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RX-P873, a Novel Protein Synthesis Inhibitor, Accumulates in Human THP-1 Monocytes and Is Active against Intracellular Infections by Gram-Positive (*Staphylococcus aureus*) and Gram-Negative (*Pseudomonas aeruginosa*) Bacteria

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The pyrrolocytosine RX-P873, a new broad-spectrum antibiotic in preclinical development, inhibits protein synthesis at the translation step. The aims of this work were to study RX-P873's ability to accumulate in eukaryotic cells, together with its activity against extracellular and intracellular forms of infection by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, using a pharmacodynamic approach allowing the determination of maximal relative efficacies (E_{max} values) and bacteriostatic concentrations (C_s values) on the basis of Hill equations of the concentration-response curves. RX-P873's apparent concentration in human THP-1 monocytes was about 6-fold higher than the extracellular one. In broth, MICs ranged from 0.125 to 0.5 mg/liter (*S. aureus*) and 2 to 8 mg/liter (*P. aeruginosa*), with no significant shift in these values against strains resistant to currently used antibiotics being noted. In concentration-dependent experiments, the pharmacodynamic profile of RX-P873 was not influenced by the resistance phenotype of the strains. E_{max} values (expressed as the decrease in the number of CFU from that in the initial inoculum) against *S. aureus* and *P. aeruginosa* reached more than 4 log units and 5 log units in broth, respectively, and 0.7 log unit and 2.7 log units in infected THP-1 cells, respectively, after 24 h. C_s values remained close to the MIC in all cases, making RX-P873 more potent than antibiotics to which the strains were resistant (moxifloxacin, vancomycin, and daptomycin for *S. aureus*; ciprofloxacin and ceftazidime for *P. aeruginosa*). Kill curves in broth showed that RX-P873 was more rapidly bactericidal against *P. aeruginosa* than against *S. aureus*. Taken together, these data suggest that RX-P873 may constitute a useful alternative for infections involving intracellular bacteria, especially Gram-negative species.

Bacterial resistance is spreading worldwide, which makes therapeutic options scarce in many circumstances and can lead to therapeutic failures. In this context, the discovery and development of antibiotics acting on novel, still unexploited targets are recognized to be priorities by both European and American agencies or by scientific societies (1–3).

The pyrrolocytosine RX-P873 (see structure, calculated ionization status, and octanol-water partition coefficients at specified pH values [log *D* values] in Fig. 1) is a new antibiotic in preclinical development that shows an innovative mode of action, inspired by the analysis of the crystal structure of the translation inhibitor blasticidin (4) in its interaction with peptidyl-tRNA. RX-P873 inhibits bacterial protein synthesis at the translation step by stabilizing a distorted binding conformer of peptidyl-tRNA (5). Preliminary data with this compound and others in the series report that they have (i) a broad spectrum of activity, including activity against multidrug-resistant Gram-positive or Gram-negative organisms as well as biodefense pathogens (6–9), and (ii) bactericidal activity (10, 11), which is classically not observed for inhibitors of protein synthesis, except aminoglycosides.

Intracellular survival is clearly part of the life cycle of biodefense pathogens, like *Bacillus anthracis* (12), *Yersinia pestis* (13), *Francisella tularensis* (14), and *Burkholderia pseudomallei* or *Burkholderia mallei* (15). It is also widely recognized to be a reason for the recurrence or persistence of infections caused by common human pathogens, like *Staphylococcus aureus* (16, 17) or *Pseudomonas aeruginosa* (18, 19).

As a first attempt to determine the potential ability of RX-P873 to act upon these intracellular bacteria, the aim of the present study was to determine the intracellular activity of this molecule (compared to its activity in broth) against *S. aureus* and *P. aeruginosa*, taken as exemplary Gram-positive and Gram-negative bacteria, respectively, in relation to its capacity to accumulate within phagocytic cells. Using multiresistant strains and previously developed *in vitro* pharmacodynamic models to assess the intracellular activities of antibiotics (18, 20), we show that the activity of RX-P873 in human THP-1 macrophages is unaffected by mechanisms of resistance to other drugs commonly used to treat infections caused by these organisms. RX-P873 proved bactericidal in broth. In cells, RX-P873 was bacteriostatic at an extracellular concentration close to its MIC and reduced by 1 to 3 log CFU the intracellular inocula of *S. aureus* and *P. aeruginosa*, irrespective of their profiles of resistance to other antibiotics. This intracellular

Received 19 February 2015 **Accepted** 24 May 2015

Accepted manuscript posted online 26 May 2015

Citation Buyck JM, Peyrusson F, Tulkens PM, Van Bambeke F. 2015. RX-P873, a novel protein synthesis inhibitor, accumulates in human THP-1 monocytes and is active against intracellular infections by Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria. Antimicrob Agents Chemother 59:4750–4758. doi:10.1128/AAC.00428-15.

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doi:10.1128/AAC.00428-15

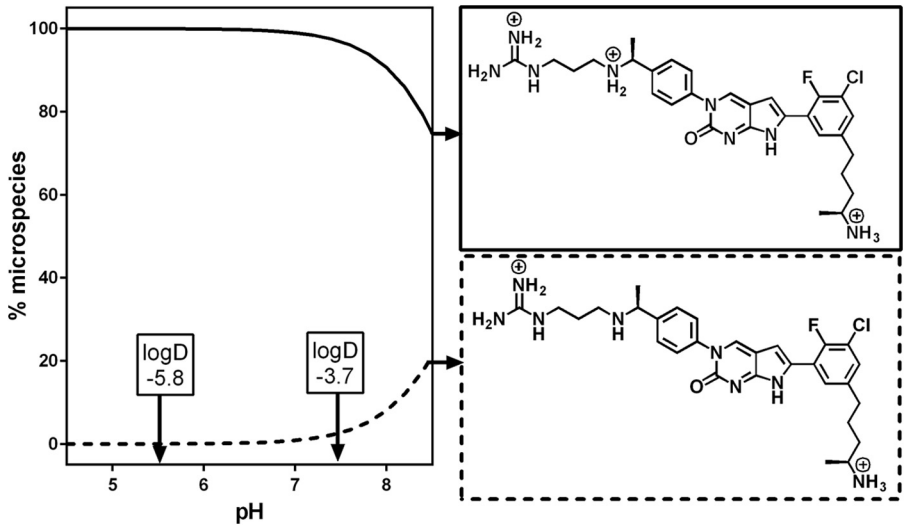

FIG 1 Chemical structure and ionization status of RX-P873. The graph shows the evolution of the proportions of the two major microspecies of the molecule in the range of pHs that it could face in biological environments, as well as log *D* values calculated at pH 5.5 and 7.4 (using Reaxys software).

activity of RX-P873 may be related to its ability to accumulate approximately 6-fold within THP-1 monocytes.

MATERIALS AND METHODS

Cells. Studies were performed using THP-1 human monocytes (21) grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere.

Cellular accumulation of RX-P873. THP-1 cells at a density of 3×10^6 cells/ml were incubated with RX-P873 for 2 h, collected by low-speed centrifugation, washed twice in cold phosphate-buffered saline (PBS), pelleted, resuspended in 1 ml H_2O, and frozen at −20°C. Samples were unfrozen on the day that they were assayed, lysed by sonication, and kept on wet ice. Standards were prepared from cell lysates spiked with known amounts of RX-P873 and treated like the samples were to construct calibration curves. RX-P873 was assayed by fluorimetry. Proteins from samples and standards were precipitated using acetonitrile (800 μl added to 400 μl of cell lysate, incubation for 1 h at −20°C). Samples were then centrifuged for 10 min at 14,000 × *g*; supernatants were collected, evaporated to dryness, and reconstituted in 100 μl acetonitrile. Fifty microliters of standards or of samples was then transferred to 96-well plates, and the fluorescence was measured (excitation λ = 280 nm, emission λ = 450 nm) using a SpectraMax reader (Molecular Devices, Sunnyvale, CA). The assay was linear in the 0.2- to 10-mg/liter concentration range (R^2 = 0.9959; lower limit of quantification, 0.2 mg/liter).

RX-P873 concentrations in samples were expressed by reference to the total protein content, as determined by the Folin-Ciocalteu/biuret method (500-0113 and 500-0114 kits; Bio-Rad, Hercules, CA).

Bacterial strains. The strains used in this study are described in Table 1. They included reference strains and clinical isolates obtained via collaborating clinical microbiology laboratories. The bacteria were routinely grown in Mueller-Hinton broth, and counting of the number of CFU was performed by plating on tryptic soy agar.

Determination of MICs and concentration-kill curve studies in extracellular medium. MICs were measured by serial 2-fold microdilution according to CLSI guidelines in cation-adjusted Mueller-Hinton broth (22). Extracellular activity was assessed with a starting inoculum of 10^6 CFU/ml in Mueller-Hinton broth, as previously described (18, 20).

Assessment of cell viability. The viability of THP-1 cells was evaluated by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) in the culture medium (Cytotoxicity Detection Kit^{Plus} [LDH]; Roche, Diagnostics GmbH, Manheim, Germany) after 24 h of incubation in the presence of increasing concentrations of RX-P873.

Intracellular infection and assessment of antibiotic intracellular activity. Intracellular infection and assessment of antibiotic intracellular activity were performed according to the procedures previously described in detail for *Staphylococcus aureus* (20) and *Pseudomonas aeruginosa* (18).

In brief, bacteria were opsonized by a 30-min (*S. aureus*) or 60-min (*P. aeruginosa*) incubation at 37°C with 10% human serum in RPMI 1640. Phagocytosis was then allowed for 1 h with an inoculum of 4 bacteria per cell for *S. aureus* and for 2 h with an inoculum of 10 bacteria per cell for *P. aeruginosa*. Medium was removed, and cells were washed once with PBS and incubated for 45 min with gentamicin at 100× its MIC to eliminate extracellular bacteria, washed 3 times with PBS to eliminate gentamicin, and reincubated for 24 h with increasing concentrations of antibiotics. At the end of the incubation period, cells were washed with PBS and collected in H_2O. The number of CFU was determined by plating, and proteins were assayed by the method of Lowry. Data are expressed as the change from the initial postphagocytosis inoculum (which was typically ~10^6 CFU/mg cell protein).

Materials. RX-P873 was provided by Melinta Therapeutics (New Haven, CT). The other antibiotics were obtained as microbiological standards from their corresponding manufacturers (ciprofloxacin [chlorhydrate; potency, 85%] and moxifloxacin [chlorhydrate; potency, 91%] were from Bayer AG; Wuppertal, Germany) or as commercial products, registered in Belgium for parenteral use, from their respective marketing authorization holders or resellers: gentamicin as Gentalline (Schering-Plough, Brussels, Belgium), vancomycin (Mylan, Hoeilaart, Belgium), linezolid as Zyvoxid (Pfizer Inc., Brussels, Belgium), daptomycin as Cubicin (Cubist, Paris, France), and ceftazidime as Glazidim (GlaxoSmithKline, Genval, Belgium). Colistin (sulfate salt; potency, 67.50%) and oxacillin (potency, 81.5%) were purchased from Sigma-Aldrich (St. Louis, MO). Unless stated otherwise, all other reagents were of analytical grade and were purchased from Sigma-Aldrich-Fluka. Cell culture or microbiology media were from Invitrogen (Paisley, Scotland) and BD Diagnostics (Sparks, MD).

Statistical analyses, curve fittings, and software. Statistical analyses, curve fittings, and calculations of the corresponding regression parameters were performed using GraphPad Prism (version 6.05) software for Windows (GraphPad Prism Software, San Diego, CA). More specifically, the Hill equations of the concentration-response curves were used to cal-

TABLE 1 Susceptibility of reference strains and clinical isolates to RX-P873 and comparators[a]

Strain	Description (reference)	MIC (mg/liter)[b]									
		RX-P873	OXA	MXF	VAN	LZD	DAP	GEN	CIP	CAZ	CST
S. aureus											
ATCC 25923	MSSA reference strain	0.5	0.25	0.06	1	2	0.125				
ATCC 33591	MRSA reference strain	0.125	**>256**	0.06	1	2	0.5				
SA618bis	Clinical isolate (35)	0.25	**256**	**4**	**4**	2	**32**				
NRS119	Clinical isolate, NARSA (36)	0.125	**>256**	**4**	1	**64**	**2**				
MG1	Clinical isolate (37)	0.125	0.125	0.5	1	2	0.5				
NRS52	Clinical isolate, NARSA (38)	0.125	0.5	**16**	1	2	1				
HMC549	Clinical isolate (39)	0.125	**128**	**8**	**4**	2	1				
CM05	Clinical isolate (40)	0.125	**>256**	**4**	1	**16**	0.5				
P. aeruginosa											
PAO1	Wild-type strain	2						2	0.125	2	1
PAO509	PAO1 Δ(*mexAB-oprM*) Δ(*mexCD-oprJ*) Δ*mexJK* Δ*mexXY* Δ(*mexEF-oprN*) (41)	0.5						0.25	0.015	1	1
PA50	Clinical isolate (42)	8						4	**16**	**32**	1
PA125	Clinical isolate (42)	4						4	0.25	8	1
PA256	Clinical isolate (43)	4						16	**64**	**32**	4
PA291	Clinical isolate (42)	2						2	**8**	**32**	1
Br667cet	Clinical isolate (44)	4						**>512**	**>64**	**64**	2

[a] Abbreviations: MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; DAP, daptomycin; GEN, gentamicin; LZD, linezolid; MXF, moxifloxacin; OXA, oxacillin; VAN, vancomycin.
[b] Values in bold are higher than the EUCAST susceptibility breakpoint for registered antibiotics (resistant strain).

culate the maximal efficacy (E_{max}; the maximal decrease in CFU counts [in $\log_{10}$ units] from those in the corresponding initial inoculum extrapolated from an infinitely large antibiotic concentration) and the bacteriostatic concentration (C_s; the extracellular concentration resulting in no apparent bacterial growth [the number of CFU is identical to that in the initial inoculum]) of each drug for each strain. The physicochemical properties of the antibiotics were calculated using Reaxys software (version 2014; Elsevier).

RESULTS

Accumulation of RX-P873 in THP-1 human monocytes. Figure 2 shows the accumulation of RX-P873 in THP-1 cells incubated with different extracellular concentrations of RX-P873 for 2 h. The cellular concentration of the antibiotic increased linearly as a function of the extracellular one over the concentration range investigated, leading to an apparent accumulation factor similar for the three extracellular concentrations tested (mean value, 6.3 ± 0.7).

MICs of RX-P873 and comparator antibiotics. Table 1 shows the MICs of RX-P873 in comparison with those of antibiotics representative of the main classes currently used in clinics (moxifloxacin, oxacillin, linezolid, daptomycin, and vancomycin for *S. aureus*; gentamicin, ciprofloxacin, ceftazidime, and colistin for *P. aeruginosa*) against a series of laboratory and clinical strains of *S. aureus* and *P. aeruginosa*. Considering first the activity against *S. aureus*, the MICs of RX-P873 ranged from 0.125 to 0.5 mg/liter, irrespective of the phenotype of resistance to other antibiotics of the strains. Against *P. aeruginosa*, the MICs were higher (0.5 to 8 mg/liter), and again, the activity was not affected by resistance to other drugs. Of note, the lowest MIC was observed for strain PAO509, which does not express active efflux systems.

Intracellular activity of RX-P873 against *S. aureus* and *P. aeruginosa*. The intracellular activity of RX-P873 against reference strains (*S. aureus* ATCC 25923 and *P. aeruginosa* PAO1) or selected clinical isolates showing multidrug resistance (vancomycin-intermediate *S. aureus* SA618bis, which is also resistant to moxifloxacin and daptomycin; methicillin-resistant *S. aureus* NRS119, which is also resistant to moxifloxacin and linezolid; *P. aeruginosa* PA256, which is resistant to ceftazidime and ciprofloxacin) or hypersusceptibility due to the absence of efflux (*P. aeruginosa* PAO509) was then evaluated. For this, infected THP-1 cells were exposed for 24 h to a broad range of antibiotic concentrations in order obtain full dose-response effects and to calculate the corresponding pertinent pharmacodynamic pa-

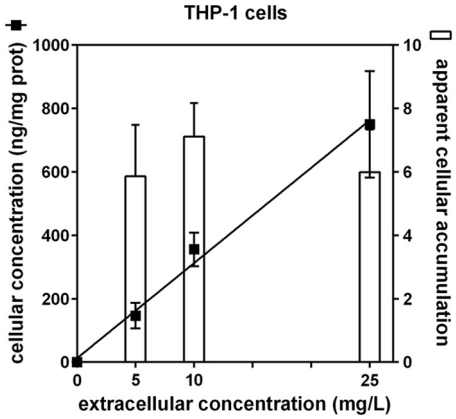

FIG 2 Apparent cellular accumulation of RX-P873 in THP-1 cells incubated for 2 h with RX-P873 at increasing extracellular concentrations. The left axis shows the cellular concentration of the drug expressed in nanograms per milligram of cell protein (prot). The data were used to fit a linear regression with a slope of 30.0 ± 1.9 ng ml^{-1}/mg $liter^{-1}$ and an R^2 value of 0.9923. The right axis shows the apparent cellular accumulation factor calculated using a conversion factor of 5 μl/mg cell protein. All values are means ± standard deviations (SDs) from three independent determinations.

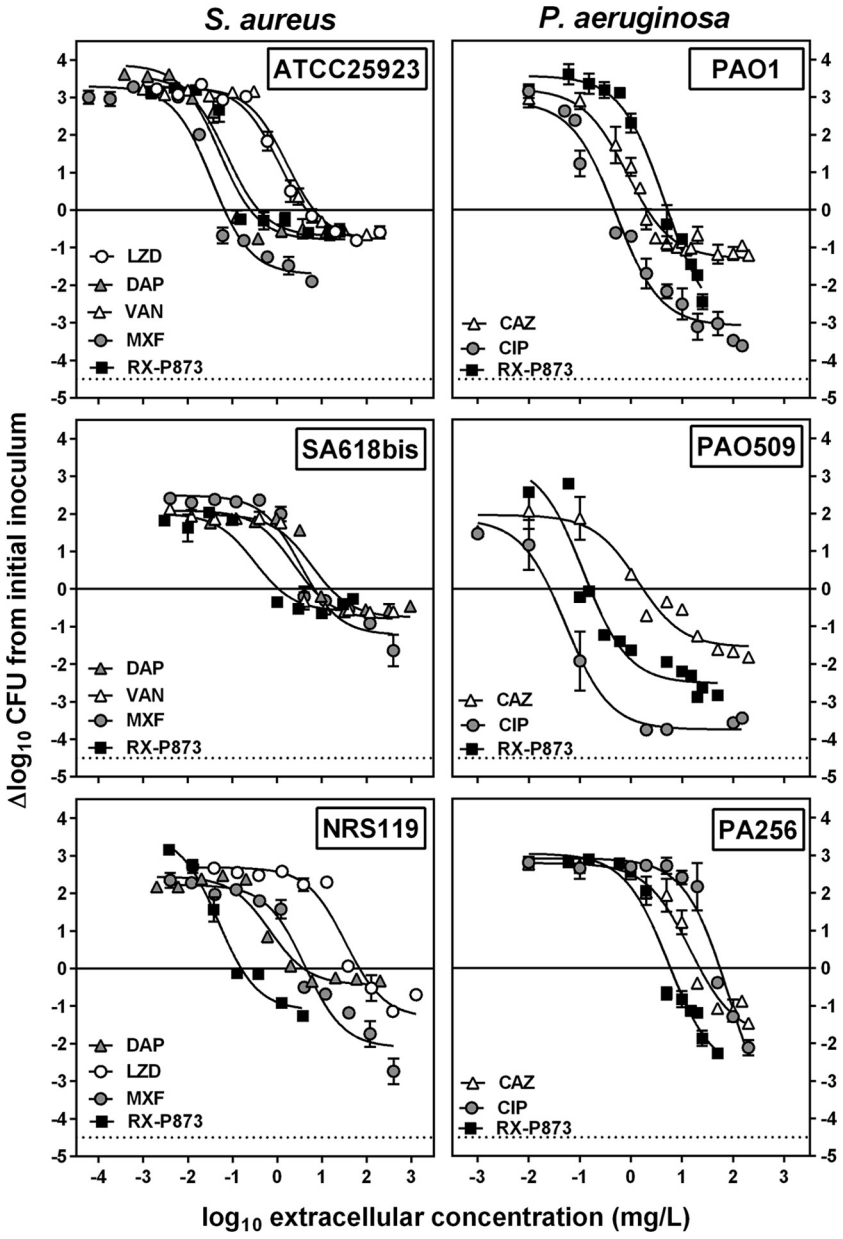

FIG 3 Intracellular activity of RX-P873 and selected comparators (linezolid [LZD], vancomycin [VAN], daptomycin [DAP], moxifloxacin [MXF] or ciprofloxacin [CIP], and ceftazidime [CAZ]) against different strains of *S. aureus* (left) or *P. aeruginosa* (right) determined after 24 h of incubation with increasing concentrations of each drug. The ordinate shows the change in the number of CFU (log scale) ($\Delta\log_{10}$ CFU) per milligram of cell protein compared to that in the initial inoculum. Solid horizontal line, apparent bacteriostatic effect; dotted horizontal line, limit of detection. All values are means ± standard errors of the means (SEMs) from 2 to 3 experiments performed in triplicate (when not visible, the SEMs are smaller than the size of the symbols).

rameters for each antibiotic-strain combination (18, 20). The concentration of RX-P873 in the culture medium of THP-1 cells was limited to 50 mg/liter to avoid undue cellular toxicity (LDH release of less than 15%, i.e., twice the value measured for control cells). The results are shown graphically in Fig. 3, and the values of the corresponding pharmacodynamic parameters are shown in Fig. 4. As described above, concentration-effect relationships followed sigmoidal responses for all antibiotics and against both bacteria.

Against *S. aureus* (Fig. 3, left), moxifloxacin was the most effective antibiotic (E_{max} values, 1.7, 1.2, and 2.1 $\log_{10}$ units against ATCC 25923, SA618bis, and NRS119, respectively). The other drugs, including RX-P873, caused globally 0.5- to 1-$\log_{10}$-unit decreases in the intracellular inoculum for all strains. While moxifloxacin was the most potent antibiotic (i.e., it had the lowest C_s) against susceptible strain ATCC 25923, RX-P873 was the most potent antibiotic against the two resistant strains.

Against *P. aeruginosa* (Fig. 3, right), ciprofloxacin reduced the intracellular inoculum by 3 $\log_{10}$ units for wild-type strain PAO1, as previously described (18), and by about 4 $\log_{10}$ units for hypersusceptible strain PAO509. For resistant strain PA256, a 2-$\log_{10}$-unit decrease at the highest concentration tested was observed (no plateau

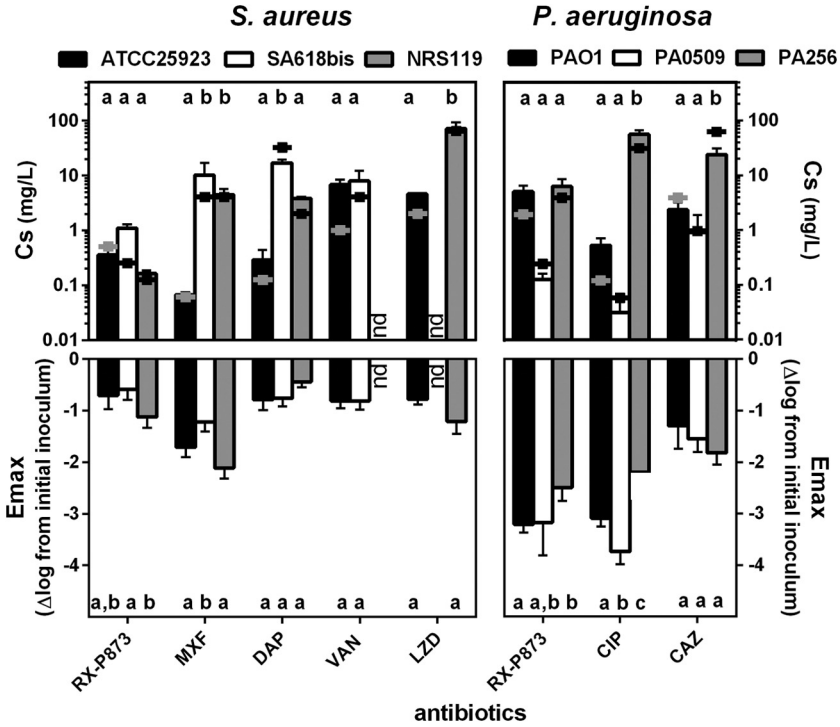

FIG 4 Comparison of intracellular C_s (top) and E_{max} (bottom) values for RX-P873 and its comparators calculated from the sigmoidal regressions of the concentration-effect studies whose results are shown in Fig. 3 (Hill slope = 1 for all antibiotics tested except ciprofloxacin against PA256, because E_{max} could not be calculated using a Hill slope of 1; the highest concentration tested was too far from that allowing the plateau value to be reached). The horizontal lines with central squares superimposed on the C_s values indicate the MIC values. Statistical analyses were performed by 2-way analysis of variance with the Tukey multiple-comparison test considering the different strains for each antibiotic. Data with different letters are significantly different from one another ($P < 0.05$). nd, not determined.

could be reached). Ceftazidime caused only a 2-$\log_{10}$-unit reduction in the intracellular inoculum for all strains. In contrast, RX-P873 was bactericidal against both susceptible strain PAO1 and hypersusceptible strain PAO509, and against multiresistant strain PA256, RX-P873 decreased the intracellular inoculum by 2.5 $\log_{10}$ units at the highest concentration tested. It was thus more effective than both ciprofloxacin and ceftazidime against this resistant strain. Focusing on relative potencies, Fig. 4 (top) shows that the bacteriostatic concentrations of each antibiotic were always close to their respective MIC values and were therefore significantly higher for antibiotics to which strains were resistant (except for vancomycin against SA618bis, probably due to the small difference in MIC [only 2 dilutions compared to the MIC for ATCC 25923]). RX-P873 bacteriostatic concentrations were similar for strains from the same species (in accordance with its almost unchanged MIC) and slightly higher for *P. aeruginosa* than *S. aureus* (again, in accordance with its higher MIC against the first species). When moving to maximal relative efficacy (Fig. 4, bottom), we did not systematically observe a reduction in this parameter in resistant strains. Fluoroquinolones were more effective against both types of intracellular bacteria than the other antibiotics used as comparators. Interestingly, RX-P873 was as effective as ciprofloxacin against *P. aeruginosa* but was less effective than moxifloxacin against *S. aureus*.

Comparison of extracellular and intracellular activities of RX-P873 against *S. aureus* and *P. aeruginosa*. Figure 5 compares the activity of RX-P873 against the extracellular and intracellular forms of the same bacterial strains. When drug concentrations were expressed in multiples of the respective MICs, the activity of RX-P873 against strains of the same species was undistinguishable. Bacteriostatic concentrations were close to the MICs for both extracellular and intracellular bacteria of both species, with a trend (not statistically significant) toward higher values against intracellular forms being detected. As was observed for all antibiotic classes in these models, maximal relative efficacies were always lower against intracellular bacteria than extracellular bacteria. Also noteworthy was the finding that the maximal relative efficacy of RX-P873 was significantly greater (more negative E_{max}) not only against the intracellular forms but also against the extracellular forms of *P. aeruginosa* than its maximal relative efficacy against both forms of *S. aureus*. More specifically, the limit of detection (5-$\log_{10}$-unit decrease) was reached at 10 times the MIC against extracellular *P. aeruginosa*, while the equivalent concentration reduced the extracellular *S. aureus* inoculum by only 4 $\log_{10}$ units.

In a last set of experiments, we compared the kinetics of RX-P873 killing of reference strains of *S. aureus* and *P. aeruginosa*. Figure 6 shows that RX-P873 was bactericidal against both pathogens as soon as its concentrations were higher than the MIC, but this effect developed more slowly against *S. aureus* than against *P. aeruginosa*, with the limit of detection being reached after more than 8 h and only 2 h, respectively.

DISCUSSION

In this paper, we document that a representative of the pyrrolocytosines, a novel class of inhibitors of bacterial protein synthesis, is active against the intracellular forms of both Gram-positive and

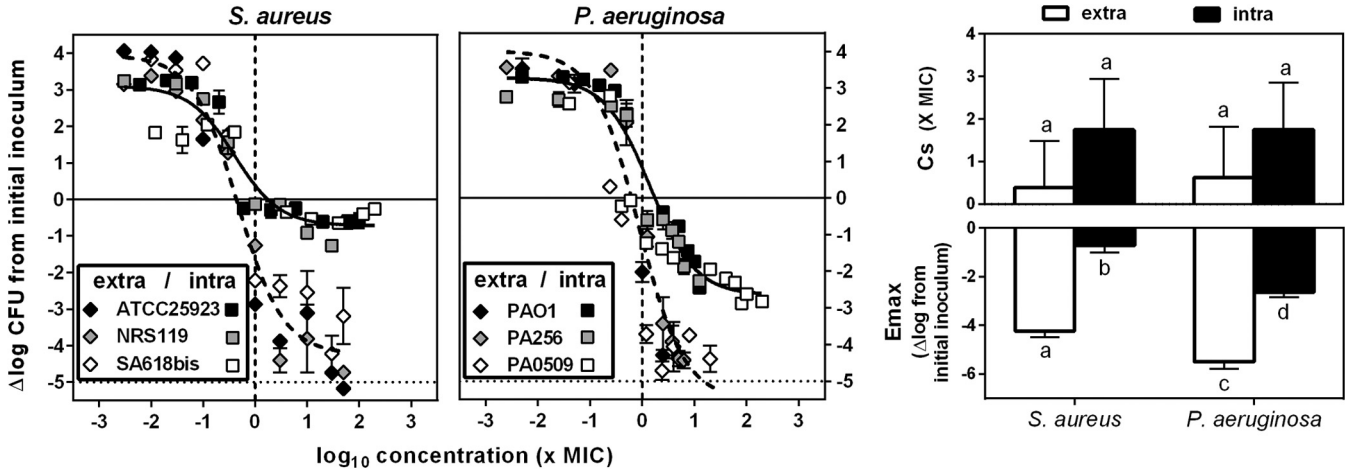

FIG 5 (Left and middle) Extracellular (dotted line) and intracellular (solid line) activity of RX-P873 against different strains of *S. aureus* (left) or *P. aeruginosa* (middle) determined after 24 h of incubation with increasing concentrations of each drug. The ordinate shows the change in the number of CFU (log scale) per milligram of cell protein of broth of broth compared to that in the initial inoculum; concentrations are expressed as multiples of the MIC for each strain. A single sigmoidal regression was fit to the whole set of data obtained for the three independent strains of each bacterial species. Solid horizontal line, apparent bacteriostatic effect; dotted horizontal line, limit of detection; dashed vertical line, MIC. All values are means ± standard errors of the means (SEMs) from 2 to 3 experiments performed in triplicate (when not visible, the SEMs are smaller than the size of the symbols). (Right) Comparison of the bacteriostatic concentration (top) and maximal efficacy (bottom) of RX-P873 against intracellular or extracellular bacteria. Statistical analyses were performed by 2-way analysis of variance with the Tukey multiple-comparison test. Data with different letters are significantly different from one another ($P < 0.05$).

Gram-negative bacteria, with its potency being unaffected by their phenotype of resistance to currently used antibiotics. The present work therefore brings new information about this class of antibiotics, which we have critically examined here.

First, we showed that the intrinsic activity of RX-P873, as determined in broth, is similar against wild-type and multiresistant strains of both *S. aureus* and *P. aeruginosa*, which is to be expected for a drug displaying a novel mode of action. Thus, our data are in accordance with and expand the observations made with other collections that included different bacterial species, but those data have so far been reported only as posters (8, 23). We also observed that RX-P873 MICs are approximately 2 dilutions higher against *P. aeruginosa* than against *S. aureus*, as was also reported in the above-mentioned studies (8, 23). It is tempting to speculate that this could be due to a modest but significant outward efflux by the broad-spectrum transporters constitutively expressed in *P. aeruginosa* because this difference vanished when we considered strain PAO509, which does not express multidrug efflux systems. Notably, however, RX-P873 seemed to be less affected by efflux than ciprofloxacin, for which the MIC was 4 dilutions lower in PAO509 than in PAO1.

Second, we showed that the intracellular activity of RX-P873 is at a level that makes it as potent and as effective against the tested bacteria whatever their phenotype of resistance to other antibiotics is. Thus, while fluoroquinolones remain globally more active intracellularly against susceptible strains, our data clearly high-

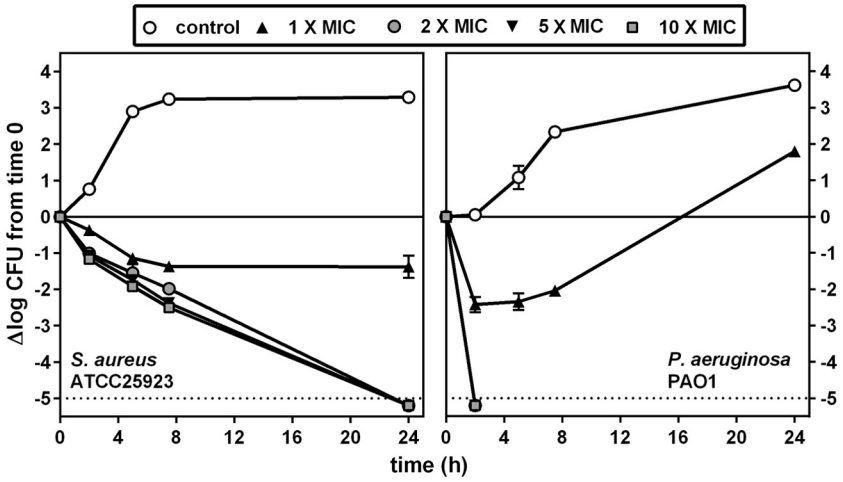

FIG 6 Influence of time on the rate and extent of activity of RX-P873 against extracellular *S. aureus* ATCC 25923 (left) or *P. aeruginosa* PAO1 (right) determined over 24 h of incubation. The ordinate shows the change in the number of CFU (log scale) per milliliter of broth compared to that in the initial inoculum; concentrations are expressed as multiples of the MIC for each strain. Solid horizontal line, apparent bacteriostatic effect; dotted horizontal line, limit of detection. All values are means ± standard deviations (SDs) from 3 independent determinations (when not visible, error bars are smaller than the size of the symbols).

light that RX-P873 may offer a clear advantage when dealing with resistant strains. However, as previously described for many other classes of antibiotics, even those accumulating within cells (18, 20, 24, 25), the intracellular bacteriostatic concentration of RX-P873 remains close to its MIC, as measured in broth. While it is premature to ascribe this to insufficient bioavailability (as was suggested for fluoroquinolones [26]), it nevertheless points again to the fact that intracellular potency and accumulation are not necessarily linked. Yet, it must remain clear that intracellular penetration and achievement of a critical concentration are the first and necessary properties for an antibiotic to express intracellular activity.

This brings us to a third observation made in this study, namely, that RX-P873 accumulates in eukaryotic cells, achieving an apparent cellular concentration 6-fold higher than the extracellular one. Of interest, recent pharmacokinetic data for mice found concentrations of RX-P873 sustainably higher in the thigh than in the serum, with the mean tissular penetration ratio being 6 (27). Although at this stage we do not have any clue about the mechanism of the cellular accumulation of RX-P873 or about its subcellular distribution, we can state that the behavior of RX-P873 differs from that of other positively charged antibiotics, such as aminoglycosides, on the one hand, and macrolides, on the other hand. Aminoglycosides enter eukaryotic cells only very slowly (28) due to their highly hydrophilic character at the extracellular pH (log distribution coefficient [log D], ~ -10 to -15 at pH 7.4). In contrast, macrolides or ketolides, which are also cationic but which are much more lipophilic at pH 7.4 (log D, close to 0 at pH 7.4), easily diffuse into cells (29, 30), where they are eventually retained in acidic compartments by proton trapping (31). The cellular accumulation of a hydrophilic drug such as RX-P873 (which is mainly in its cationic form at pH 7.4) is thus unexpected. However, the investigational hydrophilic fluoroquinolone finafloxacin accumulates about 8-fold in THP-1 cells under conditions where it is positively charged (calculated log D, -1.1 in medium at pH 5.5) (32). Thus, whatever the underlying mechanism of this accumulation is and pending further investigations, these data at least show that the quite hydrophilic character of RX-P873 is not incompatible with cell penetration and the subsequent expression of intracellular activity.

The last and probably more intriguing observation in this study is that RX-P873 has a much lower relative efficacy (a less negative E_{max}) against the intracellular forms of *S. aureus* than against the intracellular forms of *P. aeruginosa*, although its MICs against *S. aureus* are lower than those against *P. aeruginosa*. In previous work comparing the intracellular activity of different classes of drugs against the same bacterial strain, we documented that relative efficacies are not related to the intrinsic activities of antibiotics in broth (as determined by the measurement of their MICs), which is actually predictive of the relative potency, but, rather, are related to the mode of action of the drugs. Thus, highly bactericidal antibiotics tend to bring the intracellular numbers of CFU to levels lower than those achieved with slowly bactericidal or bacteriostatic ones, regardless of their respective MICs (18, 20, 33, 34). The same reasoning could possibly apply here to RX-P873, as we showed a slower bactericidal effect against *S. aureus* than *P. aeruginosa* when the strains were grown in broth (as confirmed by independent studies so far reported only as posters [10, 11]). Yet, in the present study, fluoroquinolones also showed a lower maximal relative efficacy against *S. aureus* than *P. aeruginosa*. This may denote a difference in response related to the type of bacteria studied. Thus, we previously showed that the intracellular maximal relative efficacy of ciprofloxacin is high (a more negative E_{max}) against *Listeria monocytogenes*, intermediate against *P. aeruginosa*, and lower (a less negative E_{max}) against *Legionella pneumophila* and *S. aureus* (18, 32).

Thus, taken as a whole, this study suggests that RX-P873 may constitute a useful weapon in our future armamentarium, especially against bacteria displaying multiresistance to currently available antibiotics and capable of surviving intracellularly. More specifically, our work underlines the interest in this molecule both against a Gram-positive organism (on the basis of its low MIC) and, more strikingly, against a Gram-negative organism (on the basis of its higher intracellular efficacy). This work therefore opens the door to further investigations focusing on other challenging bacterial species.

ACKNOWLEDGMENTS

We are grateful to M. C. Cambier, V. Mohymont, K. Santos, and V. Yfantis for excellent technical assistance.

J.M.B. was a postdoctoral fellow on research programs sponsored by the Région Wallonne, Belgium, and F.V.B. is maître de recherche of the Belgian Fonds National de la Recherche Scientifique. This work was supported by the Fonds de la Recherche Scientifique Médicale (grant 3.4530.12), the Interuniversity Attraction Poles Program initiated by the Belgian Science Policy Office (program IAP P7/28), and a grant from Melinta Therapeutics, New Haven, CT.

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