Increased Susceptibility of *Pseudomonas aeruginosa* to Macrolides and Ketolides in Eukaryotic Cell Culture Media and Biological Fluids Due to Decreased Expression of *oprM* and Increased Outer-Membrane Permeability

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Background. Macrolides show high minimum inhibitory concentrations (MICs) against *Pseudomonas aeruginosa* when tested in recommended media (cation-adjusted Muller-Hinton broth [CA-MHB]). Nevertheless, azithromycin is successfully used in cystic fibrosis patients, supposedly because of "nonantibiotic effects."

Methods. CA-MHB and Roswell Park Memorial Institute (RPMI) 1640 medium (used for growing eukaryotic cells) were compared for measuring azithromycin MICs (with or without Phe-Arg-β-naphthylamide [PAβN], an efflux inhibitor), [14C]-clarithromycin accumulation, azithromycin-induced protein synthesis inhibition, *oprM* (encoding the outer-membrane protein coupled with MexAB and MexXY efflux systems) expression, outer-membrane permeability (tested with 1-*N*-phenylnaphthylamine and nitrocefin), and synergy (determined by checkerboard assay) between azithromycin and outer-membrane disrupting agents. Key experiments were repeated with CA-MHB supplemented with serum, mouse bronchoalveolar lavage fluid, other macrolides, and other gram-negative bacteria.

Results. Azithromycin MICs were ≥128 mg/L in CA-MHB, compared with 1–16 mg/L in RPMI 1640 medium, CA-MHB supplemented with serum, or bronchoalveolar lavage fluid (repeated for RPMI 1640 medium with clarithromycin, other macrolides, and other gram-negative bacteria). [14C]-clarithromycin accumulation was 2.2-fold higher in RPMI 1640 medium, compared with CA-MHB. Inhibition of >95% of protein synthesis was obtained with azithromycin at 16 mg/L in RPMI 1640 medium, compared with >512 mg/L in CA-MHB. Strains not expressing *oprM* showed an MIC of 4 mg/L in CA-MHB. PAβN decreased MICs in CA-MHB but not in RPMI 1640 medium. Real-time polymerase chain reaction showed downregulation of *oprM* by azithromycin in RPMI 1640 medium. Outer-membrane permeability was 3–4.5 times higher in RPMI 1640 medium or bronchoalveolar lavage fluid, compared with CA-MHB. Azithromycin combined with outer-membrane disrupting agents were synergistic in CA-MHB but indifferent in RPMI 1640 medium.

Conclusions. Macrolides show antimicrobial activity against *P. aeruginosa* in eukaryotic media through increased uptake and reduced efflux. These data may help explain the clinical efficacy of macrolides against pseudomonal infections.

Pseudomonas aeruginosa is considered to be naturally resistant to several classes of antibiotics [1]; this is usually ascribed to low permeability [2], constitutive expression of efflux systems [3], and production of antibiotic-inactivating enzymes [4]. Thus, macrolide minimum inhibitory concentrations (MICs; measured as recommended by the Clinical Laboratory Standards Institute [5]) largely

exceed serum and tissue concentrations reachable in humans. Yet, macrolides are widely used in the treatment of respiratory tract infections caused by *P. aeruginosa*, especially in cystic fibrosis patients, for whom clinical benefit has been clearly demonstrated [6, 7]. This is assumed to result from nonantibiotic effects, such as enhancement of host defenses [8], anti-inflammatory activities [9], inhibition of the expression of virulence factors or of quorum sensing [10], alteration of the bacterial outer membrane [11], and/or destruction of biofilms [12]. During a systematic survey of antibiotic activity in media used for culturing eukaryotic cells, we noted that the azithromycin MIC against *P. aeruginosa* was considerably lower in these media as compared to the recommended cation-adjusted Mueller-Hinton broth (CA-MHB; [5]). Since this observation could be extended to other macrolides and ketolides and other biological media of eukaryotic origin, we suspected that CA-MHB could actually modulate the physiology of *P. aeruginosa,* making it apparently resistant to macrolides if tested in that medium.

Our aim was to systematize these observations and to decipher their underlying mechanism(s). We show that the permeability of the outer membrane of *P. aeruginosa* is considerably lower in microbiological broths than in eukaryotic media and that bacteria exposed to azithromycin in CA-MHB express *oprM* (encoding a protein essential for active efflux of antibiotics) to a much larger extent in broth than in eukaryotic media. These mutually supportive effects are associated with an increased accumulation of macrolides, explaining why these antibiotics can exert true antibiotic effects in eukaryotic media at much lower concentrations than what has been generally assumed on the basis of conventional antibiotic susceptibility testing.

MATERIALS AND METHODS

Bacterial Strains

P. aeruginosa American Type Culture Collection strain PAO1 was used for most experiments. Other strains are described in Supplementary Tables 1 and 2. Bacteria were grown overnight in CA-MHB (20–25 mg/L Ca^{2+}; 10–12.5 mg/L Mg^{2+}) at 37°C with constant shaking (130 rpm) and under aerobic conditions, prior to use in experiments.

MIC Determination, Time- and Concentration-Kill Studies, and Assessment of Synergy

MICs were measured by microdilution [5] in CA-MHB or another medium, as indicated in the Results. Phe-Arg-β-naphthylamide (PaβN) was used as inhibitor of active efflux [13]. Time-kill and concentration-kill studies were performed as described previously [14]. Synergy was tested using the microtiter checkerboard method [15], with data used to calculate the fractional inhibitory concentration parameter (ΣFIC; values ≤0.5 are considered synergistic).

Uptake of Clarithromycin

Overnight cultures of PAO1 were diluted to 10^9 colony-forming units (CFU)/mL into CA-MHB or Roswell Park Memorial Institute (RPMI) 1640 medium and grown for 4 or 8 hours in the same medium with 10 mg/L of [^{14}C]-clarithromycin. Bacteria were collected by centrifugation (1000g for 7 minutes at 4°C), washed 3 times with ice-cold phosphate-buffered saline, and lysed by 2 cycles of freezing (at −80°C) and thawing. Radioactivity counts (Tri-carb 2800TR, PerkinElmer, Waltham, MA) were normalized by reference to samples protein content (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA).

Protein Synthesis

[^{3}H]-leucine incorporation in proteins was evaluated in PAO1 incubated for 4 hours in CA-MHB or RPMI 1640 medium in the presence of increasing concentrations of azithromycin, with 2 μCi/mL [^{3}H]-leucine added during the last 2 hours [16].

Expression of *mexA, mexC, mexE, mexX,* and *oprM*

Overnight cultures were resuspended at an optical density (620 nm) of 0.2 in 20 mL of CA-MHB or RPMI 1640 medium (with or without 1 mg/L azithromycin), grown at 37°C for 4 or 8 hours, harvested by centrifugation (10 000g for 10min), frozen, and kept at −80°C. After total RNA extraction and reverse transcription [17], *mexC* and *mexE* were detected by polymerase chain reaction (PCR) on complementary DNA (cDNA) [17], and *mexA, mexX,* and *oprM* were quantified by real-time PCR in 25-μL reaction mixtures containing 12.5 μL of iQ SYBR Green Supermix (2x), 400 nM of forward and reverse primers, and 5 μL of cDNA in RNase/DNase-free water, using an iQ cycler (Bio-Rad Laboratories). *mexA* and *mexX* were amplified using *P. aeruginosa* MexQ-TesT (CORIS BioConcept, Gembloux, Belgium), and *oprM* was amplified with previously described primers, using *rpsl* as housekeeping gene [18].

Assessment of Outer-Membrane Permeability

Overnight cultures of PAO1 were resuspended at a density of 10^9 CFU/mL into CA-MHB, RPMI 1640 medium (without phenol red), or mouse bronchoalveolar lavage (BAL) fluid. 1-*N*-phenylnaphthylamine (NPN; validated nonpermanent tracer that becomes fluorescent when partitioned in the lipid bilayer of the inner membrane [19]) and nitrocefin (chromogenic substrate of β–lactamases [2]) were used in parallel. NPN assay was performed as previously described [20], except that the NPN concentration was 25 μM and bacteria were resuspended in the medium under study. Nitrocefin assay was performed as previously described [21], after 2-hour induction of β–lactamase expression by exposure to cefoxitin (50 mg/L; [22]). Full outer-membrane permeabilization was achieved by addition of 10 mM of ethylenediaminetetraacetic acid (EDTA) in

10 mM Tris buffer (pH 8.0), which gave a signal similar to that for bacteria lysed by sonication [23].

Source of Products

The following antibiotics were obtained as microbiological standards (potencies are specified in parentheses): clarithromycin (100%) and azithromycin (100%), from Teva, Plantex, Israel; telithromycin (99.3%), from Sanofi-Aventis, Romainville, France; and solithromycin (100%), from Cempra Pharmaceuticals, Chapel Hill, North Carolina. Erythromycin (91.6%) and clindamycin (89.2%) were purchased from Sigma-Aldrich, St. Louis, Missouri. [6-O-methyl-^{14}C]-clarithromycin (54.2 mCi/mmol) was provided by Moravek Biochemicals (Brea, CA), and L-[4,5-^{3}H]leucine (69 Ci/mmol), by Amersham (Buckinghamshire, United Kingdom). EDTA and PaβN were purchased from Sigma-Aldrich, and minimum essential medium (MEM; reference 31095), RPMI 1640 medium (reference 21875), RPMI 1640 medium without phenol red (reference 11835), RPMI 1640 medium without NaHCO$_3$ (reference 51800-035), MEM–nonessential amino acids (MEM-NEAA; reference 11140), and fetal bovine serum (reference 10270) were obtained from Invitrogen (Paisley, Scotland, United Kingdom). Human serum (reference 14-490E) was purchased from Lonza Braine SA (Braine-l'Alleud, Belgium). Broth (CA-MHB [reference 212322], brain heart infusion broth [reference 211059], Luria Bertani broth [reference 244620], and tryptic soy broth [reference 211825]) were purchased from BD Bioscience, Franklin Lakes, New Jersey. Mouse BAL fluid was kindly provided by Dr H. Tiesset (Inserm U 1019–CNRS UMR8204 INSERM, Lille, France).
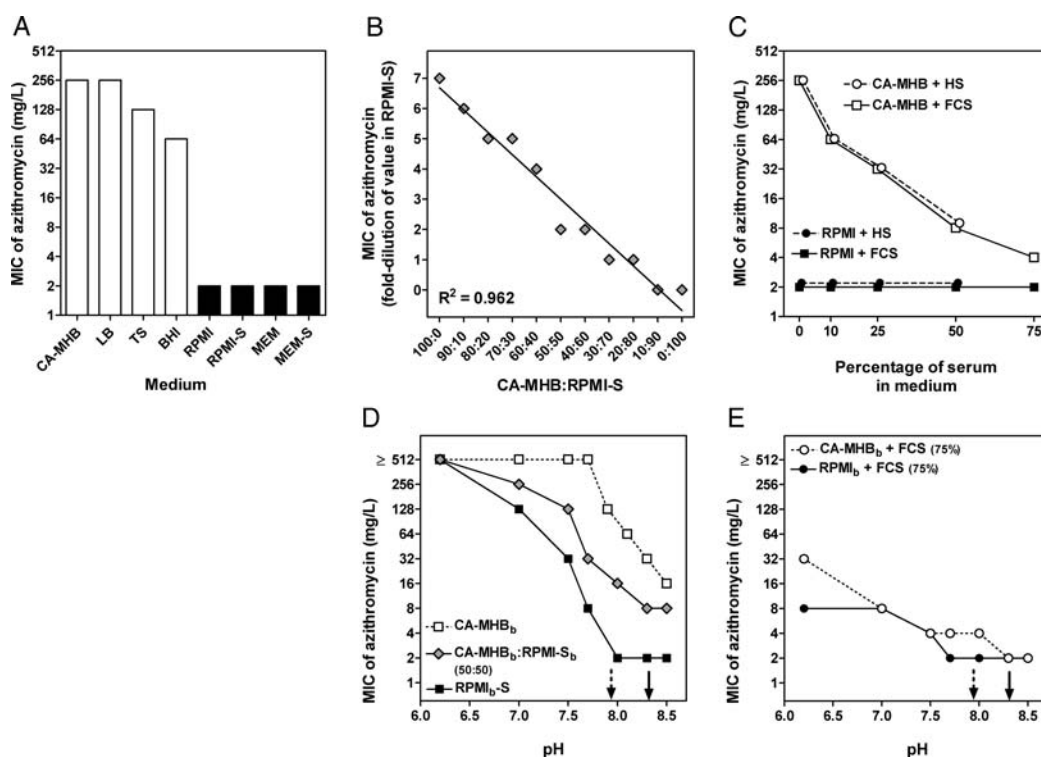
Figure 1. Influence of medium on the minimum inhibitory concentration (MIC) of azithromycin against *Pseudomonas aeruginosa* PAO1. *A*, MICs were measured in cation-adjusted Muller-Hinton broth (CA-MHB), Luria Bertani broth, tryptic soy broth, and brain heart infusion broth or in eukaryotic cell culture media without (Roswell Park Memorial Institute 1640 medium [RPMI] and minimum essential medium [MEM]) or with (RPMI-S and MEM-S) 10% of fetal calf serum (FCS). *B*, MICs were determined in CA-MHB mixed with increasing proportions of RPMI-S. *C*, Influence of FCS (solid line) or human serum (dotted line) on azithromycin MIC against *P. aeruginosa* PAO1. MICs were determined in CA-MHB (open symbols) or RPMI (closed symbols) complemented with the indicated amounts of serum. *D* and *E*, Influence of pH on MIC measured in different media buffered using 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$. *D*, Buffered CA-MHB (CA-MHB$_b$), buffered RPMI-1640 with no bicarbonate supplemented by 10% of fetal calf serum (RPMI$_b$-S), or a 50:50 buffered mixture of these media (CA-MHB$_b$:RPMI$_b$-S [50:50]). *E*, Buffered CA-MHB or buffered RPMI with no bicarbonate supplemented by 75% FCS (CA-MHB$_b$ + FCS [75%] and RPMI$_b$ + FCS [75%]). The arrows point to the pH value measured in nonbuffered CA-MHB (dotted arrow) or RPMI (plain arrow). Abbreviations: BHI, brain heart infusion broth; CA-MHB, cation-adjusted MHB; FCS, fetal calf serum; HS, human serum; LB, Luria Bertani broth; MEM, minimum essential medium; MEM-S, MEM with 10% FCS; MIC, minimum inhibitory concentration; MHB, Muller-Hinton broth; RPMI, Roswell Park Memorial Institute 1640 medium; RPMI-S, RPMI with 10% FCS; TS, tryptic soy broth.

Figure 2. Activity of azithromycin (128 mg/L) against *Pseudomonas aeruginosa* PAO1 over time during successive incubation in cation-adjusted Muller-Hinton broth (CA-MHB; 4 hours) followed by Roswell Park Memorial Institute 1640 medium (RPMI; 20 hours; top left) or in RPMI (4 hours) followed by CA-MHB (20 hours; bottom left) in comparison with continuous incubation either in RPMI (top right) or CA-MHB (bottom right). The ordinate shows the change in colony-forming units (cfu) as compared to the initial inoculum (10^6 cfu/mL). All values are means ± standard deviation (SD; n = 3; when not visible, the SD bars are smaller than the size of the symbols). The horizontal dotted line corresponds to an apparent static effect, and the vertical dotted line in the left panel corresponds to the time at which bacteria were transferred from one medium to the other. Abbreviations: CA-MHB, cation-adjusted Muller-Hinton broth; RPMI, Roswell Park Memorial Institute 1640 medium.

RESULTS

Influence of Culture Medium on the MIC of Azithromycin and Other Macrolides

Figure 1A shows that the MIC of azithromycin against PAO1 was 256 mg/L in CA-MHB and ≥64 mg/L in other broths. In contrast, its MIC was only 2 mg/L when measured in RPMI 1640 medium or MEM (alone or supplemented with 10% fetal calf serum), 2 media commonly used for culturing eukaryotic cells. Figure 1B shows that mixing CA-MHB and RPMI 1640 medium supplemented with 10% fetal calf serum in inversely related proportions resulted in MICs of almost exactly the value anticipated from their respective concentrations on the basis of the previous experiment. Constituents of RPMI 1640 medium added individually to CA-MHB did not modify the azithromycin MIC (Supplementary Table 1). All of these experiments were repeated with clarithromycin, with essentially similar results (data not shown).

Using CA-MHB and RPMI 1640 medium, we extended our observations to erythromycin and ketolides (telithromycin and solithromycin) and to other gram-negative bacteria, again with similar results (Supplementary Table 2). In contrast, no influence of media on the MIC of clindamycin (another inhibitor of protein synthesis binding to the 50S ribosomal subunit) was seen (Supplementary Table 2). Likewise, no decrease in MICs of β–lactams, fluoroquinolones, aminoglycosides, and colistin was observed when PAO1 was incubated in RPMI 1640 medium (data not shown). Last, no influence of the medium on azithromycin MIC for *S. aureus* ATCC25923 was seen (0.5 mg/L in CA-MHB or RPMI 1640 medium).

MIC of Azithromycin in CA-MHB Supplemented With Serum and in Mouse BAL

To check whether the increased susceptibility of *P. aeruginosa* to azithromycin was specific to media used to cultivate eukaryotic cells, we used CA-MHB supplemented with fetal calf serum or human serum and mouse BAL fluid. With serum, the MIC progressively decreased in proportion to the ratio of serum to broth (Figure 1*C*; MICs could not be measured in 100% serum [no bacterial growth]). In contrast, serum did not affect the MIC in RPMI 1640 medium. In BAL fluid, the azithromycin MIC was 16 mg/L. Comparable results were obtained with clarithromycin (data not shown).

Influence of pH

RPMI 1640 medium contains $NaHCO_3$, which causes a slow increase in pH, from approximately 7.4 to approximately 8.3, within 24 hours when incubation is performed in 100% air atmosphere, as done here. Figure 1*D*, however, shows that the azithromycin MIC remained elevated (32 mg/L) in CA-MHB at pH 8.3 (the pH of nonbuffered CA-MHB at 24 hours was 7.9). An MIC of 32 mg/L was already reached at pH 7.5 in RPMI 1640 medium. Intermediate MICs were observed for 50:50 mixtures of both media over the whole pH range. In the presence of 75% serum (Figure 1*E*), an MIC of 4 mg/L was observed in both CA-MHB and RPMI 1640 medium at pH 7.5, ruling out a simple pH effect as the cause of the decrease in MIC in biologically relevant media.

Influence of Culture Medium on Time-Kill Curves

To test whether the increased susceptibility of *P. aeruginosa* to azithromycin in RPMI 1640 medium was a fast-occurring phenomenon, bacteria were exposed to azithromycin first in CA-MHB or in RPMI 1640 medium for 4 hours, using a concentration (128 mg/L) that was subinhibitory in the former medium but far above the MIC in the second, and were then transferred to the other medium for the next 20 hours. As controls, we used bacteria exposed to the same concentration of azithromycin for 24 hours without medium change and bacteria unexposed to azithromycin, with or without medium change. Figure 2 shows that bacteria exposed to azithromycin
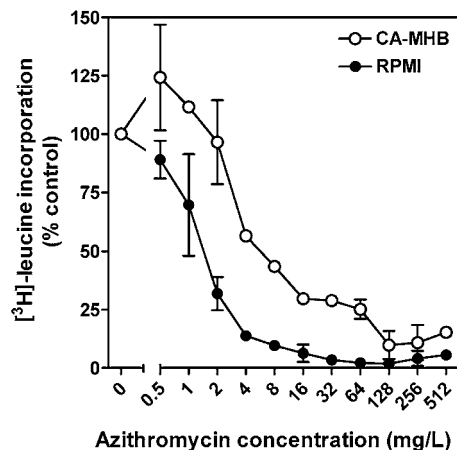

Figure 3. Incorporation of [³H]-leucine in proteins of PAO1 incubated for 4 hours in the presence of increasing concentration of azithromycin in cation-adjusted Muller-Hinton broth (CA-MHB; open symbols) or in Roswell Park Memorial Institute 1640 medium (RPMI; closed symbols); 2 μCi/mL [³H]-leucine was added during the 2 last hours. The pH of the media at the end of the experiment was 7.4 (for CA-MHB) and 7.7 (for RPMI). Data are expressed as the percentage of the value measured in the same medium in the absence of antibiotic and are the mean of triplicates ± standard error of the mean from 2 independent experiments. $P < .001$, by 2-way analysis of variance, with respect to either medium effect or concentration effect. Abbreviations: CA-MHB, cation-adjusted Muller-Hinton broth; RPMI, Roswell Park Memorial Institute 1640 medium.

in CA-MHB for the first 4 hours remained unaffected but were quickly killed after transfer to RPMI 1640 medium. Conversely, bacteria exposed to azithromycin in RPMI 1640 medium for the first 4 hours suffered a loss of about 1.5 $\log_{10}$ cfu but, when transferred to CA-MHB, started growing essentially like the control. No difference in control inoculum was noted between the 2 media at that time point, demonstrating that differences in activity were not related to variations in bacterial growth. Experiments with no medium change showed that bacteria grew slightly slower in RPMI 1640 medium than in CA-MHB and that azithromycin was poorly effective over time in CA-MHB but strongly bactericidal in a time-dependent fashion in RPMI 1640 medium (experiments made at 16 mg/L yielded similar results).

Influence of Culture Medium on the Accumulation of Clarithromycin

The influence of medium on macrolide accumulation in *P. aeruginosa* was tested using [¹⁴C]-labeled clarithromycin (which was the only radiolabeled macrolide available to us). Clarithromycin accumulation measured after 4 and 8 hours of contact was 1.6- and 2.2-fold higher in bacteria incubated in RPMI 1640 medium than in those incubated in CA-MHB.
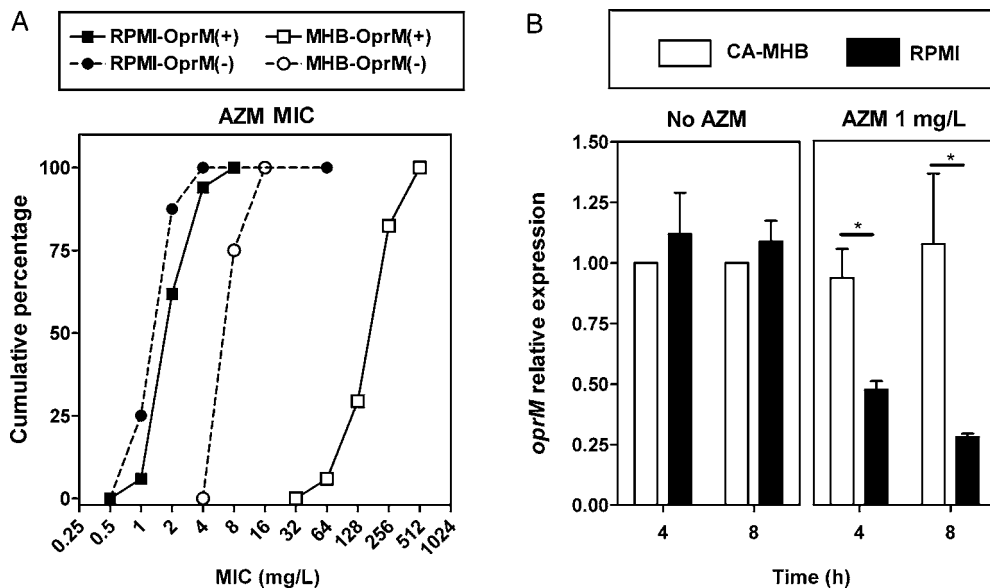
Figure 4. *A*, Cumulative minimum inhibitory concentration (MIC) of azithromycin (AZM) against 42 strains of *Pseudomonas aeruginosa* with different levels of expression of the genes encoding the main efflux systems (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM). Strains have been stratified for positive (squares; solid lines) or negative (circles; dotted lines) expression of *oprM* and for results obtained in cation-adjusted Muller-Hinton broth (CA-MHB; open symbols) or in Roswell Park Memorial Institute 1640 medium (RPMI; closed symbols). Individual MICs and strain phenotypes are presented in Supplementary Table 3. *B*, expression of *oprM* (relative to that measured after 4 hours in CA-MHB in the absence of AZM) in bacteria grown in CA-MHB (open bars) or in RPMI (closed bars) in the absence (left) or presence (right) of 1 mg/L AZM. The pH of the media at the end of the experiment was 7.4 (for CA-MHB) and 7.7 (for RPMI). *P < .05, by the *t* test, comparing expression in the 2 media at the same time point. Abbreviations: CA-MHB, cation-adjusted Muller-Hinton broth; MIC, minimum inhibitory concentration; RPMI, Roswell Park Memorial Institute 1640 medium.

Influence of Culture Medium on the Inhibition of Protein Synthesis by Azithromycin

Figure 3 shows that [^{3}H]-leucine incorporation in newly synthesized proteins was inhibited by lower concentrations of azithromycin with PAO1 incubated in RPMI 1640 medium versus CA-MHB, indicating a more effective inhibition of protein synthesis in the first medium.

Influence of *oprM* Expression and of the Addition of PaβN on MICs of Macrolides and Ketolides

The MICs of azithromycin and other macrolides and ketolides was measured against a panel of 42 strains with distinct expression of the main efflux systems in *P. aeruginosa* (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM; Supplementary Table 3). Strains with a deletion in *oprM* all showed a low MIC in CA-MHB (illustrated for azithromycin in Figure 4A). In contrast, strains with deletions in other genes all had a high MIC for azithromycin (and for other macrolides and ketolides) in CA-MHB. Of interest, some of the strains with the *oprM* deletion still showed MICs that were 1 or 2 dilutions lower in RPMI 1640 medium than those measured for strains with no OprM deletion (Figure 4A and Supplementary Table 3). This indicates that OprM-dependent efflux could still operate but at a low level in RPMI 1640

medium (this was even more evident for other macrolides and ketolides; Supplementary Table 3). Last, addition of the broad-spectrum efflux inhibitor PaβN to PAO1 in CA-MHB reduced the MIC of azithromycin to 4 mg/L but had no effect on its MIC in RPMI 1640 medium (data not shown).

Impairment of *oprM* Expression by Azithromycin in RPMI 1640 Medium

The expression of *oprM* in PAO1 was measured after 4 or 8 hours of incubation in CA-MHB or in RPMI 1640 medium (Figure 4B). There was no difference in *oprM* expression for bacteria cultivated in the absence of antibiotic. However, the addition of a subinhibitory concentration of azithromycin (1 mg/L) caused a marked decrease of *oprM* expression in bacteria grown in RPMI 1640 medium but no change in those grown in CA-MHB.

Influence of the Culture Medium on Outer-Membrane Permeability

Figure 5 shows that the permeability of PAO1 to NPN and nitrocefin was markedly increased in RPMI 1640 medium, reaching 90% and 60%, respectively, of the maximal value recorded in the presence of EDTA. NPN permeability was also increased in bacteria grown in mouse BAL. In an independent approach, we
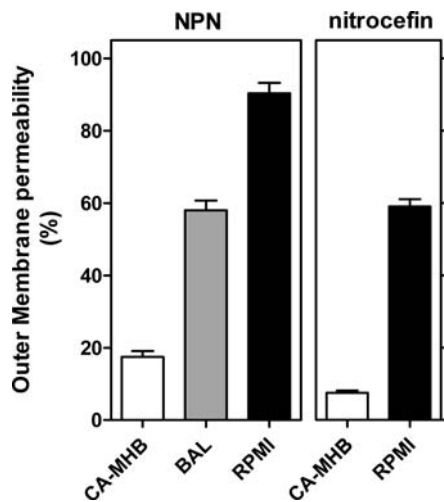
Figure 5. Outer-membrane permeability of *Pseudomonas aeruginosa* PAO1 to 1-*N*-phenylnaphthylamine (NPN; 25 µM; left panel) or to nitrocefin (100 µM; right panel). Bacteria were incubated for 4 hours in cation-adjusted Muller-Hinton broth (CA-MHB; open bars), bronchoalveolar lavage fluid (BAL; grey bar), or Roswell Park Memorial Institute 1640 medium (RPMI; black bar). Data are expressed as a percentage of the maximal value recorded in the presence of 10 mM EDTA in 10 mM Tris buffer (pH 8) and are the mean ± standard deviation of 3 independent determinations. No significant difference was found when permeability to nitrocefin was measured in RPMI without $NaHCO_3$ and buffered at pH 7.4 as compared to nonbuffered RPMI. $P < .001$, by analysis of variance and the Tukey post-hoc test, for comparisons of RPMI and CA-MHB for both NPN and nitrocefin and for comparison of BAL with CA-MHB for NPN. Abbreviations: BAL, bronchoalveolar lavage fluid; MHB, Muller-Hinton broth; NPN, 1-N-phenylnaphthylamine; RPMI, Roswell Park Memorial Institute 1640 medium.

examined whether agents disrupting the outer membrane of *P. aeruginosa* would be synergistic with azithromycin in CA-MHB. This was the case for colistin [24], the ceragenin CSA-13 [25], and EDTA [26], with ΣFIC indices (± standard deviation) of 0.52 ± 0.03, 0.23 ± 0.04, and 0.07 ± 0.01, respectively. These combinations were indifferent in RPMI 1640 medium.

DISCUSSION

This article shows that macrolides and ketolides but not any of the other antibiotics tested (ie, clindamycin and antipseudomonal drugs) have considerably lower MICs against *P. aeruginosa* when assayed in media used for culturing eukaryotic cells, broth supplemented with serum, or BAL fluid, compared with CA-MHB (recommended for in vitro susceptibility testing studies [5]) or other broths. Since antibiotic susceptibility is a key property to consider when assessing their clinical usefulness [27], our observations open potentially important

perspectives concerning the clinical benefit of macrolides and ketolides for patients with pseudomonal infections.

New, unanticipated observations with the potential to modify current therapeutic approaches need critical examination concerning reproducibility, consistency and specificity, underlying mechanism, and scope of application.

Our studies yielded reproducible and consistent observations when comparing conventional microbiological media and media with a composition close to that of eukaryotic environments. They could be extended to a large array of clinical strains of *P. aeruginosa*, other gram-negative organisms, and macrolides and ketolides but not to other antibiotics or gram-positive bacteria. Taken globally, they show that the activity of macrolides and ketolides is impaired in broths as compared to media closer to the eukaryotic environment. The data shows that MICs in CA-MHB are not decreased by addition of individual constituents found in RPMI 1640 medium (making unlikely a mechanism based on simple drug complexation) and that a progressive replacement of CA-MHB by serum brings MICs to values close to those measured in RPMI 1640 medium, whereas serum does not affect MICs measured in RPMI 1640 medium. RPMI 1640 medium and serum effects are observed over a broad pH range, demonstrating that macrolide protonation state is not the main determinant in the increased activity observed in biological media but is rather another, independent mechanism that modulates macrolide activity. Inhibition of activity in CA-MHB requires direct contact and is transient, since it appears and/or is quickly lost when bacteria are transferred from CA-MHB to RPMI 1640 medium or vice versa, and is independent of bacterial growth rate.

Two nonmutually exclusive molecular mechanisms may account for these effects, namely, impairment of efflux of macrolides and increased outer-membrane permeability to these antibiotics.

Evidence for an active efflux in CA-MHB can be deduced from the facts that the efflux pump inhibitor PAβN brings MICs measured in CA–MHB to values close to those measured in RPMI 1640 medium and that disruption of *oprM* (encoding the outer-membrane protein working in conjunction with the 2 constitutively expressed multidrug efflux pumps, MexB and MexY [28]) drastically reduces MICs in CA-MHB. Erythromycin is a substrate for MexAB-OprM, MexCD-OprJ, and MexXY-OprM but not for MexEF-OprN in *P. aeruginosa* [29, 30], and MexAB-OprM and MexXY-OprM are involved in intrinsic resistance of *P. aeruginosa* to macrolides [30]. In line with these observations, we noted a significant reduction in the expression of *oprM* for bacteria incubated with a sub-MIC of azithromycin (1 mg/L) in RPMI 1640 medium. As OprM works in conjunction with the 2 constitutive transporters involved in macrolide efflux, reduction in its expression is anticipated to have important effects. Indeed,
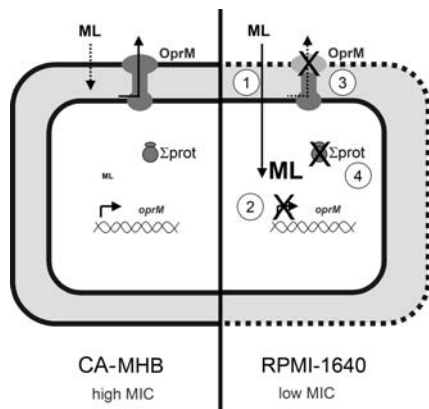
Figure 6. Model depicting the proposed mechanisms contributing to the differences in susceptibility of *Pseudomonas aeruginosa* to macrolides (MLs) in cation-adjusted Muller-Hinton broth (CA-MHB; left) and in a culture medium of eukaryotic cells (Roswell Park Memorial Institute 1640 medium [RPMI]). In CA-MHB (the recommended medium to assess *P. aeruginosa* susceptibility to antibiotics, according to the Clinical and Laboratory Standards Institute), MLs show a high minimum inhibitory concentration because of a poor diffusibility through the outer membrane (depending on the protonation state of the macrolide) and because of active efflux by the constitutively expressed efflux systems MexAB-OprM and MexXY-OprM, which in combination ensures a low intrabacterial antibiotic concentration. In RPMI, the outer membrane becomes more permeable, which favors macrolide penetration within bacteria (1), allows them to impair, through a still undefined mechanism, the expression of *oprM* (2), and therefore reduces the activity of the efflux systems MexAB-OprM and MexXY-OprM (3). In combination, steps 1 and 4 allows for an increased intracellular accumulation of MLs and allows them to impair protein synthesis at lower extrabacterial concentrations (4) and, thereby, to exert true antibacterial activity. Abbreviations: MHB, Muller-Hinton broth; MIC, minimum inhibitory concentration; ML, macrolides; NPN, 1-N-phenylnaphthylamine; RPMI, Roswell Park Memorial Institute 1640 medium.

we showed an increased drug accumulation in bacteria incubated in RPMI 1640 medium versus CA-MHB (demonstrated for clarithromycin), together with an inhibition of protein synthesis upon exposure to lower antibiotic concentrations, as previously described [31]. The increase in clarithromycin bacterial accumulation may seem too modest to account for the change in MIC observed. Yet, similar values were observed when comparing MICs and accumulation of ciprofloxacin in *P. aeruginosa* expressing efflux systems at different levels [32]. However, the presence of azithromycin (presumably in bacteria) is necessary to induce downregulation of *oprM*. This implies that an additional mechanism must first ensure a larger initial penetration of the antibiotic in bacteria grown in eukaryotic media versus CA-MHB.

Evidence of an increased permeability of the outer membrane of *P. aeruginosa* when grown in RPMI 1640 medium or BAL fluid versus CA-MHB was documented by the increased

accumulation of NPN and nitrocefin. While the magnitude of the effect depends on the size of the marker, it remains significant for a molecule with a molecular weight of 515 Da (nitrocefin), which is close to that of macrolides (around 740 Da). Indirect confirmation of the low outer-membrane permeability of *P. aeruginosa* in CA-MHB as compared to RPMI 1640 medium stems from the synergistic effect seen between membrane-destabilizing agents (colistin and CSA-13) with azithromycin in CA-MHB (but not or only to a minimal extent in RPMI 1640 medium).

Macrolides poorly diffuse across the outer membrane of most gram-negative bacteria [21]. Our experiments bring complementary pieces of evidence supporting the concept that high susceptibility of *P. aeruginosa* to macrolides in RPMI 1640 medium and other eukaryotic media as compared to broths is probably related to an increase in their accumulation within the bacteria, consecutive to an alteration of the outer-membrane integrity (mediated by the nature of the medium in contact with bacteria) combined with an impairment of their active efflux. Figure 6 presents a schematic view of these proposed mechanisms, which, notably, have been evidenced at concentrations (0.5–4 mg/L) relevant to those reached in the serum or tissues of patients receiving azithromycin at the registered dosage [33].

Pending further confirmatory studies, the present work opens new perspectives as to why macrolides may exert in vivo antibacterial properties against *P. aeruginosa* that would not be predicted on the basis of their reported activities in conventional antibiotic susceptibility testing systems. Our work, therefore, also suggests the need to actively develop more-pertinent in vitro models for susceptibility testing of antibiotics.

Supplementary Data

Notes

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Increased Susceptibility of *Pseudomonas aeruginosa* to Macrolides and Ketolides in Eukaryotic Cell Culture Media and Biological Fluids (Serum, Bronchoalveolar Lavage) by Decreased Expression of *oprM* (efflux impairment) and Increased Outer Membrane Permeability

Supplementary Material

Table S1. MIC of azithromycin against *P. aeruginosa* PAO1 as measured in CA-MHB supplemented with specific constituents of RPMI medium.

RPMI constituent added to CA-MHB[a]	concentration	MIC (mg/L)
none	-	128
D-glucose	2000 mg/L	64
Ca(NO3)$_2$	0.424 mg/L	128
MgSO$_4$	0.407 mg/L	128
KCl	5.33 mg/L	128
NaCl	103.45 mg/L	128
thiamine hydrochloride	0.00297 mg/L	128
niacinamide	0.0082 mg/L	128
pyridoxine	0.00485 mg/L	128
non essential amino acids	1 %	256
salt mixture [Ca(NO3)$_2$, MgSO$_4$, KCl, NaCl]	same as above [b]	128
vitamin mixture [thiamine, niacinamide, pyridoxine]	same as above [b]	64
phenol red	0.0133 mg/L	128

[a] each constituent was added to CA-MHB (for mixtures, in the order indicated in the Table), and the supplemented medium carefully mixed before being used for MIC determination.

[b] with respect to individual constituents

Table S2: MIC (mg/L) of macrolides, ketolides and clindamycin against representative Gram-negative bacteria, as determined in CA-MHB (MHB) and RPMI.

Strains	erythromycin		clarithromycin		azithromycin		telithromycin		solithromycin		clindamycin	
	MHB	RPMI	MHB	RPMI	MHB	RPMI	MHB	RPMI	MHB	RPMI	MHB	RPMI
P. aeruginosa ATCC PAO1*	512	16	512	16	128	2	64	4	32	2	>512	>512
E. coli ATCC 25922*	64	4	64	4	84	4	16	8	8	8	256	256
A. baumannii ACA001[†]	16	0.25	16	0.5	0.25	0.125	1	0.25	2	0.25	2	2
K. pneumoniae ATCC700603*	256	16	512	16	128	8	128	16	128	32	512	512
K. pneumoniae 21823917[†]	128	4	256	8	8	8	32	16	32	16	512	256
E. aerogenes ATCC13048*	256	16	512	16	16	8	32	16	32	16	256	256
E. aerogenes 21824238[†]	128	8	512	8	16	8	32	16	16	16	256	256
S. marcescens 21823969[†]	256	16	512	16	64	8	128	32	128	16	256	256

* Wild-type strain, [†] Clinical strain kindly provided by H. Rodriguez, Microbiology laboratory, cliniques universitaires St Luc, UCL, Brussels.

Table S3. MIC (mg/L) of macrolides and ketolides against 42 strains of *P. aeruginosa* with different expression levels for multidrug efflux pumps, as determined in CA-MHB or in RPMI. Strains highlighted in gray are disrupted in *oprM.*

Strains	Reference or origin	Description	AB	CD	EF	XY	ERY MHB	ERY RPMI	CLR MHB	CLR RPMI	AZM MHB	AZM RPMI	TEL MHB	TEL RPMI	SOL MHB	SOL RPMI
Br667	[1]	Clinical strain	≥ 2	+	-	≥ 5	512	128	512	128	512	4	128	4	128	8
63	-	Clinical strain	< 2	-	-	≥ 5	512	64	512	32	256	2	128	4	64	4
207	-	Clinical strain	nd	nd	nd	nd	512	128	512	64	512	4	128	4	32	4
48	-	Clinical strain	< 2	+	-	≥ 5	512	64	512	64	256	2	128	8	128	4
49	-	Clinical strain	< 2	+	-	≥ 5	512	64	512	32	256	2	128	4	128	4
11	-	Clinical strain	≥ 2	-	-	≥ 5	256	16	512	16	128	2	64	1	16	2
266B	-	Clinical strain	nd	nd	nd	nd	512	64	512	64	256	2	256	4	256	4
333A	-	Clinical strain	≥ 2	-	-	< 5	512	64	512	64	256	2	64	2	128	2
335	-	Clinical strain	nd	nd	nd	nd	512	64	512	64	512	4	128	4	128	2
16	-	Clinical strain	nd	nd	nd	nd	512	32	512	64	256	4	128	4	32	4
68	-	Clinical strain	< 2	-	-	≥ 5	512	64	512	64	256	2	128	4	64	4
168B	-	Clinical strain	< 2	-	-	< 5	512	256	512	256	512	4	256	4	128	4
133	-	Clinical strain	nd	nd	nd	nd	512	64	512	64	256	4	128	4	64	4
156	-	Clinical strain	< 2		+	< 5	512	16	512	32	512	4	128	2	64	2
JFL 30	[2]	MexAB overproducer	≥ 2	-	-	< 5	512	8	512	32	128	4	128	4	128	2
PT629	[3]	MexAB overproducer	≥ 2	-	-	< 5	512	64	512	64	256	2	64	2	128	4
JFL 28	[2]	MexCD overproducer	< 2	+	-	< 5	512	256	512	128	512	4	256	16	256	8
eryR	[3]	MexCD overproducer	< 2	+	-	< 5	512	64	512	64	256	2	256	4	256	4
JFL 05	[2]	MexEF overproducer	< 2	-	+	< 5	256	8	256	16	64	2	64	0.25	8	1
PAO7 H	[3]	MexEF overproducer	< 2	-	+	< 5	256	32	256	16	256	2	64	2	32	4
JFL 10	[2]	MexXY overproducer	< 2	-	-	≥ 5	512	32	512	32	256	2	128	4	128	4
MutGr1	[3]	MexXY overproducer	< 2	-	-	≥ 5	512	32	512	32	256	2	128	2	64	4
PAO1	ATCC		< 2	-	-	< 5	512	32	512	32	256	4	256	2	128	4

Strain	Ref	Genotype					ERY		CLA		AZM		TEL		SOL	
FB1	[2]	PAO1 Δ (mexb::FRT)					256	16	512	8	128	2	128	2	64	1
PAO1 mexAB	[4]	PAO1 Δ (mexAB::FRT)	0	-	+	< 5	256	4	512	4	256	1	128	1	16	1
PAO 200	[4]	PAO1 Δ (mexAB-oprM)	0	-	-	< 5	16	2	16	2	8	1	8	0.03	2	1
CB 536	[5]	PAO1 Δ (mexCD-oprJ)	< 2	-	+	< 5	256	16	256	16	128	2	32	0.5	16	1
PAO 238	[6]	PAO1 Δ (mexAB-OprM, MexCD-oprJ)	0	-	-	< 5	16	4	32	4	16	1	8	0.25	4	2
CB603	[5]	PAO1 Δ (mexEF-oprN)	< 2	-	+	< 5	256	32	256	16	128	2	64	2	32	2
CB602	[5]	PAO1 Δ (mexXY::FRT)	< 2	-	-	< 5	256	8	512	8	256	8	64	2	16	2
PAO 280	[6]	PAO1 Δ (mexAB-OprM, MexXY::FRT)	0	-	-	≥ 5	16	4	16	4	8	2	8	0.25	4	1
CB 494	[5]	PAO1 Δ (mexJK)	≥ 2	-	-	< 5	512	32	512	32	256	4	64	1	32	4
CB462	[5]	PAO1 Δ (mexHI-opmD)	< 2	-	-	< 5	256	32	256	32	128	2	32	2	64	2
CB480	[5]	PAO1 Δ (opmH)	< 2	-	+	< 5	128	16	64	16	64	2	16	0.25	8	2
PAO 1048	[4]	PAO1 Δ (mexAB, opmH::FRT)	0	-	+	< 5	256	4	512	4	256	1	128	1	16	1
PAO 386	[6]	PAO1 Δ (mexAB-oprM, nfxB, mexCD-oprJ, mexJKL, mexXY, opmH ::FRT)	0	-	-	~ 5	8	4	8	2	8	2	4	1	2	2
PAO 255	[6]	PAO7 H Δ (MexAB-oprM)::FRT, mexEF-oprN)	0	-	-	< 5	16	4	16	4	16	2	4	0.25	8	1
PAO 509	[4]	PAO1 Δ (MexAB-oprM), MexCD-oprJ, MexJK, MexXY, MexEF-oprN)	0	-	-	< 5	16	4	16	4	8	2	4	0.5	4	1
PAO 509.5	[4]	PAO 509 TriABC+	0	-	-	≥ 5	8	4	8	4	8	2	8	0.25	4	1
4098	[7]	PAO1					256	64	128	32	128	2	128	4	64	4
4098[E]	[7]	Overexpression of MexAB-oprM					256	256	256	32	128	8	128	32	64	16
4098ET	[7]	Δ OprM					128	64	32	8	8	4	8	4	8	1

ERY: erythromycin; CLA: clarithromycin; AZM: azithromycin; TEL: telithromycin; SOL: solithromycin

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