenting its presence and localization in our strain of THP-1 macrophages in comparison with MDCK and MDCK-MDR1 cells using immunological techniques. As shown in Fig. 4, the bulk of immunodetected P-gp was in both cases located at the periphery of the cells in close vicinity to actin (used to visualize the cytoskeleton, located as a submembraneous network in association with integrins and other membrane proteins, and determining the cell shape) (41, 44).

Influence of the impairment of P-gp expression through siRNA (gene silencing) on daptomycin accumulation in THP-1 macrophages. Having demonstrated the presence of immunoreactive P-gp in THP-1 (with a localization that could account for an efflux of daptomycin), we examined whether the silencing of its gene (and the corresponding decrease in its cell content) would modulate the accumulation of daptomycin. For this purpose, cells were electroporated with duplex human *mdr1* siRNAs and examined thereafter for the presence of P-gp (by Western blot analysis; performed on lysates from cells incubated for 30 h in control medium after electroporation) and for daptomycin accumulation (measured in cells incubated for 30 h in control medium after electroporation and further incubated for 24 h

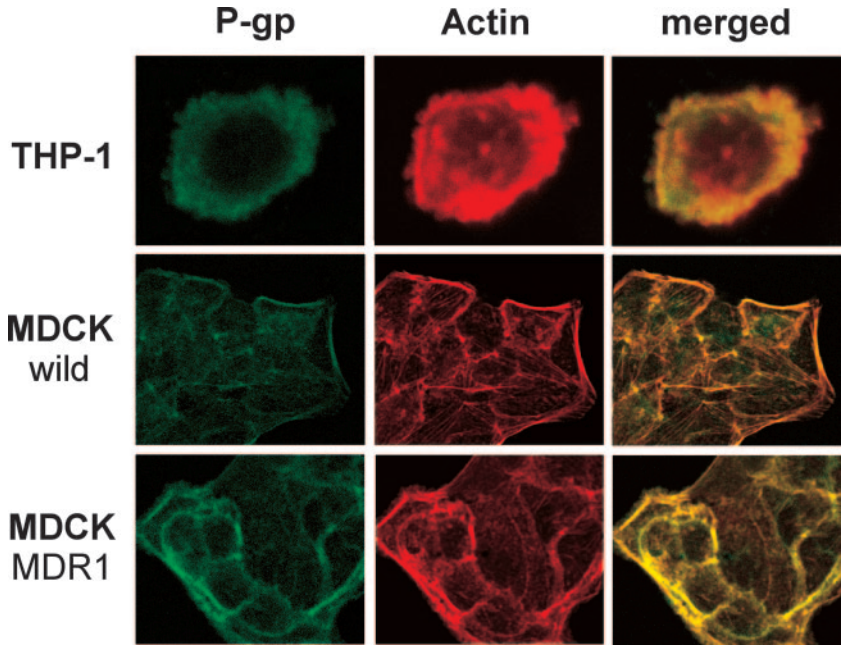

FIG. 4. Immunohistodetection and localization of P-gp in THP-1 macrophages and in MDCK cells (wild type and MDCK-MDR1) by confocal microscopy. Formalin-fixed and saponin-permeabilized cells were exposed to mouse monoclonal anti-P-gp antibodies. Green channel, detection of mouse antibodies with goat fluorescein isothiocyanate-labeled anti-mouse antibodies; red channel, detection of actin with rhodamine-labeled phalloidin; merged images, the two labels are largely but not entirely colocalized.

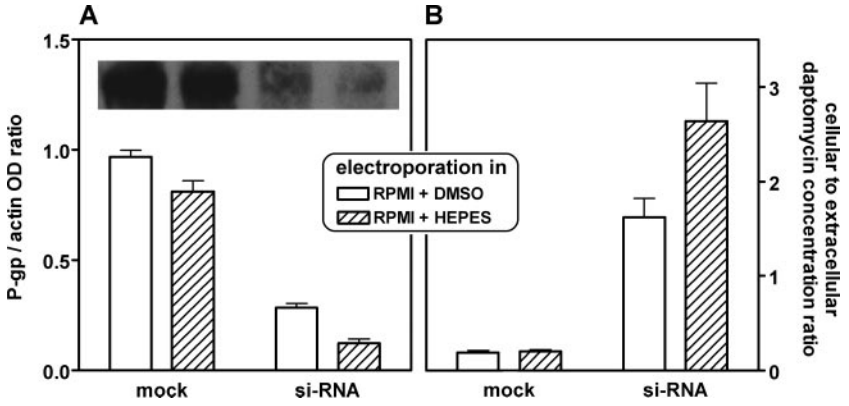

FIG. 5. Modulation of the cell content in P-gp and of daptomycin accumulation in THP-1 macrophages subjected to electroporation without (mock) or with duplex human *mdr1* siRNAs in two different media (RPMI + DMSO, RPMI 1640 medium supplemented with 2.5% DMSO; RPMI + HEPES, RPMI medium supplemented with 25 mM HEPES). (A) Western blot analysis of lysates of cells collected 30 h after electroporation. The picture shows the bands corresponding to the protein as detected by mouse monoclonal anti-P-gp and polyclonal horseradish peroxidase-labeled anti-mouse antibody. The ordinate shows the absorbance ratio of the corresponding band in each condition to that of actin (not shown). OD, optical density. (B) Measurement of the apparent ratio of cellular to extracellular daptomycin concentration in cells incubated in drug-free medium for 30 h after electroporation and thereafter incubated for 24 h with 250 mg/liter daptomycin.

with 250 mg/liter daptomycin). Results are shown in Fig. 5. Electroporation with the duplex human *mdr1* siRNAs (in either of the two media used) markedly reduced the cell content in immunoreactive P-gp, and, in parallel, allowed for a marked increase in daptomycin accumulation.

Correlations between P-gp expression, daptomycin potency, and daptomycin accumulation. Figure 6 shows the correlations established between the amounts of immunoreactive P-gp detected in control THP-1 macrophages, MDCK, and MDCK-MDR1 cells, and THP-1 macrophages subjected to *mdr1* silencing (as determined by Western blot analysis) versus (i) the daptomycin relative potencies (EC_{50}) towards phagocytized *S. aureus* (data of Table 1) and (ii) daptomycin accumulation (data of Table 2 and of Fig. 5) in these cells. In both cases, the correlation was highly significant in spite of the small number of data points. This figure also suggests that the high levels of cellular accumulation of daptomycin noted for THP-1 cells subjected to *mdr1* silencing (see Fig. 5) could only be observed because the P-gp cell content in these cells was much lower than in untreated THP-1 or in wild-type MDCK cells.

Influence of verapamil on cell Ca^{2+} content. Because the antibacterial activity of daptomycin is critically dependent upon a sufficient Ca^{2+} concentration (2, 11), we examined whether the enhancing effect of verapamil on the intracellular activity of daptomycin could not be due to an increase in its intracellular cell Ca^{2+} content. For this purpose, the concentration of free intracellular Ca^{2+} was monitored by means of the calcium sensor Fluo-3 dye in cells exposed to verapamil (100 μM) or gemfibrozil (250 μM). Compared to control cells, verapamil caused a ≈25% reduction of the free Ca^{2+} cellular concentration over 60 min, whereas gemfibrozil was without influence. Ionomycin, an antibiotic also known as a calcium ionophore (6) and used as positive control, caused a 75% increase in apparent free intracellular Ca^{2+} under the same conditions.

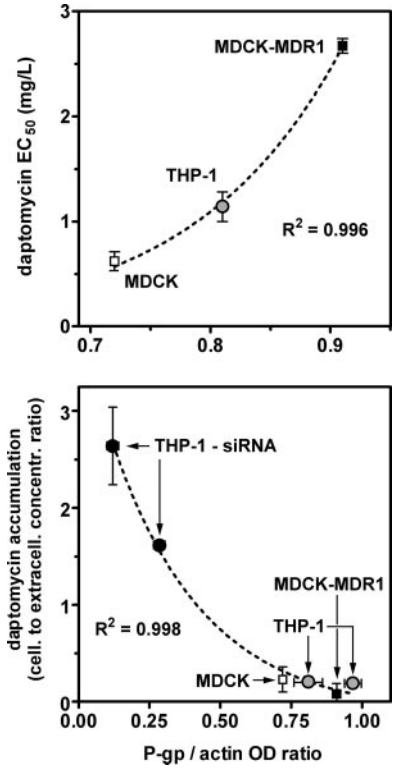

FIG. 6. Correlations between the expression of P-gp (as determined by Western blot analysis and expression as the P-gp/actin optical density [OD] ratio as in Fig. 5) and (i) daptomycin intracellular relative potency and (ii) daptomycin cellular accumulation. (Top) Wild-type MDCK, THP-1, and MDCK-MDR1 cells; EC_{50} values (ordinate) are those listed in Table 1. (Bottom) Comparison between wild-type MDCK, MDCK-MDR1, and THP-1 cells and THP-1 cells subjected to *mdr1* silencing (THP-1-siRNA); accumulation values (ordinate) are those listed in Table 2 and shown in Fig. 5; P-gp expression values (abscissa) are those of the top panel of this figure for MDCK, MDCK-MDR1, and THP-1 cells, and those of Fig. 5 for THP-1-siRNA cells. All values are means ± standard deviations (n = 3; when not visible, the standard deviation bars are smaller than the symbols). The dotted lines show the regression curve obtained by using exponential growth (top) or decay (bottom) functions.

DISCUSSION

The present data extend our previous observations underlining the role of the ATP-energized efflux transporters for modulation of the accumulation and activity of antibiotics in macrophages (33, 34, 46, 47). Specifically, we show here that the activity of P-gp in THP-1 human macrophages decreases the relative potency of daptomycin towards phagocytized *S. aureus* by reducing its cellular concentration. This conclusion, and its specificity with respect to P-gp versus other eukaryotic efflux transporters, stems from five converging pieces of evidence gained from independent approaches, namely (i) the use of two well-known inhibitors of P-gp activity, verapamil (42) and elacridar (GF 120918) (17), in comparison with gemfibrozil, a preferential inhibitor of MRP efflux transporters, which are also present in macrophages (34); (ii) the comparison of the behavior of THP-1 macrophages with that of MDCK (wild-type) and of MDCK-MDR1 (overexpressing P-gp) cell lines; (iii) the direct measurement of the cell content in daptomycin and in $DiOC_2$, a well-known substrate of P-gp (55), in the presence of molecules known to impair or to increase P-gp activity (viz. the inhibitors mentioned above and ouabain, an inducer of *mdr1* expression) (7); and (iv) the silencing of *mdr1* (the gene encoding P-gp in humans) expression by specific siRNA. It is intriguing, at first glance, that *mdr1* silencing causes a much larger accumulation of daptomycin than P-gp inhibitors. This may actually indicate that neither verapamil nor elacridar is capable of fully blocking daptomycin efflux. This conclusion is in accordance with the growing evidence for the presence of multiple binding sites in P-gp (9) and with the observation that substrates and inhibitors of the related half-transporter ABCG2 are effluxed by multiple pathways so that inhibitors remain intrinsically poorly effective at pharmacologically achievable concentrations (1).

Originally described in tumor cells in relation to pleiotropic cross-resistance to a wide range of amphiphilic drugs (18), P-gp is also expressed in the apical surface of a variety of epithelia, where it exerts a function of protection against the toxicity of xenobiotics (by excreting them, for instance, in the intestinal lumen or the urine). P-gp has been described in THP-1 macrophages (17), and our observation that the bulk of the protein is located at the cell surface is consistent with a detoxification function. We, however, are facing two difficulties in the context of our study, namely, (i) how daptomycin could be effluxed by P-gp, based on what we know about preferences of this transporter for truly amphiphilic compounds, and (ii) how this efflux could modulate its intracellular activity towards bacteria that sojourn and thrive not in the cytosol but within phagolysosomes.

Association of drugs to P-gp appears to be mediated mainly by lateral diffusion within the inner monolayer of the plasma, thereby maximizing efflux to a wide range of xenobiotics (56). Even though specific structural determinants appear critical for optimal recognition of drugs by P-gp (40, 65), binding constants remain usually weak, since it is the high partition coefficients of the drugs into lipid bilayers that allow them to reach sufficient values in the vicinity of the protein. These considerations should actually make daptomycin a very unlikely substrate for P-gp, since (i) its overall structure is very remote from that of typical P-gp substrates or inhibitors such as doxorubicin and $DiCO_2$ or verapamil and elacridar, and (ii) the calculated daptomycin octanol/water partition coefficient (log *P*) and distribution coefficient (log *D*, a more correct descriptor for predicting lipid solubility of ionizable compounds) are very negative (-4.073 ± 0.25 and -9.56 at pH 7) (Advanced Chemistry Development [ACD/Labs] software, version V8.19 for Solaris, Advanced Chemistry Development, Inc., Toronto, Ontario, Canada, 2007), in sharp contrast to the positive values calculated for most substrates or inhibitors of P-gp (28). The name "peptolide," originally coined to designate daptomycin (11) but used later on also for cyclosporine and related drugs (22), may erroneously suggest a high lipophilicity, which is not true for daptomycin. Actually, the cyclic peptide moiety of daptomycin contains several anionic amino acids that confer a high polarity to this part of the molecule, which is in contrast to the highly hydrophobic amino acids constitutive of the cyclic peptide moiety of cyclosporine. The confusion is further compounded by the fact that cyclosporine and related peptolides are well-known inhibitors of P-gp (21). Whereas daptomycin in solution spontaneously adopts a conformation that does not confer amphiphilic properties, it oligomerizes in the presence of physiological concentrations of calcium to form micelles, which tend to dissociate when interacting with membranes (53). In bacteria, the high content in the negatively charged phospholipid phosphatidylglycerol allows for a deep, Ca^{2+}-facilitated insertion of daptomycin, leading to membrane leakage and cell death. While a similar, toxic process does not occur in eukaryotic cells (as evidenced by the lack of membrane-permeabilizing properties of daptomycin towards the cells used in our study), interaction of Ca^{2+}-daptomycin micelles with other phospholipids is possible and could lead to its recognition as a phospholipid-drug complex by P-gp. Phospholipids, indeed, are among the physiological substrates of P-gp, the transporter acting then as a flippase (70). A similar model of drug-phospholipid complexation for transport of nonlipophilic drugs by efflux proteins has been presented recently to explain the recognition of aminoglycosides (which have calculated log *P* and log *D* [pH 7] values similar to those of daptomycin) (ACD/Labs software, version V8.19) by the AcrB multidrug efflux pump of *Escherichia coli* (69). Interestingly enough, MDR3, another member of the MDR family, is also known to promote phosphatidylcholine translocation through the plasma membrane of fibroblasts (52).

Efflux of daptomycin at the level of the plasma membrane does not directly explain, however, the reduction of its potency against intracellular *S. aureus*, since the bacterium is not located in the cytosol but in the phagolysosomes (26). It could be argued that P-gp localized in the plasma membrane diverts part of the daptomycin from entering cells by diffusion and reduces, therefore, the amount of drug that can thereafter reach the phagolysosomes, as proposed to explain the defeating effect of P-gp towards azithromycin in J774 macrophages (46). But the biophysical properties of daptomycin discussed above are not compatible with the mechanism of diffusion/segregation observed for azithromycin (8), which is an amphiphilic, basic drug. The difficulty is actually compounded by our ignorance, at this stage, of the pathway and mechanism allowing daptomycin to enter cells and reach the phagolysosomes. Further studies will need to examine these aspects of the cellular pharmacokinetics of dap-

tomycin, but their performance is dependent upon the availability of more sensitive bioanalytical tools than are available to us so far. Yet, since the intracellular activity of daptomycin is concentration dependent, the most likely explanation for the effects observed here is that impairing or increasing the activity of P-gp effectively modulates its concentration at the site of infection. We know that daptomycin activity is enhanced by increasing the calcium concentration (10, 11). But such a calcium effect is unlikely to take place here, since verapamil decreased the cell free Ca^{2+} concentration, as anticipated from its known action on calcium channels in macrophages (68).

Moving now to the chemotherapeutic significance of our observations, we first see that daptomycin is active against intracellular *S. aureus*, in spite of its limited cellular accumulation (with apparent cellular-to-extracellular concentration ratios remaining lower than 1). This conclusion, already reached from early studies of daptomycin with *S. aureus*-infected polymorphonuclear neutrophils (63), reinforces our previous conclusions that cellular accumulation per se is not a sufficiently predictive factor of antibiotic intracellular activity (60). The explanation could be that cell-associated daptomycin is accumulated in the same subcellular compartment that phagocytized *S. aureus*. The localization of daptomycin in macrophages has not yet been established, but in vivo studies suggest that daptomycin taken up by proximal tubular cells is partially localized in lysosomes by the endocytosis route (5). Second, we also see that daptomycin is a concentration-dependent antibiotic towards intracellular *S. aureus* as described for extracellular bacteria using appropriate pharmacodynamic models (62). Yet, in spite of this, and in sharp contrast to what is observed with extracellular bacteria, daptomycin never yields a truly intracellular bactericidal effect, since its E_{max} remains lower than the 3 $\log_{10}$ CFU decrease considered the minimum for a drug to be called bactericidal by CLSI criteria. This loss of E_{max} against intracellular *S. aureus* has been a common observation for all antibiotics tested in macrophage models so far disregarding their subcellular localization (see reference 60 for a review) and still needs to receive a satisfactory explanation. Third, our data clearly show that P-gp reduces the potency of daptomycin against intracellular bacteria in relation with its level of expression. Extrapolation to the in vivo situation cannot be ascertained at this stage, since our experiments were conducted under conditions designed to maximize the effects described and used an immortalized cell line, the behavior of which may differ from that of normal macrophages. Yet, this may now help to develop appropriate models to test for such an impact of P-gp expression in vivo. It could also be suggested that concomitant administration of P-gp inhibitors should favorably modulate the activity of daptomycin in difficult-to-treat staphylococcal infections where intracellular survival may play an important role in the maintenance of the infection. This approach, however, seems very unpractical today, in view of the present lack of availability of very safe P-gp inhibitors. Yet, the observation that some fluoroquinolones may act as potent inhibitors of P-gp-mediated efflux in MDCK-MDR1 cells (50) may open new perspectives in this context. A possible caveat, however, is that daptomycin myopathy (one of the drug's most serious side effects) (38) is driven by drug concentrations remaining above some threshold level for an extended period of time (hence directing its clinical use on a once-daily schedule) (37). We cannot exclude that a P-gp inhibitor could amplify this adverse effect. The design and/or selection of new derivatives within the class of acidic lipopeptide antibiotics with a lack of or weak recognition by P-gp could, therefore, also appear as a more useful and a safer approach.

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REFERENCES

1. **Acharya, P., T. T. Tran, J. W. Polli, A. Ayrton, H. Ellens, and J. Bentz.** 2006. P-glycoprotein (P-gp) expressed in a confluent monolayer of hMDR1-MDCKII cells has more than one efflux pathway with cooperative binding sites. Biochemistry **45:**15505–15519.
2. **Andrew, J. H., M. C. Wale, L. J. Wale, and D. Greenwood.** 1987. The effect of cultural conditions on the activity of LY146032 against staphylococci and streptococci. J. Antimicrob. Chemother. **20:**213–221.
3. **Barcia-Macay, M., S. Lemaire, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke.** 2006. Evaluation of the extracellular and intracellular activities (human THP-1 macrophages) of telavancin versus vancomycin against methicillin-susceptible, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant Staphylococcus aureus. J. Antimicrob. Chemother. **58:**1177–1184.
4. **Barcia-Macay, M., C. Seral, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke.** 2006. Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. Antimicrob. Agents Chemother. **50:**841–851.
5. **Beauchamp, D., P. Gourde, M. Simard, and M. G. Bergeron.** 1994. Subcellular distribution of daptomycin given alone or with tobramycin in renal proximal tubular cells. Antimicrob. Agents Chemother. **38:**189–194.
6. **Beeler, T. J., I. Jona, and A. Martonosi.** 1979. The effect of ionomycin on calcium fluxes in sarcoplasmic reticulum vesicles and liposomes. J. Biol. Chem. **254:**6229–6231.
7. **Brouillard, F., D. Tondelier, A. Edelman, and M. Baudouin-Legros.** 2001. Drug resistance induced by ouabain via the stimulation of MDR1 gene expression in human carcinomatous pulmonary cells. Cancer Res. **61:**1693–1698.
8. **Carlier, M. B., I. Garcia-Luque, J. P. Montenez, P. M. Tulkens, and J. Piret.** 1994. Accumulation, release and subcellular localization of azithromycin in phagocytic and non-phagocytic cells in culture. Int. J. Tissue React. **16:**211–220.
9. **Clark, R., I. D. Kerr, and R. Callaghan.** 2006. Multiple drugbinding sites on the R482G isoform of the ABCG2 transporter. Br. J. Pharmacol. **149:**506–515.
10. **Ehlert, F., and H. C. Neu.** 1987. In vitro activity of LY146032 (daptomycin), a new peptolide. Eur. J. Clin. Microbiol. **6:**84–90.
11. **Eliopoulos, G. M., S. Willey, E. Reiszner, P. G. Spitzer, G. Caputo, and R. C. Moellering, Jr.** 1986. In vitro and in vivo activity of LY 146032, a new cyclic lipopeptide antibiotic. Antimicrob. Agents Chemother. **30:**532–535.
12. **Evers, R., M. Kool, A. J. Smith, L. van Deemter, M. de Haas, and P. Borst.** 2000. Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. Br. J. Cancer **83:**366–374.
13. **Foster, T. J.** 2005. Immune evasion by staphylococci. Nat. Rev. Microbiol. **3:**948–958.
14. **French, G. L.** 2006. Bactericidal agents in the treatment of MRSA infections—the potential role of daptomycin. J. Antimicrob. Chemother. **58:**1107–1117.
15. **Fuchs, P. C., A. L. Barry, and S. D. Brown.** 2000. Daptomycin susceptibility

tests: interpretive criteria, quality control, and effect of calcium on in vitro tests. Diagn. Microbiol. Infect. Dis. **38:**51–58.
16. **Hyafil, F., C. Vergely, V. P. Du, and T. Grand-Perret.** 1993. In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. Cancer Res. **53:**4595–4602.
17. **Ichibangase, Y., M. Yamamoto, M. Yasuda, N. Houki, and M. Nobunaga.** 1998. Induction of drug resistance to gold sodium thiomalate in a monocyte cell line, THP-1. Clin. Rheumatol. **17:**214–218.
18. **Juliano, R. L., and V. Ling.** 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim. Biophys. Acta **455:**152–162.
19. **Kao, J. P., A. T. Harootunian, and R. Y. Tsien.** 1989. Photochemically generated cytosolic calcium pulses and their detection by fluo-3. J. Biol. Chem. **264:**8179–8184.
20. **Katragadda, S., B. Budda, B. S. Anand, and A. K. Mitra.** 2005. Role of efflux pumps and metabolising enzymes in drug delivery. Expert Opin. Drug Deliv. **2:**683–705.
21. **Keller, R. P., H. J. Altermatt, P. Donatsch, H. Zihlmann, J. A. Laissue, and P. C. Hiestand.** 1992. Pharmacologic interactions between the resistance-modifying cyclosporine SDZ PSC 833 and etoposide (VP 16–213) enhance in vivo cytostatic activity and toxicity. Int. J. Cancer **51:**433–438.
22. **Lawen, A., R. Traber, and D. Geyl.** 1991. In vitro biosynthesis of [Thr2, Leu5, D-Hiv8, Leu10]cyclosporin, a cyclosporin-related immunosuppressive peptolide. Biomed. Biochim. Acta **50:**S260–S263.
23. **Lee, S. Y., H. W. Fan, J. L. Kuti, and D. P. Nicolau.** 2006. Update on daptomycin: the first approved lipopeptide antibiotic. Expert. Opin. Pharmacother. **7:**1381–1397.
24. **Leith, C. P., I. M. Chen, K. J. Kopecky, F. R. Appelbaum, D. R. Head, J. E. Godwin, J. K. Weick, and C. L. Willman.** 1995. Correlation of multidrug resistance (MDR1) protein expression with functional dye/drug efflux in acute myeloid leukemia by multiparameter flow cytometry: identification of discordant MDR−/efflux+ and MDR1+/efflux− cases. Blood **86:**2329–2342.
25. **Lemaire, S., T. Bogdanovitch, S. Chavez-Bueno, P. D. Savage, F. Van Bambeke, P. Appelbaum, and P. M. Tulkens.** 2006. Bactericidal activity of ceragenin CSA-13 against intracellular MSSA, hospital-acquired (HA) and community-acquired (CA) MRSA, and VISA in THP-1 macrophages: relation to cellular toxicity?, abstr. A-0633. Abstr. 46th Intersci. Conf. Antimicrob. Agents Chemother., San Francisco, CA, 27 to 30 September 2006.
26. **Lemaire, S., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2005. Activity of three β-lactams (ertapenem, meropenem and ampicillin) against intraphagocytic Listeria monocytogenes and Staphylococcus aureus. J. Antimicrob. Chemother. **55:**897–904.
27. **Leslie, E. M., R. G. Deeley, and S. P. Cole.** 2005. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol. Appl. Pharmacol. **204:**216–237.
28. **Liu, X., M. Tu, R. S. Kelly, C. Chen, and B. J. Smith.** 2004. Development of a computational approach to predict blood-brain barrier permeability. Drug Metab. Dispos. **32:**132–139.
29. **Louvard, D.** 1980. Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. Proc. Natl. Acad. Sci. USA **77:**4132–4136.
30. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
31. **Lowy, F. D.** 2000. Is Staphylococcus aureus an intracellular pathogen? Trends Microbiol. **8:**341–343.
32. **Melkonyan, H., C. Sorg, and M. Klempt.** 1996. Electroporation efficiency in mammalian cells is increased by dimethyl sulfoxide (DMSO). Nucleic Acids Res. **24:**4356–4357.
33. **Michot, J. M., M. F. Heremans, N. E. Caceres, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke.** 2006. Cellular accumulation and activity of quinolones in ciprofloxacin-resistant J774 macrophages. Antimicrob. Agents Chemother. **50:**1689–1695.
34. **Michot, J. M., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens.** 2004. Active efflux of ciprofloxacin from J774 macrophages through an MRP-like transporter. Antimicrob. Agents Chemother. **48:**2673–2682.
35. **Minderman, H., U. Vanhoefer, K. Toth, M. B. Yin, M. D. Minderman, C. Wrzosek, M. L. Slovak, and Y. M. Rustum.** 1996. DiOC2(3) is not a substrate for multidrug resistance protein (MRP)-mediated drug efflux. Cytometry **25:**14–20.
36. **Minta, A., J. P. Kao, and R. Y. Tsien.** 1989. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. J. Biol. Chem. **264:**8171–8178.
37. **Oleson, F. B., Jr., C. L. Berman, J. B. Kirkpatrick, K. S. Regan, J. J. Lai, and F. P. Tally.** 2000. Once-daily dosing in dogs optimizes daptomycin safety. Antimicrob. Agents Chemother. **44:**2948–2953.
38. **Papadopoulos, S., A. M. Ball, S. E. Liewer, C. A. Martin, P. S. Winstead, and B. S. Murphy.** 2006. Rhabdomyolysis during therapy with daptomycin. Clin. Infect. Dis. **42:**e108–e110.
39. **Ramagli, L. S.** 1999. Quantifying protein in 2-D PAGE solubilization buffers. Methods Mol. Biol. **112:**99–103.
40. **Raub, T. J.** 2006. P-glycoprotein recognition of substrates and circumvention through rational drug design. Mol. Pharm. **3:**3–25.
41. **Robertson, D., H. F. Paterson, P. Adamson, A. Hall, and P. Monaghan.** 1995. Ultrastructural localization of ras-related proteins using epitope-tagged plasmids. J. Histochem. Cytochem. **43:**471–480.
42. **Safa, A. R.** 1988. Photoaffinity labeling of the multidrug-resistance-related P-glycoprotein with photoactive analogs of verapamil. Proc. Natl. Acad. Sci. USA **85:**7187–7191.
43. **Saleh, M., J. C. Mathison, M. K. Wolinski, S. J. Bensinger, P. Fitzgerald, N. Droin, R. J. Ulevitch, D. R. Green, and D. W. Nicholson.** 2006. Enhanced bacterial clearance and sepsis resistance in caspase-12-deficient mice. Nature **440:**1064–1068.
44. **Sandig, M., E. Negrou, and K. A. Rogers.** 1997. Changes in the distribution of LFA-1, catenins, and F-actin during transendothelial migration of monocytes in culture. J. Cell Sci. **110:**2807–2818.
45. **Seral, C., M. Barcia-Macay, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke.** 2005. Comparative activity of quinolones (ciprofloxacin, levofloxacin, moxifloxacin and garenoxacin) against extracellular and intracellular infection by Listeria monocytogenes and Staphylococcus aureus in J774 macrophages. J. Antimicrob. Chemother. **55:**511–517.
46. **Seral, C., S. Carryn, P. M. Tulkens, and F. Van Bambeke.** 2003. Influence of P-glycoprotein and MRP efflux pump inhibitors on the intracellular activity of azithromycin and ciprofloxacin in macrophages infected by Listeria monocytogenes or Staphylococcus aureus. J. Antimicrob. Chemother. **51:**1167–1173.
47. **Seral, C., J. M. Michot, H. Chanteux, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke.** 2003. Influence of P-glycoprotein inhibitors on accumulation of macrolides in J774 murine macrophages. Antimicrob. Agents Chemother. **47:**1047–1051.
48. **Seral, C., F. Van Bambeke, and P. M. Tulkens.** 2003. Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) activities against intracellular *Staphylococcus aureus* in mouse J774 macrophages. Antimicrob. Agents Chemother. **47:**2283–2292.
49. **Shitara, Y., T. Horie, and Y. Sugiyama.** 2006. Transporters as a determinant of drug clearance and tissue distribution. Eur. J. Pharm. Sci. **27:**425–446.
50. **Sikri, V., D. Pal, R. Jain, D. Kalyani, and A. K. Mitra.** 2004. Cotransport of macrolide and fluoroquinolones, a beneficial interaction reversing P-glycoprotein efflux. Am. J. Ther. **11:**433–442.
51. **Silverman, J. A., L. I. Mortin, A. D. Vanpraagh, T. Li, and J. Alder.** 2005. Inhibition of daptomycin by pulmonary surfactant: in vitro modeling and clinical impact. J. Infect. Dis. **191:**2149–2152.
52. **Smith, A. J., J. L. Timmermans-Hereijgers, B. Roelofsen, K. W. Wirtz, W. J. van Blitterswijk, J. J. Smit, A. H. Schinkel, and P. Borst.** 1994. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. FEBS Lett. **354:**263–266.
53. **Straus, S. K., and R. E. Hancock.** 2006. Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: comparison with cationic antimicrobial peptides and lipopeptides. Biochim. Biophys. Acta **1758:**1215–1223.
54. **Takara, K., M. Tsujimoto, M. Kokufu, N. Ohnishi, and T. Yokoyama.** 2003. Up-regulation of MDR1 function and expression by cisplatin in LLC-PK1 cells. Biol. Pharm. Bull. **26:**205–209.
55. **Tarasova, N. I., R. Seth, S. G. Tarasov, T. Kosakowska-Cholody, C. A. Hrycyna, M. M. Gottesman, and C. J. Michejda.** 2005. Transmembrane inhibitors of P-glycoprotein, an ABC transporter. J. Med. Chem. **48:**3768–3775.
56. **Tran, T. T., A. Mittal, T. Aldinger, J. W. Polli, A. Ayrton, H. Ellens, and J. Bentz.** 2005. The elementary mass action rate constants of P-gp transport for a confluent monolayer of MDCKII-hMDR1 cells. Biophys. J. **88:**715–738.
57. **Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada.** 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer **26:**171–176.
58. **Tyteca, D., S. P. Van Der, M. Mettlen, F. Van Bambeke, P. M. Tulkens, M. P. Mingeot-Leclercq, and P. J. Courtoy.** 2002. Azithromycin, a lysosomotropic antibiotic, has distinct effects on fluid-phase and receptor-mediated endocytosis, but does not impair phagocytosis in J774 macrophages. Exp. Cell Res. **281:**86–100.
59. **Van Bambeke, F., E. Balzi, and P. M. Tulkens.** 2000. Antibiotic efflux pumps. Biochem. Pharmacol. **60:**457–470.
60. **Van Bambeke, F., M. Barcia-Macay, S. Lemaire, and P. M. Tulkens.** 2006. Cellular pharmacodynamics and pharmacokinetics of antibiotics: current views and perspectives. Curr. Opin. Drug Discov. Dev. **9:**218–230.
61. **Van Bambeke, F., J. M. Michot, and P. M. Tulkens.** 2003. Antibiotic efflux pumps in eukaryotic cells: occurrence and impact on antibiotic cellular pharmacokinetics, pharmacodynamics and toxicodynamics. J. Antimicrob. Chemother. **51:**1067–1077.
62. **Vance-Bryan, K., T. A. Larson, J. C. Rotschafer, and J. P. Toscano.** 1992. Investigation of the early killing of *Staphylococcus aureus* by daptomycin by using an in vitro pharmacodynamic model. Antimicrob. Agents Chemother. **36:**2334–2337.
63. **Van der Auwera, P., G. Petrikkos, T. Matsumoto, and M. Husson.** 1988. Influence of LY146032 on human polymorphonuclear leucocytes in vitro. J. Antimicrob. Chemother. **21:**57–63.
64. **van der Heijden, J., M. C. de Jong, B. A. Dijkmans, W. F. Lems, R.**

Oerlemans, I. Kathmann, C. G. Schalkwijk, G. L. Scheffer, R. J. Scheper, and G. Jansen. 2004. Development of sulfasalazine resistance in human T cells induces expression of the multidrug resistance transporter ABCG2 (BCRP) and augmented production of TNFalpha. Ann. Rheum. Dis. **63:**138–143.

65. **Vandevuer, S., F. Van Bambeke, P. M. Tulkens, and M. Prevost.** 2006. Predicting the three-dimensional structure of human P-glycoprotein in absence of ATP by computational techniques embodying crosslinking data: insight into the mechanism of ligand migration and binding sites. Proteins **63:**466–478.
66. **Varma, M. V., O. P. Perumal, and R. Panchagnula.** 2006. Functional role of P-glycoprotein in limiting peroral drug absorption: optimizing drug delivery. Curr. Opin. Chem. Biol. **10:**367–373.
67. **Wise, R., T. Gee, J. M. Andrews, B. Dvorchik, and G. Marshall.** 2002. Pharmacokinetics and inflammatory fluid penetration of intravenous daptomycin in volunteers. Antimicrob. Agents Chemother. **46:**31–33.
68. **Woehlck, H. J., and F. V. McCann.** 1986. Action potentials in human macrophages are calcium spikes. Cell Biol. Int. Rep. **10:**517–525.
69. **Yu, E. W., J. R. Aires, and H. Nikaido.** 2003. AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. J. Bacteriol. **185:**5657–5664.
70. **Zhou, Y., M. M. Gottesman, and I. Pastan.** 1999. Studies of human MDR1-MDR2 chimeras demonstrate the functional exchangeability of a major transmembrane segment of the multidrug transporter and phosphatidylcholine flippase. Mol. Cell. Biol. **19:**1450–1459.