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N-Acetylcysteine Selectively Antagonizes the Activity of Imipenem in *Pseudomonas aeruginosa* by an OprD-Mediated Mechanism

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The modulating effect of *N*-acetylcysteine (NAC) on the activity of different antibiotics has been studied in *Pseudomonas aeruginosa*. Our results demonstrate that, in contrast to previous reports, only the activity of imipenem is clearly affected by NAC. MIC and checkerboard determinations indicate that the NAC-based modulation of imipenem activity is dependent mainly on OprD. SDS-PAGE of outer membrane proteins (OMPs) after NAC treatments demonstrates that NAC does not modify the expression of OprD, suggesting that NAC competitively inhibits the uptake of imipenem through OprD. Similar effects on imipenem activity were obtained with *P. aeruginosa* clinical isolates. Our results indicate that imipenem-susceptible *P. aeruginosa* strains become resistant upon simultaneous treatment with NAC and imipenem. Moreover, the generality of the observed effects of NAC on antibiotic activity was assessed with two additional bacterial species, *Escherichia coli* and *Acinetobacter baumannii*. Caution should be taken during treatments, as the activity of imipenem may be modified by physiologically attainable concentrations of NAC, particularly during intravenous and nebulized regimes.

Although its benefit has been questioned (1), *N*-acetylcysteine (NAC) is being used for the treatment of numerous disorders, including paracetamol intoxication, doxorubicin cardiotoxicity, ischemia-reperfusion cardiac injury, acute respiratory distress syndrome, bronchitis, chemotherapy-induced toxicity, HIV/AIDS, heavy metal toxicity, and psychiatric disorders (2). This compound is also sold as a dietary supplement, with claims of antioxidant and liver-protecting effects. The antioxidant activity of NAC has been attributed to its reactivity with ·OH, ·NO2, $CO_3{\cdot}^-$, and thiyl radicals, to its capacity for repair of oxidized key cellular molecules, and to its activity as a precursor for glutathione biosynthesis (2). Because of this mucolytic capacity, it has also been used to facilitate the processing of sputum specimens in bacteriology laboratories (3). The ability of NAC to reduce biofilms, alone or in combination with antimicrobials, has been addressed in several bacterial species (4–6). Furthermore, NAC has been proposed as a treatment in *Helicobacter pylori* infections (7). NAC utility in reducing sputum viscosity in patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (8), likely due to its ability to break disulfide bonds, has also been claimed, although its mechanism of action is not well understood.

As a result of an imbalance between the production of reactive oxygen species (ROS) caused by inflammation and their inactivation by the impaired antioxidant systems, CF patients with chronic *Pseudomonas aeruginosa* lung infection have increased oxidative stress. Therefore, antioxidant interventions, including the use of NAC, have been proposed to reduce the extent of oxidative lesions and the rate of lung deterioration (for a review, see reference 9). However, although controversial results on the improvement of lung function have been obtained in clinical trials (9–11), NAC is still being considered positively by many clinicians for the treatment of CF patients.

It has recently been shown that NAC is an important modulator of antibiotic activity, reducing the antibacterial activity of aminoglycosides, fluoroquinolones, and erythromycin and enhancing that of ampicillin, with little or no effect on the activity of chloramphenicol, tetracycline, and penicillin, on different bacterial species, including *P. aeruginosa* (12). Also, an older study (13) reported that *P. aeruginosa* strains were inhibited synergistically by NAC and carbenicillin or ticarcillin and that NAC antagonized the activity of gentamicin and tobramycin. Interestingly, in that study low concentrations of NAC showed a growth-inhibitory effect on *P. aeruginosa* (13). Nevertheless, the effect of NAC on the activity of most antipseudomonal drugs has not been addressed.

Since NAC is so widely used, knowledge of the putative effects of NAC on antibiotic activity on *P. aeruginosa* is of capital importance. Therefore, we decided to evaluate the effect of NAC on the activity of different antipseudomonal drugs, including imipenem (IMI), meropenem, aztreonam, ceftazidime, ciprofloxacin, tobramycin, and colistin, on *P. aeruginosa*. Interestingly, only imipenem activity was clearly affected by NAC. Consequently, the molecular basis of this phenomenon and its generality in other *P. aeruginosa* clinical isolates have been studied. Furthermore, we tested the generality of the NAC modulating effect on other bacterial species such as *Escherichia coli* and *Acinetobacter baumannii*.

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MATERIALS AND METHODS

Bacterial strains and media. *P. aeruginosa* strains PAO1 and PAOD1 (14), PA14 and FQSE21-1003 (15), and Pamb18 and Pamb91 (16), *E. coli* K-12 strain BW25113 (17), and *A. baumannii* strain ATCC 17978 were used in this study. A pharmaceutical preparation of NAC (Hidonac 100; Zambon, Barcelona, Spain), containing NAC (100 mg/ml), disodium edetate (3 mg/ml) as a preservative, and sodium hydroxide (pH 6.5), was used. This formulation is very similar to other preparations dispensed elsewhere (e.g., Mucomyst in the United States). Pure NAC powder was purchased from Sigma-Aldrich as *N*-acetyl-L-cysteine, Sigma grade ≥99% (TLC), and dissolved in sterilized double-distilled water. The pH was adjusted with sodium hydroxide when indicated. Antibiotic and NAC solutions were made fresh daily, filtered through a sterile 0.22-μm membrane filter, and used within 12 h of preparation.

Growth with NAC. Exponential cultures of *P. aeruginosa* strains were diluted 1:1,000 in Mueller-Hinton broth (MHB) and grown in 96-well plates (Nunclon delta surface; Thermo Scientific). When appropriate, MHB was supplemented with NAC. Plates were incubated at 37°C with regular orbital shaking in a microplate reader (Infinite F200; Tecan, Switzerland). Absorbance at 595 nm was recorded in at least 6 biological replicates.

MIC estimation. MICs of different antibiotics and NAC were determined in MHB as outlined by the Clinical and Laboratory Standards Institute (CLSI) (18). The effect of NAC on bacterial susceptibility to different antibiotics was studied by adding NAC to the broth. A concentration of 10 mM NAC (equivalent to 1.6 mg/ml or 0.16%) was used to match that in a previous study on NAC-based modulation of antibiotic susceptibility (12). For experiments involving preincubation with NAC, exponentially growing cultures of PAO1 and PAOD1 were incubated with 10 mM NAC for 2.5 h. NAC was eliminated by saline washing, and MICs of imipenem were determined as indicated above. Interactions between NAC and imipenem were studied by the use of the checkerboard method and analyzed according to EUCAST recommendations (19).

OMP analysis. Cultures of *P. aeruginosa* were grown overnight at 37°C in 5 ml of MHB medium and then diluted 100-fold into fresh medium. Bacterial cells were incubated with different *N*-acetylcysteine (Hidonac) concentrations (0 mg/ml, 0.8 mg/ml, and 1.6 mg/ml) for 4 h with shaking at 37°C. Outer membrane proteins (OMPs) were obtained using a previously reported method (20), OMPs were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. OprD profiles of PAO1 and the PAO1 *oprD* mutant with and without NAC were compared.

RESULTS AND DISCUSSION

Effect of NAC on growth of *P. aeruginosa*. Because low concentrations of NAC were previously shown to be inhibitory for the growth of *P. aeruginosa* (13), we first tested the effect of 10 mM NAC (Hidonac), a concentration that match that in the previous study on NAC-based modulation of antibiotic susceptibility (12), on the growth of several strains of *P. aeruginosa*. According to that report (12), this concentration can be reached in patients with severe respiratory disorders treated with high doses of NAC. Moreover, it has been proposed that the concentration of active NAC in the lower airways after its administration via nebulization of a single dose of 300 mg may be as high as 29 mM (21). Therefore, 10 mM is a realistic concentration of NAC to be tested. Figure 1 shows that after 16 h of growth, this concentration of NAC produced little or no effect on the growth of the tested strains, a result that does not fit with those previously published (13).

pH is the key parameter in the NAC effect on the activity of antibiotics in *P. aeruginosa* PAO1, except on that of imipenem. Our data demonstrate that the addition of a 10 mM concentration of the pharmaceutical preparation of NAC (Hidonac) had little or no effect on *P. aeruginosa* PAO1 susceptibility (Table 1, MH and NAC-P), except in the case of imipenem (see below). As stated in Materials and Methods, the pharmaceutical formulation contained, in addition to NAC (100 mg/ml), disodium edetate (3 mg/ml) and sodium hydroxide (pH 6.5). This formulation is very similar to other preparations dispensed elsewhere (e.g., Mucomyst in the United States). Therefore, the NAC-mediated modification of MICs observed by others (12) could be due to the absence of these additional compounds.

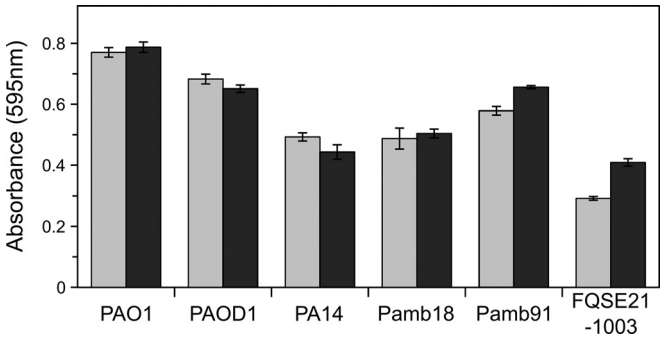

FIG 1 Effect of 10 mM NAC on *P. aeruginosa* growth. For the six strains tested, absorbance at 595 nm was measured after 16 h of growth in MHB with (black bars) or without (gray bars) 10 mM NAC.

To test this possibility, pure NAC powder purchased from Sigma was dissolved in sterilized double-distilled water and used to measure its effect on the susceptibility of *P. aeruginosa* to the antibiotics described above. Table 1 shows that this NAC preparation (NAC-σ) decreased the activity of most antibiotics against *P. aeruginosa* PAO1. The activities of ciprofloxacin, tobramycin, and imipenem were the most affected. Finally, the activities of ceftazidime, aztreonam, meropenem, and colistin were slightly changed or not modified.

To study the importance of the other compounds present in the pharmaceutical formulation of NAC for the antibiotic activity, a simulated pharmaceutical formulation was prepared with the NAC powder purchased from Sigma. NAC (100 mg/ml) and disodium edetate (Sigma) (3 mg/ml) were dissolved in sterilized double-distilled water. Sodium hydroxide was added to adjust the pH to 6.5. As shown in Table 1 (NAC-σ-pH), the MICs of the tested antibiotics, except imipenem, showed levels similar to those reached without NAC or with the pharmaceutical formulation.

When the NAC powder from Sigma was dissolved in sterilized double-distilled water, the pH of the NAC solution was equal to 1.8, far below that of the pharmaceutical preparation (pH 6.5). When the pH of this NAC solution was adjusted to 6.5 with sodium hydroxide, its addition to Mueller-Hinton broth did not modify the final pH of the broth (pH 7.3); i.e., the pH of the Mueller-Hinton broth containing NAC is close to neutrality (Table 1) in all cases, except for that containing the pure NAC powder dissolved in water without pH adjustment (final pH 5.7). of Table 1 (NAC-σ-pH and NAC-σ-EDTA-pH) shows that the activity of antibiotics, previously modified by the solution of NAC powder, was almost completely recovered when the pH of this NAC preparation was adjusted to 6.5.

Again, our results do not match those previously published by others (12, 13), as NAC, under our experimental conditions, does not modify the activity of most antibiotics tested against *P. aerugi-*

TABLE 1 MICs of antibiotics in Mueller-Hinton broth containing different NAC preparations against *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 *oprD*-deficient derivative PAOD1

P. aeruginosa strain	Antibiotic	MIC (μg/ml) in[a]:				
		MH (pH 7.3)	NAC-P (pH 7.2)	NAC-σ (pH 5.7)	NAC-σ-pH (pH 7.2)	NAC-σ-EDTA-pH (pH 7.3)
PAO1	Ciprofloxacin	0.06	0.06	**0.25**	0.06	0.06
	Tobramycin	0.25	0.25	**8**	0.25	0.25
	Ceftazidime	1	1	2	1	1
	Aztreonam	4	8	8	4	4
	Imipenem	1	**16**	**16**	**16**	**16**
	Meropenem	0.25	**1**	0.25	0.5	0.5
	Colistin	0.5	0.5	1	0.5	0.5
PAO1 *oprD*	Ciprofloxacin	0.06	0.06	**0.25**	0.06	0.125
	Tobramycin	0.125	0.125	**8**	0.125	0.125
	Ceftazidime	1	1	2	1	1
	Aztreonam	4	8	8	4	4
	Imipenem	16	32	16	32	32
	Meropenem	2	**8**	2	4	4
	Colistin	0.5	0.5	1	0.5	0.5

[a] The final pH of the broth is indicated. Values representing significant MIC modifications (≥4-fold) are in bold. In all cases, the concentration of NAC was adjusted to 10 mM (1.6 mg/ml). MH, only Mueller-Hinton broth; NAC-P, pharmaceutical preparation of NAC (Hidonac); NAC-σ, solution of NAC powder (Sigma) in water; NAC-σ-pH, solution of NAC powder in water where the pH was adjusted to 6.5 with sodium hydroxide; NAC-σ-EDTA-pH, solution of NAC powder in water supplemented with edetate disodium (3 mg/ml) as a preservative and sodium hydroxide (pH 6.5).

nosa. We do not presently have an appropriate explanation for these discrepancies, although different variables, including pH, bacterial inoculum, and growth broth, could be argued as being responsible for the observed differences.

Apart from the indicated discrepancies, the main conclusion from our results is that the presence of NAC resulted in a 16-fold increase in the MIC of imipenem (from 1 to 16 μg/ml), placing the susceptible PAO1 strain clearly above the breakpoint for resistance according to the clinical EUCAST breakpoints, v4.0 (http://www.eucast.org). The activity of meropenem is also affected, though only slightly, by the NAC concentration used in this assay.

The effect of NAC on imipenem activity in *P. aeruginosa* is mediated by OprD. Since the porin OprD facilitates the uptake of imipenem and some amino acids in *P. aeruginosa* (14), we performed a rapid double-disk synergy test to see if this porin is involved in the NAC effect on imipenem activity. Figure 2 shows that the antagonistic effect of NAC observed in the wild-type (WT) strain *P. aeruginosa* PAO1 is drastically reduced in the *oprD*-deficient strain, suggesting that OprD is mediating this phenomenon.

To gain insight into the involvement of OprD in the antagonistic effect of NAC on imipenem activity, we first quantitatively evaluated the interactions between the drugs by the use of the checkerboard method according to the EUCAST recommendations (19). For this assay, only the pharmaceutical formulation of NAC (pH-independent effect) was used. The data shown in Fig. 3A demonstrate an antagonistic interaction between NAC and imipenem in *P. aeruginosa* PAO1. The fractional inhibitory concentration index (FICi) (22) was 129. This value is 64 to 32 times above the limit for antagonism, which has been suggested to be 2

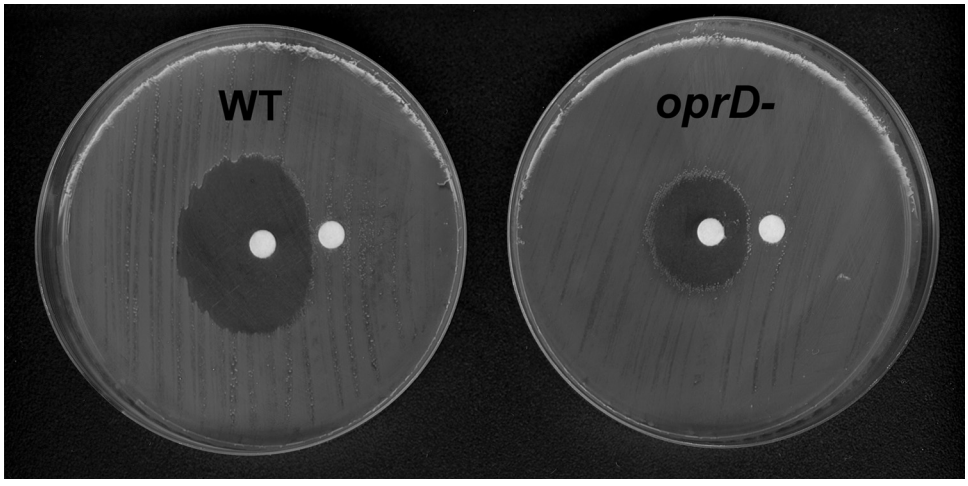

FIG 2 Antagonistic effect of NAC on imipenem activity. Different antagonistic effects of NAC on imipenem activity in *P. aeruginosa* PAO1 (WT) and its mutant derivative PAO1 *oprD* were demonstrated by a double-disk synergy test. Disks of NAC (right) and IMI (left) contained 5 mg and 80 μg, respectively. Note that the distance between disks is shorter in the *oprD* plate. This is to show that an antagonism, although small, exists in the *oprD* mutant.

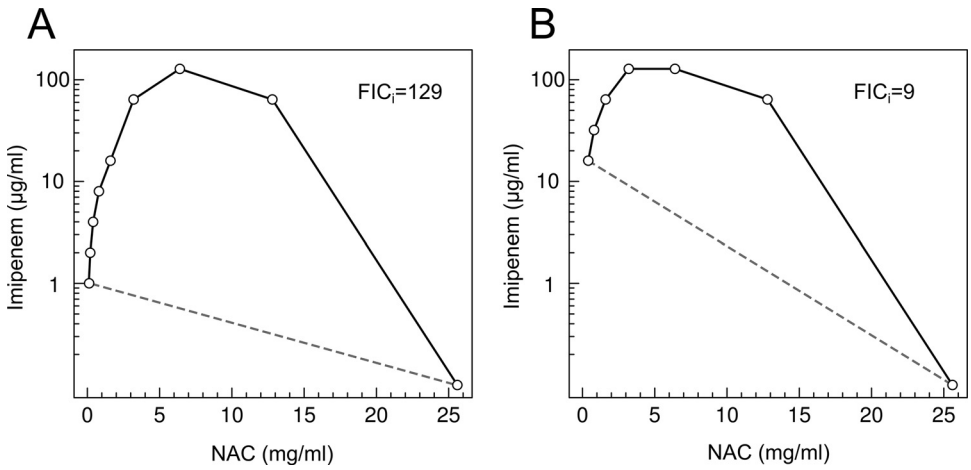

FIG 3 Quantification of the effect of NAC on imipenem activity. The effect of combined treatment of NAC and imipenem on *P. aeruginosa* PAO1 (A) and PAOD1 (B) as analyzed by the checkerboard method is shown. The solid line separates the wells in which the concentration of both drugs allowed growth (below the line) from those with no growth (above). The dashed line indicates the expected effect if the combination of NAC and imipenem were exclusively additive. The values of the fractional inhibitory concentration index (FICi) are shown.

or 4 (19, 22). Interestingly, a large effect on imipenem activity can be observed at concentrations of NAC as low as 0.8 mg/ml (equivalent to 5 mM), which are in the range of concentrations attainable in plasma after intravenous administration (23) or in lungs after its administration via nebulization (21). However, the mean plasma concentration of NAC after oral administration is far below the concentration of NAC necessary to affect imipenem activity (24), even in patients with end-stage renal disease (25).

When the NAC/IMI antagonism was studied in the *oprD* mutant derivative, the PAOD1 variant, the FICi value was 9 (Fig. 3B), slightly over the cited antagonism limit. This result indicates that more than 90% of the observed NAC effect is mediated by OprD. We can conclude, therefore, that OprD is the principal factor involved in the NAC-mediated decreased activity of imipenem in *P. aeruginosa*, although another mechanism(s) may be additionally involved in this effect.

It has been reported that some amino acids may negatively regulate *oprD* transcription (26). Therefore, given that it is an amino acid derivative, NAC could negatively regulate *oprD* transcription, thus decreasing OprD production. To test this hypothesis, we performed an analysis of OprD expression through SDS-PAGE of outer membrane proteins (OMPs). As can be observed in Fig. 4, the presence of NAC at 0.8 mg/ml or 1.6 mg/ml did not modify the expression of OprD, ruling out this hypothesis.

Amino acids may also act as competitors of imipenem for OprD (27, 28), competitively reducing the uptake of this carbapenem. Table 1 shows that the effect of NAC on the antibiotic activities in the *P. aeruginosa oprD*-defective mutant PAOD1 is similar to that in the wild-type strain PAO1, except for imipenem, where only a marginal effect on the activity is produced. These results also eliminate the possibility of the inhibition of imipenem activity by a direct interaction with NAC. If this were the case, the MIC of imipenem should have been increased in the *oprD*-defective mutant in a proportion similar to that observed in the wild-type strain (i.e., about 16-fold). Therefore, our data strongly suggest that the effect in *P. aeruginosa* is exerted mainly via competition of NAC with imipenem by the OprD channel. Obviously, this mechanism requires the simultaneous presence of both compounds. Consequently, if NAC is eliminated (oxidized or metabolized, for instance), bacterial cells should recover their previous susceptibility to IMI. To test this hypothesis, cultures of PAO1 and PAOD1 were incubated with 10 mM NAC for 2.5 h.

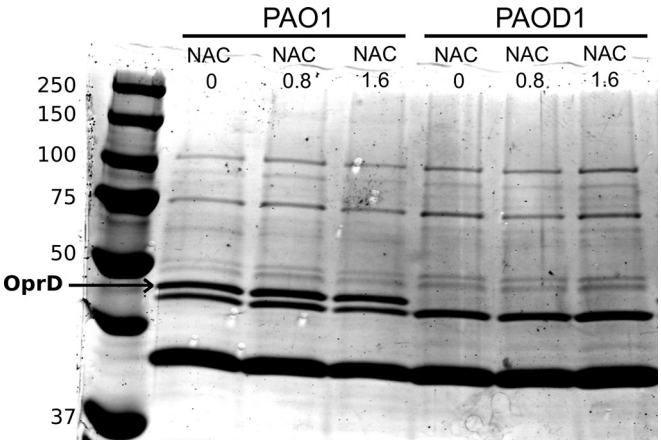

FIG 4 Effect of NAC on OprD expression. SDS-PAGE of outer membrane proteins (OMPs) extracted from *P. aeruginosa* PAO1 and PAOD1 (*oprD*-defective mutant) treated with NAC at 0.8 mg/ml and 1.6 mg/ml is shown. Controls without NAC are also shown. The bands corresponding to OprD and protein sizes of the molecular mass marker (in kDa) are shown.

TABLE 2 MICs of imipenem in Mueller-Hinton broth containing different NAC preparations against different *P. aeruginosa* strains

Strain	MIC (μg/ml) in[a]:				
	MH	NAC-P	NAC-σ	NAC-σ-pH	NAC-σ-EDTA-pH
PA14	0.5	**16**	**2**	**8**	**4**
Pamb18	2	**32**	**16**	**32**	**16**
Pamb91	2	**16**	**8**	**16**	**8**
FQSE21-1003	1	**8**	**4**	**8**	**4**

[a] Abbreviations and the final pH of the broth in each case are as described for Table 1. Values representing significant MIC modifications (≥4-fold) are in bold. In all cases, the concentration of NAC was adjusted to 10 mM (1.6 mg/ml).

TABLE 3 MICs of antibiotics in Mueller-Hinton broth containing different NAC preparations against *E. coli* K-12 BW25113 and *A. baumannii* ATCC 17978

Strain	Antibiotic	MIC (μg/ml) in[a]:				
		MH	NAC-P	NAC-σ	NAC-σ-pH	NAC-σ-EDTA-pH
E. coli K-12 BW25113	Ciprofloxacin	0.008	0.008	**0.25**	0.008	0.015
	Gentamicin	0.25	0.25	**4**	0.125	0.5
	Tobramycin	0.5	0.5	**8**	0.5	0.5
	Ampicillin	4	4	2	2	4
	Ceftazidime	0.125	0.125	0.25	0.25	0.25
	Imipenem	0.5	**8**	**0.5**	**4**	**2**
	Meropenem	0.5	**2**	0.5	0.5	0.5
	Colistin	0.5	0.5	0.5	0.5	0.5
A. baumannii ATCC 17978	Ciprofloxacin	0.125	0.125	**1**	0.125	0.125
	Ceftazidime	8	16	16	16	16
	Imipenem	0.5	**8**	**2**	**8**	**8**
	Meropenem	0.25	0.25	0.125	0.25	0.25
	Colistin	0.06	0.125	**2**	0.06	0.06
	Tigecycline	≤0.015	<0.015	**1**	<0.015	<0.015

[a] Abbreviations and the final pH of the broth in each case are as described for Table 1. Values representing significant MIC modifications (≥4-fold) are in bold.

NAC was eliminated by saline washing, and the susceptibility to IMI was compared to that of nonpretreated cultures. No differences were observed between the two conditions (data not shown), indicating that the antagonistic effect is produced only when both drugs are present simultaneously. This result reinforces the hypothesis that NAC directly competes with IMI by OprD.

The NAC effect on imipenem activity is a general phenomenon in *P. aeruginosa*. The generality of the NAC-modulating effect on imipenem activity was tested on other *P. aeruginosa* strains from different clinical origins, including cystic fibrosis and bacteremia (14). Table 2 shows that imipenem activity is clearly reduced for all these strains.

Effects of NAC on antibiotic activity in *E. coli* and *A. baumannii*. The results with *P. aeruginosa* clearly demonstrate that is the pH of the solution, not NAC by itself, which produces the modulating effects on the activity of most antibiotics, with the exception of imipenem. To analyze whether this is an exclusive effect on *P. aeruginosa*, we performed identical studies with *E. coli*, a species also used in the work of Goswami and Jawali (12), and *A. baumannii*. Antibiotics currently used in the treatment of infections caused by these two species were tested.

Table 3 shows that, as for *P. aeruginosa*, the effect of NAC on the activity of most antibiotics is fully attributable to the pH of the solution in both *E. coli* and *A. baumannii*. Again, a clear pH-independent effect on imipenem activity was observed in both species. Meropenem activity is also slightly affected by NAC-P when tested in *E. coli* but is not in *A. baumannii*. Interestingly, in the latter species, the activities of the two last-resource antibiotics used for the treatment of frequent multidrug-resistant strains, colistin and tigecycline, were also affected by pH.

Conclusions. Our data show that the effect of NAC on the activity of most antibiotics is due exclusively to the pH of the NAC solution, and not to the NAC itself, in *P. aeruginosa*. It is already known that factors such as divalent cation concentration, partial pressure of oxygen, and pH can largely affect antibiotic activity (29, 30). Particularly, an acidic pH compromises the activity of aminoglycosides, quinolones, and erythromycin (31, 32).

Interestingly, the only activity clearly affected by NAC is that of imipenem. This effect is independent of pH and is mediated mainly by the OprD porin of *P. aeruginosa*. The main mechanism underlying this effect in this species is likely the competitive inhibition of imipenem uptake by the OprD channel, since the expression of OprD itself is not modified. Additional mechanisms of inhibition cannot be ruled out, as the elimination of OprD does not completely abolish the antagonistic NAC effect. The effect of NAC on imipenem activity has been demonstrated for different *P. aeruginosa* isolates. Finally, the generality of the pH-mediated NAC effect has been demonstrated in two additional bacterial species, *E. coli* and *A. baumannii*.

Although the clinical implications of the results presented here remain to be confirmed, clinicians should take caution that the susceptibility to imipenem determined in the microbiology laboratory may be modified when this carbapenem is coadministered with NAC, particularly in intravenous and nebulized regimes.

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