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Article

The Natural Alkaloid Berberine Can Reduce the Number of *Pseudomonas aeruginosa* Tolerant Cells

Gianmarco Mangiaterra,*[,‖] Nicholas Cedraro,[‖] Emiliano Laudadio, Cristina Minnelli, Barbara Citterio, Francesca Andreoni, Giovanna Mobbili, Roberta Galeazzi, and Francesca Biavasco

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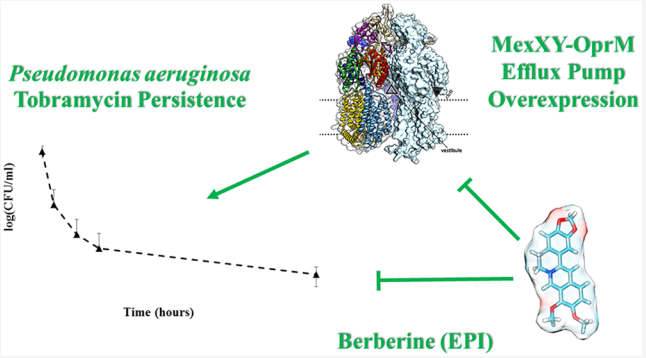
ABSTRACT: The eradication of recurrent *Pseudomonas aeruginosa* (PA) lung infection in cystic fibrosis (CF) patients may be hampered by the development of persistent bacterial forms, which can tolerate antibiotics through efflux pump overexpression. After demonstrating the efflux pump inhibitory effect of the alkaloid berberine on the PA MexXY-OprM efflux pump, in this study, we tested its ability (80/320 μg/mL) to enhance tobramycin (20xMIC/1000xMIC) activity against PA planktonic/biofilm cultures. Preliminary investigations of the involvement of MexY in PA tolerance to tobramycin treatment, performed on the isogenic pair PA K767 (wild type)/K1525 (Δ*mexY*) growing in planktonic and biofilm cultures, demonstrated that the Δ*mexY* mutant K1525 produced a lower (100 and 10 000 times, respectively) amount of tolerant cells than that of the wild type. Next, we grew broth cultures of PAO1, PA14, and 20 PA clinical isolates (of which 13 were from CF patients) in the presence of 20xMIC tobramycin with and without berberine 80 μg/mL. Accordingly, most strains showed a greater (from 10- to 1000-fold) tolerance reduction in the presence of berberine. These findings highlight the involvement of the MexXY-OprM system in the tobramycin tolerance of PA and suggest that berberine may be used in new valuable therapeutic combinations to counteract persister survival.



INTRODUCTION

Antibiotic failure against bacterial infections is a growing threat to human health.[1] In addition to antibiotic resistance, antibiotic persistence, though described since the first half of the 1900s, has only recently begun to be characterized. Persister cells were first described by Hobby[2] as a bacterial subpopulation characterized by survival to antibiotic concentrations hundreds of times higher than the minimal inhibitory concentration (MIC).[3] They are different from stationary phase and tolerant cells, because they are a small fraction (<1%) of an antibiotic-susceptible bacterial population,[4] which results in multimodal killing curves.[5] In this, they seem more similar to heteroresistant cells, which are characterized by transient genetic changes and less marked resistance levels.[6] A key feature of persisters is their low metabolic activity, which is likely responsible for their tolerance to high doses of antibiotics[7] and, consequently, for therapeutic failures. Unlike antibiotic-resistant cells, persisters are not genetically different from their antibiotic-susceptible siblings; accordingly, in the absence of antibiotics, a persister cell gives rise to an antibiotic-susceptible progeny.[4] Recurrent infections are characterized by an apparent clearance of bacteria, due to the action of the antibiotic treatment on the susceptible cells, representing most of the bacterial population, followed by symptom exacerbation, due to a reason other than the insurgence of antibiotic resistance, even to the reactivation of the small antibiotic-persistent and/or tolerant subpopulation.[8] The presence of different types of persisters, including viable but non-culturable cells,[9−11] appears to be a major cause of antibiotic treatment failure,[12] as demonstrated by the recurrence of cystic fibrosis (CF) *Pseudomonas aeruginosa* lung infections, characterized by the isolation over time of the same bacterial strain showing a still-susceptible phenotype.[13,14]

Several studies have tried to identify the genes and regulation pathways directly involved in the development of the persistent phenotype.[15] In *P. aeruginosa*, a role in the persistent phenotype has been claimed for quorum sensing[16] and toxin−antitoxin modules, which by interfering with protein synthesis, can shift the cell to a low metabolic state, called "passive persistence".[17] Moreover, a key role in persister induction has been reported for the alarmone guanosine

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tetraphosphate, which can generate persisters through ribosome inactivation and dimerization.[18] A direct correlation between drug tolerance and efflux pumps activity has been also proposed and labeled as "active persistence". It comes from the observed marked overexpression by persisters of multidrug resistance efflux pumps (EPs).[19] Accordingly, overexpression of the *mexXY-oprM* gene cluster is a hallmark of *P. aeruginosa* isolates from chronic CF patients.[20] Because infectious biofilms are known to contain persistent bacterial forms, this could be also related to a role for MexXY-OprM in biofilm formation, as reported for other EPs.[21]

Identifying novel compounds capable of contrasting persister formation and survival is a new strategy against chronicity. Recent *in silico* studies predicted the ability of the natural alkaloid berberine to inhibit tobramycin efflux by binding MexY, the inner membrane channel of the MexXY-OprM system of *P. aeruginosa*, an observation which is further corroborated by *in vitro* studies showing a decreased tobramycin MIC in different strains.[22]

In this study, we explore the possible influence of berberine in counteracting the development of *P. aeruginosa* persistent subpopulations in both planktonic and biofilm cultures, and we consider that, as a persistent phenotype, those cells are able to grow on culture media after exposure to tobramycin concentrations several times (20× or 1000×) the MIC as suggested by Kim et al.[10]

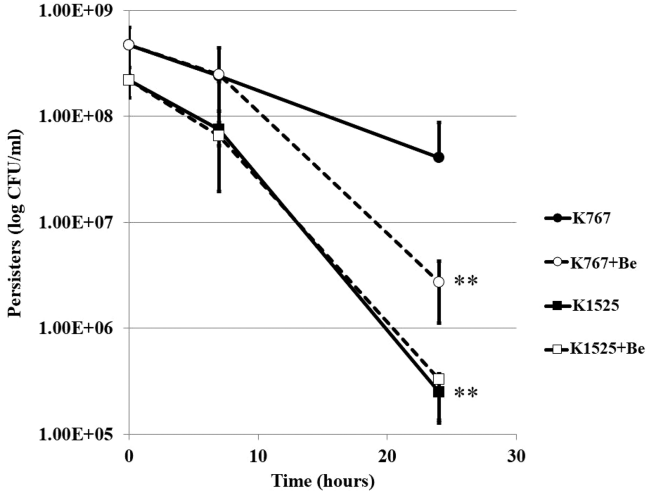

Figure 1. *P. aeruginosa* K767/K1525 tobramycin persistence assays in broth cultures. Overnight cultures of the isogenic pair *P. aeruginosa* K767/K1525 were exposed for 24 h to tobramycin alone (20xMIC) or combined with berberine (Be; 80 μg/mL). Tolerant cells (i.e., the survivors to high-dose tobramycin) were quantified by plate count on antibiotic-free medium 0, 7, and 24 h after exposure. The results are reported as the average of two biological replicates ± standard deviation. ** $p < 0.01$.

RESULTS AND DISCUSSION

Tobramycin Persistence Assays. The contribution of the MexXY-OprM system to the ability of *P. aeruginosa* to survive in the presence of tobramycin concentrations several times the MIC was evaluated by exposing the isogenic pair *P. aeruginosa* K767 (WT)/K1525 (Δ*mexY*) to 20xMIC tobramycin for 24 h and counting the survivors. The CFU reduction was 1 log (from 4.70×10^8 to 4.11×10^7 CFU/ml) in the WT strain and 3 log (from 2.2×10^8 to 2.5×10^5 CFU/ml) in the *mexY* mutant. The obtained results actually resemble the behavior of a tolerant rather than persistent population, as survivors accounted for about 10% of the whole bacterial population;[5] this is not surprising, as in persistence assays, we used stationary phase cultures, thus enhancing the proportion of cells able to tolerate tobramycin. This significant ($p < 0.01$) greater reduction of tolerant cells in the absence of a functional EP (Figure 1) suggests a role for a functional MexXY-OprM system in the tobramycin tolerance. The same behavior was not observed when stationary phase cultures were exposed to high doses of ceftazidime or colistin, which are not substrates of MexXY-OprM,[23,24] further confirming the specificity of the EP to the aminoglycoside drugs.

The involvement of MexY in bacterial survival to tobramycin exposure was then confirmed by exposing the two strains to the same tobramycin concentration (20xMIC) used above in the presence of 80 μg/mL berberine. Its action as an efflux pump inhibitor (EPI) was first confirmed by ethidium bromide efflux assays with the WT strain which showed a lower decrease of the cell fluorescence in the presence of berberine (2%) compared to that of the untreated control (10%). After 24 h exposure, the CFU count of the WT strain was 10 times lower than that after exposure to tobramycin alone, whereas berberine failed to exert a significant effect on the Δ*mexY* strain. Because the two strains differ only by the presence/absence of *mexY*, a mechanism leading to a decrease of antibiotic survivors different from the binding of berberine to MexY can be excluded. This further indicates the importance of a functional MexXY-OprM system in the survival of a culturable *P. aeruginosa* subpopulation to high tobramycin concentrations.

Growth Rate of *P. aeruginosa* K767/K1525 in the Absence/Presence of Berberine. To exclude an influence of berberine on *P. aeruginosa* fitness, the isogenic pair K767/K1525 was grown in its absence/presence (80 μg/mL), and the growth rate was monitored for 24 h. When grown without berberine, the *mexY* mutant K1525 strain showed a slower growth compared with that of the WT, which became detectable after 6 h incubation. This highlights the role of a functional efflux pump MexXY-OprM in the fitness of the strain, as confirmed by the assays performed in the presence of berberine, where the growth was slower for both strains, but especially for *P. aeruginosa* K767, whose growth rate was similar to that of the mutant strain without berberine (Figure S1).

Biofilm Amount and Tolerant Cell Abundance in *P. aeruginosa* K767 and K1525 Biofilms in the Absence/Presence of Berberine. To establish whether the role of MexY in persister development could be related to its involvement in biofilm production, as reported for other EPs,[21] the amount of biofilm produced by the isogenic pair *P. aeruginosa* K767/K1525 was evaluated in the absence/presence of berberine (Figure 2).

Unexpectedly, we found that, in the absence of berberine, the two strains did not exhibit significant differences in biofilm production; in its presence, the production was significantly enhanced in both strains, but more markedly (100%, $p < 0.001$) in the WT strain than in the mutant (50%, $p < 0.01$). The ability of berberine to enhance biofilm production is to be explained. It does not seem to be related with its interference with MexXY-OprM activity, because the *mexY* mutant did not exhibit any significant difference in biofilm production compared to that of the WT.

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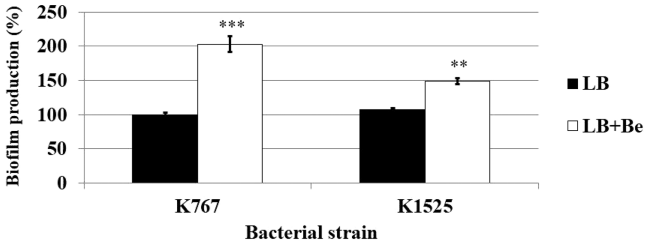

Figure 2. Effect of berberine on *P. aeruginosa* biofilm production. Biofilm production by *P. aeruginosa* K767 (WT) and K1525 (Δ*mexY*) grown in LB in the absence/presence of berberine 80 μg/mL (Be). Biofilm production/bacterial cell was determined as OD_{570}/OD_{600} of the sessile/planktonic bacteria in each well; its variation is reported as % of the value of *P. aeruginosa* K767 (100%). ** $p < 0.01$, *** $p < 0.001$.

To gain further insight into the involvement of the MexXY-OprM system in the persistence of biofilm-growing *P. aeruginosa*, we evaluated the amount of tolerant cells in *P. aeruginosa* K767/K1525 biofilms exposed to tobramycin 1000xMIC for 24 h and in *P. aeruginosa* K767 biofilms exposed to 1000xMIC tobramycin and 80 or 320 μg/mL berberine for 24 h.

Exposure to tobramycin induced the production of a significantly lower amount (3 log, $p < 0.001$) of tolerant cells by the *mexY* mutant compared to the WT (Figure 3), consistent with the results obtained with the planktonic cultures (Figure 1).

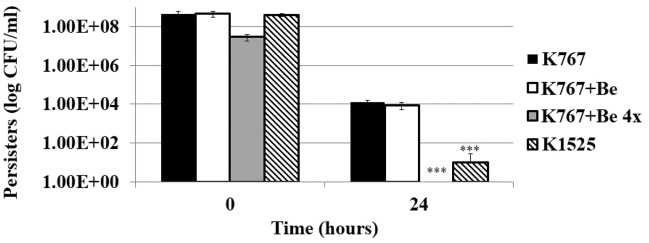

Figure 3. *P. aeruginosa* K767/K1525 tobramycin persistence assays in biofilm cultures. Tolerant cells of *P. aeruginosa* K767 and K1525 biofilms cultured in LB were counted after 24 h exposure to tobramycin 1000xMIC alone or with berberine (Be), 80 or 320 (Be 4x) μg/mL. Results are reported as the average of three biological replicates ± standard deviation. ** $p < 0.01$, *** $p < 0.001$.

When testing the association tobramycin 1000xMIC/berberine 80 μg/mL against *P. aeruginosa* K767, no antipersister activity was noted, probably due to the poor penetration of berberine into the thick biofilm layers, as reported for several hydrophilic compounds including tobramycin.[25,26] On the other hand, a 4-fold higher berberine concentration lead to the clearance of *P. aeruginosa* biofilm and the recent demonstration that berberine at 320 μg/mL lacks appreciable cytotoxic effects[22] supports its suitability to be used in combination with tobramycin.

Altogether, these findings lend further support to the role of the MexXY-OprM system in *P. aeruginosa* unresponsiveness to tobramycin and exclude the involvement of berberine in bacterial tolerance except through its EP inhibitor (EPI) activity. Indeed, MexXY-OprM overexpression has been related to early persister selection, resulting in adaptive resistance to aminoglycosides[27] and to a mutation in the regulatory gene *mexZ*, leading to *mexXY-oprM* overexpression in clonally related strains of *P. aeruginosa* repeatedly isolated from the same patient.[28] Conversely, the MexXY-OprM system, differently from MexAB-OprM[21], seems to play no role in biofilm production, as suggested by the absence of significant differences between the WT and the *mexY* mutant.

Effect of Berberine on the Reduction of the Tolerant Subpopulation of Clinical Strains and Comparison with the Reduction of the Tobramycin MIC. The effect of berberine (80 μg/mL) on the amount of antibiotic tolerant cells after exposure to 20xMIC tobramycin was assessed in 22 additional strains of *P. aeruginosa* (2 laboratory and 20 clinical, of which 13 were CF isolates). The findings (Figure 4) highlighted a variety of different responses to berberine and a strain-specific behavior.

After exposure to tobramycin alone, 6/22 strains (27.3%) exhibited a 100- to 10 000-fold reduction of the starting inoculum, and 9/22 strains (40.9%) showed a 10-fold reduction; 4/22 strains (18%) showed a more limited (<10-fold) reduction, and 3/22 (13.6%) showed no CFU reduction, i.e., 100% survivors. These three isolates (*P. aeruginosa* C15, NC04, and NC10) were all recovered from patients affected by CF. Combined with tobramycin, berberine 80 μg/mL induced a reduction of the starting inoculum 1−3 log greater than the one induced by tobramycin alone in 16/22 (72.7%) strains, while the remaining isolates were unaffected. Notably, in the case of *P. aeruginosa* C61, tobramycin induced a 1 log reduction of the bacterial population, whereas the drug combination left no survivors (≥7 log CFU reduction). Remarkably, 6/7 (85.7%) isolates, where berberine induced the most limited reduction, were from CF patients.

These results were then compared with the decrease of the tobramycin MIC in the presence of the same berberine concentration (Table S1). Berberine reduced the tobramycin MIC and tolerant cell abundance in 7/23 (30.4%) strains; in 10/23 strains (43.5%), it only reduced the tolerant population, and in 3/23 (13.05%), it only reduced the MIC. Finally, 3/23 (13.04%) strains were unaffected both in terms of tobramycin susceptibility and of tolerant cell amount.

Though difficult to explain and regarding a small amount of strains, these results suggest an even greater influence of berberine on the development of cells able to tolerate high tobramycin concentrations rather than on the tobramycin MIC. In an attempt to elucidate the mechanisms underlying these different responses, we analyzed the possible involvement of MexY polymorphisms.

MexY Protein Sequence Analysis. To gain insight into the heterogeneity of the bacterial responses to berberine, we analyzed the gene (Figure S2) and the protein (Figure S3) sequences of eight *P. aeruginosa* strains selected on the basis of their different response to berberine in terms of the tobramycin MIC (Table S1) and of tolerant cell amount (Figure 4). Accordingly, strains C25 and NC06 showed a reduction in both resistance and tolerance; strains C30 and C54 showed only reduced tolerance; strains C84 and AR48 showed only a reduced MIC (and no or very limited reduction in survivor abundance), and strains C31 and C59 showed no change in either susceptibility or tolerance. Two internal sequences of the *mexY* gene, measuring 270 bp (sequence 1) and 588 bp (sequence 2), were selected based on preliminary *in silico* investigations (data not shown), which had suggested that the corresponding putative amino acid sequences could be involved in MexY binding to berberine. The alignment of the two sequences to the corresponding sequences of *P.*

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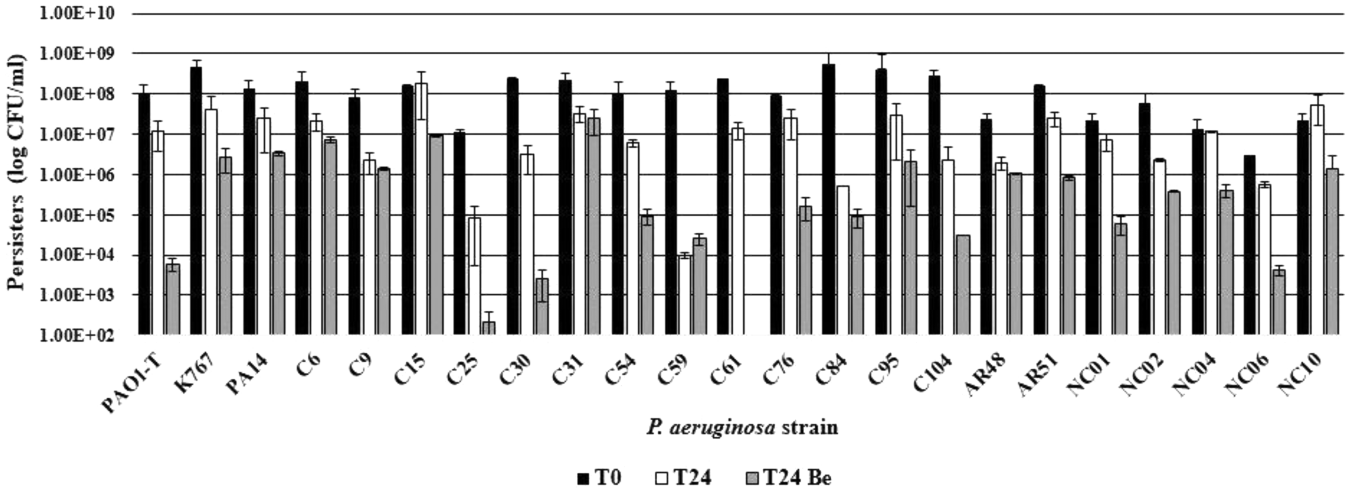

Figure 4. Tobramycin tolerance in the absence/presence (T24 Be) of berberine. The survival of 23 *P. aeruginosa* isolates, including laboratory (PAO1-T, PA14, and K767), clinical (C54, C59, C61, C76, C84, C95, C104) and CF (C6, C9, C115, C25, C30, C31, AR48, AR51, NC01, NC02, NC04, NC06, NC10) strains, to tobramycin 20xMIC was evaluated in the absence or presence of berberine (80 μg/mL). Plate counts performed immediately before tobramycin exposure (T0) and after 24 h (T24) incubation at 37 °C are reported as the average of two biological replicates ± standard deviation.

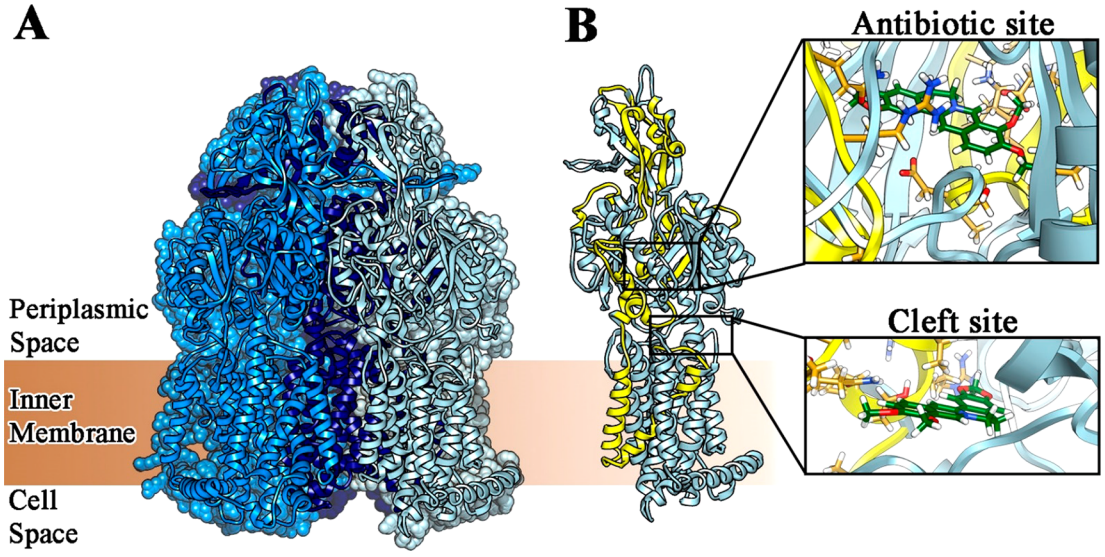

Figure 5. MexY protein (trimer) from *P. aeruginosa* PA01. (A) The three monomers of the MexY trimer are shown as sky blue, light blue, and dark blue van der Waals (vdW) spheres and ribbons. (B) The amino acids involved in the polymorphisms are shown as yellow ribbons of the MexY monomer. (Insets) The bound berberine molecules in the PAO1 model are shown as green sticks; around the berberine molecules, the amino acids involved in the polymorphisms are shown as yellow sticks.

aeruginosa PAO1 (accession number, AB015853.1) highlighted a small number of point mutations (Figure S4). We found six single nucleotide polymorphisms (SNPs) in the 270 bp sequence and 27 SNPs in the 588 bp sequence, as follows. Sequence 1: c.71C > T, c.80G > A, c.98G > A, c.107T > C, c.113T > C, c.146G > A; sequence 2: c.110C > G, c.112G > C, c.113C > G/A, c.114G > C, c.179G > C, c.294C > G, c.295G > C, c.296A > G, c.298G > A, c.317C > G, c.331A > G, c.332A > C, c.343C > G, c.344A > C, c.345T > A, c.388T > C, c.428T > C, c.451C > G, c.511A > G, c.521C > G, c.522T > C, c.523G > T, c.524G > C, c.525T > C, c.526C > A, c.527G > C, c.531T > A. The CF strains *P. aeruginosa* C30, C31, AR48, and NC06 carried the highest number of SNPs in both sequences. The alignment of the putative amino acid sequences (Figure S5) confirmed their possible involvement in MexY polymorphism, because the 3D structure of the pump (Figure 5) and *in silico* modeling localized the mutations in correspondence to the binding sites of berberine.

Amino acid substitutions were more numerous in sequence 2 than in sequence 1 (Figure S5), where the I112Y mutation was shared by all strains.

Sequence 2 exhibited 22 substitutions (Figure 6); of these, 12 (R771G, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, L841I, M842D, Q843A, and I849H) were shared by all strains, and T845P and R772A were shared by all strains but one (C54 and C84, respectively). R772A was detected in 7 strains. Q840G and A794P were detected, respectively, in 6 and 5 strains. Q840A was found in 2 strains. R772T, L908A, L909A, G910H, and V911G were found in a single strain.

The correlation between amino acid substitutions and berberine response was investigated by comparing amino acid variations and the reduction of both MIC and tolerant

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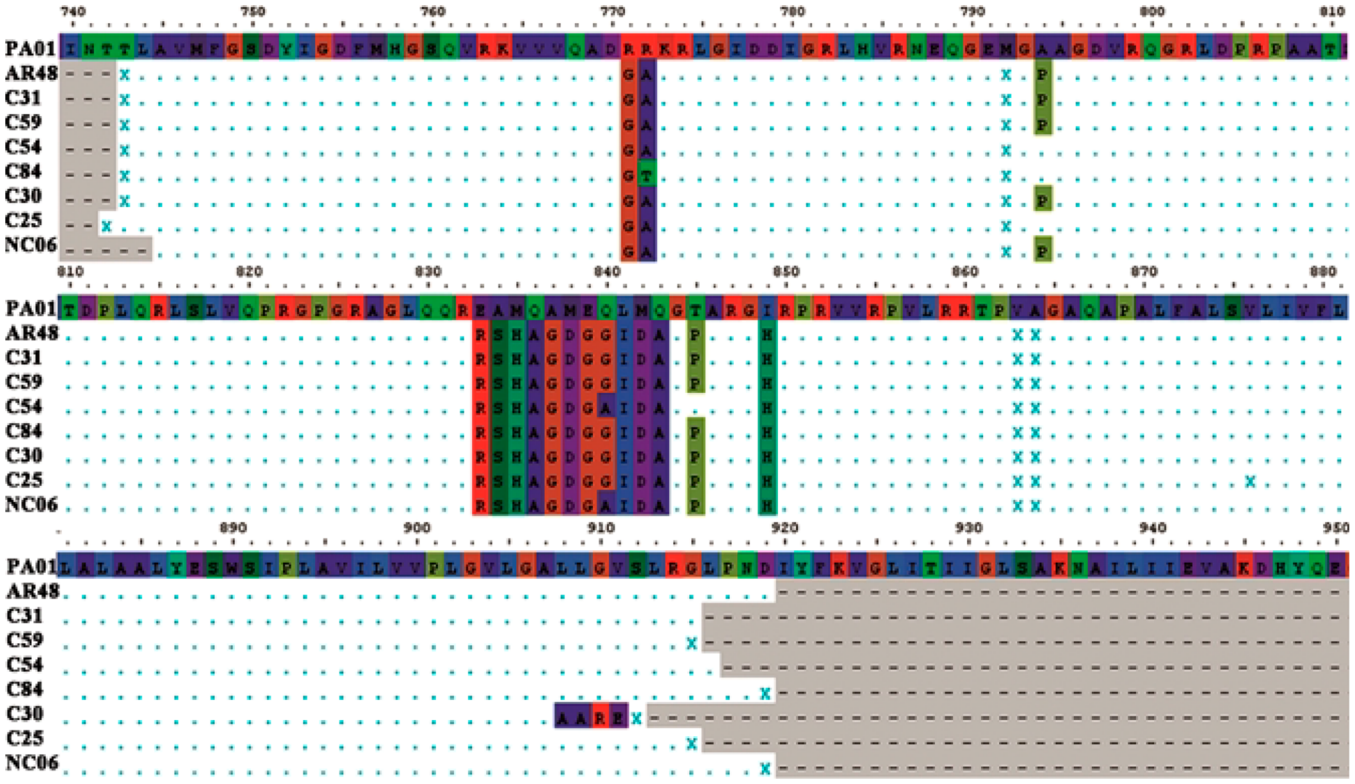

Figure 6. Amino acid sequence alignment of the putative MexY fragment retrieved from the 588 bp nucleotide sequence of eight *P. aeruginosa* strains. The different amino acids are shown in different colors.

Table 1. Correlation between Specific Amino Acid Substitutions and Tobramycin Susceptibility/Tolerance[a]

bacterial strain[b]	amino acid substitution	MIC (μg/mL) reduction[c]	tolerant cell reduction (log)
P. aeruginosa C25 (CF)	R771G, R772A. E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H,	16-fold	2.5
P. aeruginosa C30 (CF)	R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H, L908A, L909A, G910H, V911G	no	3
P. aeruginosa C31 (CF)	R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H,	no	no
P. aeruginosa C54 (C)	R771G, R772A, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840A, L841I, M842D, Q843A, I849H,	2-fold	1.5
P. aeruginosa C59 (C)	R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H,	2-fold	no
P. aeruginosa C84 (C)	R771G, R772T, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H,	4-fold	0.5
P. aeruginosa AR48 (CF)	R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H,	4-fold	no
P. aeruginosa NC06 (CF)	R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840A, L841I, M842D, Q843A, T845P, I849H,	4-fold	2

[a]In the presence of berberine in the eight *P. aeruginosa* strains selected for sequence analysis. [b]CF, cystic fibrosis; C, clinical. [c]No, no reduction.

cells amount exerted by berberine. Though difficult to explain analyzing a little amount of strains, these results suggest an even greater influence of berberine on the decrease of cells able to survive high tobramycin concentrations than on the tobramycin susceptibility level (Table 1).

Consistent with its nucleotide sequence, *P. aeruginosa* C30 showed the highest number of substitutions. Although no clear correlations were observed between the amino acid substitutions and the tobramycin tolerance and susceptibility phenotypes caused by berberine, the former could actually influence the EPI activity, particularly when the mutation involved amino acids characterized by different chemical–physical properties (polar–apolar). This would probably result in different numbers and types of interactions between berberine and MexY, with a possible influence on the stability of the Michaelis complex, as suggested by the behavior of strains *P. aeruginosa* C31 and C59, which carried the same four polar–apolar amino acid substitutions (Figure 6) and exhibited no changes in tobramycin susceptibility or persistence after exposure to berberine.

In addition, the substitutions may affect berberine binding not only directly, through changes in binding interactions, but also indirectly, via changes in receptor conformation; hence, the ligand binding pose would partly explain the less effective MexY inhibition.

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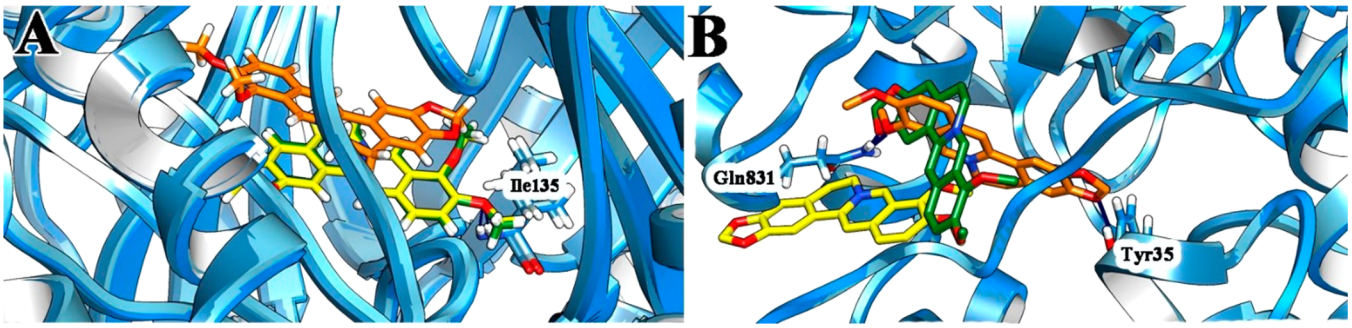

Figure 7. Binding mode of berberine to MexY to the antibiotic (A) and the cleft site (B). The overlapping MexY structures of *P. aeruginosa* C25, C30, and AR48 are shown in light blue, blue, and dark blue, respectively. Berberine molecules are shown in green, yellow, and orange, in the binding model of *P. aeruginosa* C25, C30, and AR48, respectively.

Binding Mode of Berberine. To detect a possible correlation between berberine binding modes and the different *P. aeruginosa* strain responses to the alkaloid, we used a molecular docking approach. MexY structures of *P. aeruginosa* C30 (which showed reduced tolerance), AR48 (which showed a reduced MIC), C25 (which showed a reduction in both resistance and tolerance), C31, and C59 (which showed no reduction in either resistance or tolerance) have been analyzed. Blind docking identified two berberine binding sites in all five MexY models, one at the periplasmic cleft and the other at the antibiotic binding site (Figure 5). The focused docking results showed high binding affinities at the antibiotic binding site in *P. aeruginosa* C25 (Eb = −8.05 kcal/mol; K_i = 1.25 μM) and C30 (Eb = −8.02 kcal/mol; K_i = 1.33 μM) and a lower berberine affinity (Eb = −7.31 kcal/mol; K_i = 4.36 μM) in *P. aeruginosa* AR48. The structures of the C25 and C30 shared the same berberine binding pose with the hydrogen bond formation with the Ile135 residue (Figure 7A).

In contrast, in regards to the berberine affinity for the cleft site, the greatest values were observed in *P. aeruginosa* AR48 (Eb = −7.77 kcal/mol; K_i = 2.28 μM), probably corresponding to a deeper localization of the binding pose, with the formation of two important hydrogen bonds with Tyr35 and Gln 831 (Figure 7B). In *P. aeruginosa* C25, berberine displayed an intermediate behavior (Eb = −7.62 kcal/mol; K_i = 2.33 μM), with one hydrogen bond with Gln 831 and less deep positioning, while the lowest binding values (Eb = −7.31 kcal/mol; K_i = 4.36 μM) were recorded in *P. aeruginosa* C30 with no hydrogen bonds. This is in agreement with its observed inability to reduce the tobramycin MIC (Table 1). In spite of their different amounts of tobramycin MIC decrease in the presence of berberine, both strains showed a similar, reduced number of tolerant cells, suggesting that the ability of berberine to lower drug tolerance might depend on its strong binding at the antibiotic binding site by interaction with Ile135. A reduced berberine binding affinity at both antibiotic and cleft binding sites was observed in *P. aeruginosa* C31 (−6.51 and −5.98 kcal/mol, respectively) and C59 (Eb = −7.03 and −6.93 kcal/mol, respectively), as shown in Figure S6. Notably, at both binding sites, the berberine binding poses were different from those of all the other strains and lacked H-bond interactions, suggesting a less specific berberine binding, mostly based on hydrophobic interactions. Because a hydrophilic contribution is required for a strong berberine binding, these data can explain the lack of inhibitory activity of berberine observed in these strains.

Overall, the obtained results suggest a direct correlation between the reduction of tolerant cells and a specific, highly stable binding of berberine to MexY at the antibiotic binding site through hydrogen bonding. On the contrary, the specific binding of berberine through hydrogen bonds, at the cleft site, seems to be related to an MIC decrease. Further *in silico* and *in vitro* work to determine the involvement of specific MexY polymorphisms in berberine binding is in progress in our laboratory.

CONCLUSIONS

In conclusion, our findings demonstrate the involvement of the MexXY-OprM system in *P. aeruginosa* tolerance to high tobramycin concentrations and suggest that EPs might influence antibiotic survival not only through their efflux activity but also through additional mechanisms still to be elucidated; they also highlight that mechanisms underlining antibiotic persistence are, at least partially, different from those responsible for antibiotic resistance and suggest the need for further investigation of EPs as targets for antipersistence strategies. Interestingly, new drug combinations harnessing natural compounds with EPI activity may go some way toward treating persistent *P. aeruginosa* infections.

EXPERIMENTAL SECTION

Bacterial Strains, Media, and Chemicals. We used 19 previously characterized *P. aeruginosa* strains[22] which included 15 clinical isolates; the isogenic pair *P. aeruginosa* K767 (wild type, WT)/K1525 (Δ*mexY*), kindly provided by Prof. Keith Poole (Queen's University, Kingston, ON, Canada); and *P. aeruginosa* PAO1-T and PA14, kindly provided by Prof. Olivier Jousson (Integrated Biology Center, University of Trento, Trento, Italy) as well as 5 new CF isolates (Table 2). Strains were grown in Luria−Bertani broth (LB) or cystine-lactose-electrolyte-deficient (CLED) agar plates (Oxoid SpA, Milano, Italy) and stored as stock cultures in LB supplemented with 20% glycerol at −80 °C. Tobramycin, ceftazidime, and colistin were purchased from Sigma-Aldrich SRL (Milano, Italy), and berberine was purchased from SPECs (www.specs.net).

Antibiotic Susceptibility Tests. Susceptibility to tobramycin and to the tobramycin−berberine combination was determined by broth microdilution according to CLSI guidelines.[29] The association was considered synergistic when, in the presence of berberine, the MIC was ≥4 times lower than the MIC of tobramycin alone.[22]

Biofilm Production Assays. Biofilm production was assessed as described previously,[30] with some modifications. Briefly, *P. aeruginosa* biofilms were developed in flat-bottom microtiter plates in LB without/with berberine 80 μg/mL. After overnight incubation at 37 °C, the planktonic phase was removed, and its optical density (OD_{600}) was recorded; the sessile phase, after it was washed with sterile deionized water (DW), was stained with 1% crystal violet for

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Table 2. *P. aeruginosa* Strains Used in the Study and Their Tobramycin MICs Without and With Berberine 80 μg/mL

bacterial strain			MIC (μg/mL)	
code	origin[a]	source	tobramycin	tobramycin+ Be[b]
P. aeruginosa PAO1	L	22	1	0.5
P. aeruginosa K767	L	22	0.25	0.125
P. aeruginosa PA14	L	22	0.125	0.125
P. aeruginosa C6	CF	22	2	0.5
P. aeruginosa C9	CF	22	2	1
P. aeruginosa C15	CF	22	8	8
P. aeruginosa C25	CF	22	16	1
P. aeruginosa C30	CF	22	8	8
P. aeruginosa C31	CF	22	8	8
P. aeruginosa C54	C	22	32	16
P. aeruginosa C59	C	22	64	32
P. aeruginosa C61	C	22	64	16
P. aeruginosa C76	C	22	32	16
P. aeruginosa C84	C	22	0.5	0.125
P. aeruginosa C95	C	22	32	4
P. aeruginosa C104	C	22	32	8
P. aeruginosa AR48	CF	22	32	8
P. aeruginosa AR51	CF	22	16	8
P. aeruginosa NC01	CF	this study	16	8
P. aeruginosa NC02	CF	this study	32	16
P. aeruginosa NC04	CF	this study	0.5	<0.125
P. aeruginosa NC06	CF	this study	2	0.5
P. aeruginosa NC10	CF	this study	1	0.25

[a]L, laboratory; CF, cystic fibrosis; C, clinical. [b]Be, berberine.

15 min. After removing the dye, biofilms were then washed with DW, resuspended in 96% ethanol, and quantified by measuring the OD at 570 nm. Biofilm production was normalized by calculating OD_{570}/OD_{600}. These tests were run in triplicate.

Ethidium Bromide Efflux Inhibition Assays. The EPI activity of berberine was assessed in ethidium bromide efflux inhibition assays of *P. aeruginosa* K767, performed as previously described.[31] Efflux inhibition was evaluated according to the fluorescence decrement observed in the absence/presence of the natural compound after 25 min dynamic. All assays were performed in biological triplicates.

Planktonic Persistence Assays. Overnight cultures of *P. aeruginosa* grown in LB and incubated at 37 °C were exposed to tobramycin or ceftazidime or colistin 20xMIC,[19] alone or combined with berberine 80 μg/mL, and incubated for 24 h at 37 °C. After antibiotic exposure for 0, 7, and 24 h, 1 mL aliquots of the cultures were washed with phosphate buffered saline (PBS), serially 10-fold diluted and plated on antibiotic-free CLED agar. After 24 h incubation at 37 °C, the CFUs were counted as antibiotic persisters. These tests were run in biological duplicate.

***P. aeruginosa* K767 and K1525 Growth Curves in Presence/Absence of Berberine.** Overnight cultures in LB broth were diluted to OD_{600} 0.1 and incubated for 24 h at 37 °C with shaking (140 rpm), in the absence/presence of berberine 80 μg/mL. OD_{600} was recorded after 2, 4, 6, 8, and 24 h. Assays were performed in biological triplicates.

Biofilm Persistence Assays. *P. aeruginosa* biofilms developed overnight in LB in 35 mm Petri dishes at 37 °C were incubated for 24 h at 37 °C in LB supplemented with tobramycin 1000xMIC, alone or combined with berberine (80 or 320 μg/mL). Immediately before tobramycin exposure (T_0) or after 24 h incubation (T_{24}), biofilms were mechanically detached, resuspended in PBS, and serially 10-fold diluted. Suitable dilutions were plated on CLED agar and incubated for 24 h at 37 °C before the CFU counts. These tests were run in biological triplicate.

***mexY* Gene Sequence Analysis.** The main variable regions of the *mexY* gene were identified by comparing the gene sequence of *P. aeruginosa* PAO1 (accession no. AB015853.1) to a number of strain-specific sequences. Two amplicons of these regions (respectively of 270 and 588 bp) were obtained by PCR using the primer pairs mexY-F 5′-TGGAAGTGCAGAACCGCCTG-3′/mexY-R 5′-AGGTCAGCTTGGCCGGGTC-3′[32] and YF 5′-CGTGAGCATGGACGAGATCA-3′/YR 5′-ATGATGGTGATCAGGCCGAC-3′ (this study). The amplicons were purified using Gene Elute PCR Cleanup kit (Sigma-Aldrich SRL) and directly sequenced using BigDye Terminator v.1.1 Cycle Sequencing kit according to the manufacturer's instructions. Sequences were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The consensus sequences thus obtained were compared to the PAO1 sequence. The MAFFT algorithm implemented in AliView software (https://github.com/andersla/Assseq[33]) was used to align the nucleotide sequences analyzed with the corresponding *mexY* regions of *P. aeruginosa* PAO1.

Protein Sequence Analysis and 3D Modeling. Conversion of the nucleotide sequences to the corresponding amino acid sequences (one for each *P. aeruginosa* strain tested) was performed with Aliview.[34] The 3D structure of the MexY trimer of PA01[22] was used as a template to visualize the mutated amino acid positions with respect to the putative berberine binding sites. Any differences in protein surface and 3D structure were investigated using Chimera software.[31,35] Three-dimensional structures of the MexY proteins were modeled as described previously, using MexAB (pdb code 2V50) and AcrB (pdb code 2HRT) as templates,[22] and employed to investigate the binding modes of berberine. The 3D MexY structures were minimized using AMBER99SB-ILDNP Force Field[36,37] implemented in the GROMACS 5.0 software package.[38,39] A robust energy minimization protocol consisting of 10 000 cycles with the steepest descent minimization algorithm was then applied, followed by 5000 cycles using the conjugate gradient algorithm until the threshold (Fmax < 100 kJ mol^{-1}) was reached.

Molecular Docking. Starting from the minimized MexY structures, a molecular docking procedure was used to investigate the binding modes of berberine to the five tobramycin extrusion protein variants. Automated molecular docking of the berberine−MexY complexes of different *P. aeruginosa* strains was performed with the docking program Autodock 4.2.[40] A blind docking approach confirmed the presence of two specific binding sites, corresponding to the two pockets found in *P. aeruginosa* PA01 MexY in our recent work.[22] Two focused docking procedures were used for each MexY model, and the Lamarckian genetic algorithm (GA) was applied to handle ligand−protein interactions. A grid map centered in the ligand and extending around the cleft, with points spaced equally at 0.375 Å intervals, was generated using the Autogrid4 module, to estimate the binding energy between berberine and the MexY forms. The docking parameters were set to default values except for the number of GA runs (100), the energy evaluations (25 000 000), the maximum number of top individuals that automatically survive (0.1), and the step size for translation (0.2 Å). The redocked berberine−MexY complexes were ranked according to the predicted binding energy and arranged into clusters according to root-mean-square deviation (RMSD) values. The cluster of each complex (containing 90% of the docked structures found by the procedure) characterized by the lowest energy was then used for refinement using a validated protocol.[41,42]

Statistical Analysis. The significance of the change in persister amount and biofilm production was assessed by the Student's *t* test (threshold, 0.05).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c01151.

Growth curves of the isogenic pair *P. aeruginosa* K767/K1525 with and without berberine, nucleotide and amino acid sequences of MexY in *P. aeruginosa* PAO1 and in investigated strains, berberine binding mode in *P.*

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aeruginosa C31 and C59, and effect of berberine on tobramycin MIC and tolerant cell abundance in analyzed *P. aeruginosa* strains (PDF)

AUTHOR INFORMATION

Corresponding Author

Gianmarco Mangiaterra − *Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona 60131, Italy*; Phone: +39 071 220 4622; Email: g.mangiaterra@pm.univpm.it; Fax: +39 071 220 4316

Authors

Nicholas Cedraro − *Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona 60131, Italy*

Emiliano Laudadio − *Department of Materials, Environmental Sciences and Urban Planning, Polytechnic University of Marche, Ancona 60131, Italy*

Cristina Minnelli − *Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona 60131, Italy*

Barbara Citterio − *Department of Biomolecular Sciences, sect. Biotechnology, University of Urbino "Carlo Bo", Fano 61032, Italy*

Francesca Andreoni − *Department of Biomolecular Sciences, sect. Biotechnology, University of Urbino "Carlo Bo", Fano 61032, Italy*

Giovanna Mobbili − *Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona 60131, Italy*

Roberta Galeazzi − *Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona 60131, Italy*; orcid.org/0000-0003-1792-654X

Francesca Biavasco − *Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona 60131, Italy*; orcid.org/0000-0002-8582-2227

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jnatprod.0c01151

Author Contributions

[‖]G.M. and N.C. are co-first authors.

Notes

The authors declare no competing financial interest.

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