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Tetracycline improved the efficiency of other antimicrobials against Gram-negative multidrug-resistant bacteria

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Summary Treatment of infectious diseases with antimicrobials constituted a great achievement in the history of medicine. Unfortunately, the emergence of resistant strains of bacteria to all classes of antimicrobials limited their efficacy. The present study was aimed at evaluating the effect of combinations of antibiotics on multi-drug resistant Gram-negative (MDRGN) bacteria.

A liquid micro-broth dilution method was used to evaluate the antibacterial activity of 10 different classes of antimicrobials on 20 bacterial strains belonging to six different species. The antimicrobials were associated with phenylalanine β-naphthylamide (PAβN), an efflux pump inhibitor, and with other antimicrobials at their sub-inhibitory concentrations. The effectiveness of each combination was monitored using the minimal inhibitory concentration (MIC) and the fractional inhibitory concentration (FIC).

Most of the antimicrobials tested showed low antibacterial activity with a MIC value of 128 mg/L on a majority of the bacterial strains, justifying their multidrug-resistant (MDR) profile. Synergistic effects were mostly observed (FIC $\leq$ 0.5) when ampicillin (AMP), cloxacillin (CLX), erythromycin (ERY), chloramphenicol (CHL), kanamycin (KAN) and streptomycin (STR) were combined with tetracycline (TET) at the sub-inhibitory concentration of MIC/5 or MIC/10.

The results of the present work suggest that the association of several antimicrobials with TET could improve the fight against MDRGN bacterial species.

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Introduction

In the twenty-first century, infectious diseases continue to ravage the human population, and they account for approximately half of the mortality rates in tropical countries. These alarming statistics indicate their devastating nature. Unfortunately, the worldwide dissemination of multi-drug-resistant bacteria has severely reduced the efficacy of antibacterial agents, thus increasing therapeutic failures [1]. One of the major antibiotic resistance mechanisms utilized by bacteria is active efflux. Efflux pumps (EPs) involved in this type of resistance are membrane-associated active transporters promoting the extrusion of toxic compounds, including antimicrobials. This extrusion decreases the intracellular concentration of antimicrobials and reduces the susceptibility of bacterial strains to these drugs. Therefore, antibiotic therapy has more than ever become a challenge for scientists, and new means of tackling resistant bacteria are urgently needed. The use of synergistic antibiotic association is an appealing way to optimize good results during antibiotherapy [2]. Although antibiotic combinations have long been used against multiple potential pathogens in the initial empirical treatment of critically ill patients, the selection of such drugs and their potential for providing increased activity in combination should be made with particular attention to minimize any negative interactions [3]. This work was aimed at evaluating the effect of antibiotic combinations against MDRGN bacteria.

Material and methods

Chemicals for antimicrobial assays

Tetracycline (TET), doxycycline (DOX), cefepime (FEP), streptomycin (STR), ciprofloxacin (CIP), norfloxacin (NOR), chloramphenicol (CHL), cloxacillin (CLX), ampicillin (AMP), erythromycin (ERY), and kanamycin (KAN) (Sigma-Aldrich, St Quentin Fallavier, France) were used as reference antibiotics. *p*-Iodonitrotetrazolium chloride (INT) and phenylalanine arginine β-naphthylamide (PAβN) were used as microbial growth indicators and efflux pump inhibitors (EPIs), respectively.

Bacterial strains and culture media

The studied microorganisms included reference (American Type Culture Collection) and clinical (laboratory collection) strains of *Providencia stuartii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter aerogenes* and *Enterobacter cloacae*. The bacterial strains and their characteristics were previously reported [4], as shown in Table S2. The preliminary treatment of these organisms and the culture media were prepared as described in a previous study [5].

Antibiotic susceptibility tests

Antibiotic susceptibility tests using single antimicrobials and in combination with PAβN

The MICs of each of the 10 antimicrobials used were determined for 20 bacterial strains using the rapid INT colorimetric assay [6,7]. One hundred microliters of prepared antimicrobial was added in the first well of each column of a micro titer plate containing 100 μl of broth in each well and were than serially diluted twofold. Next, 100 μl of the inoculum prepared in Mueller Hinton broth (MHB, Sigma-Aldrich) was added to have a final concentration of 10^6 UFC/ml. The final concentration of DMSO was 2.5% and did not affect the microbial growth. The plates were covered with a sterile plate sealer and were agitated to mix the content of the wells using a shaker. The MICs of the samples were determined after 18 h of incubation at 37 °C, following the addition (40 μl) of 0.2 mg/ml INT and incubation at 37 °C for 30 min [8,9]. The MIC was defined as the lowest concentration of antimicrobial that prevented color change of the medium and exhibited complete inhibition of microbial growth [6,8].

The role of efflux pumps in the susceptibility of Gram-negative bacteria to the antimicrobials used in this study

The antibacterial susceptibility test in the presence of the efflux pump inhibitor was carried out after the twofold serial dilution by adding 20 μl of PAβN (final concentration of PAβN 20 mg/L) to 100 μl of MHB in the wells of the micro titer plate. Then, 80 μl of the inoculum was introduced to obtain a final concentration of 10^6 UFC/ml. Wells containing MHB, 100 μl of inoculum, and DMSO at a final concentration of 2.5% served as negative controls (this internal control was systematically added). The total volume in each well was 200 μl. The MICs of the antimicrobials were obtained after 18 h of incubation at 37 °C, as mentioned above.

The inhibitory activity of the combined antimicrobials

The MICs of the two combined antimicrobials were monitored as follows: 100 μl of the antibiotic, A1,

Table 1 The minimal inhibitory concentration of the studied antimicrobials on the different bacterial species.

Bacteria	Antimicrobials and MICs (mg/L)									
	B-lactams		Tetracycline		Quinolones		Macrolides	Phenicol	Aminosides	
	AMP	CLOX	TET	DOX	NOR	CIP	ERY	CHL	KAN	STR
E. coli										
ATCC8739	—	—	64	64	2	64	128	—	—	128
AG100Atet	—	—	64	64	8	64	64	—	—	64
AG100	64	32	8	1	128	2	32	8	16	64
MC4100	—	32	8	8	128	64	64	<1	—	—
E. aerogenes										
ATCC13048	—	—	16	8	<1	32	64	16	4	16
EA3	—	—	16	32	128	64	—	64	—	64
EA289	—	128	64	32	128	128	64	—	<1	32
EA298	—	—	16	128	nd	2	128	32	—	64
EA27	—	128	32	16	32	32	32	—	16	32
K. pneumoniae										
ATCC11296	—	—	32	32	<1	<1	—	16	128	—
KP63	128	128	16	8	8	32	64	64	—	64
K2	—	—	32	16	32	128	64	64	128	128
K24	—	—	32	32	64	64	128	32	—	64
P. aeruginosa										
PA01	—	—	16	16	128	32	64	32	—	128
PA124	—	—	16	16	64	128	64	128	64	64
E. cloacae										
ECCI69	—	—	32	<1	<1	128	64	—	—	64
BM47	—	128	32	16	—	128	128	—	—	8
BM67	—	—	16	32	64	—	128	64	64	32
P. stuartii										
PS2636	—	128	—	64	128	64	—	—	64	—
PS299645	—	—	—	16	8	64	32	16	<1	64
NAE16	—	64	32	16	32	128	64	64	2	128

'—' represents >128 mg/L.
MIC, Minimal inhibitory concentration; AMP, Ampicillin; CLOX, Cloxacillin; TET, Tetracycline; DOX, Doxycyclin; NOR, Norfloxacin; CIP, Ciprofloxacin; ERY, Erythromycin; CHL, Chloramphenicol; KAN, Kanamycin; STR, Streptomycin; *E. coli*, *Escherichia coli*; *K. pneumonia*, *Klebsiella pneumoniae*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *E. cloacae*, *Enterobacter cloacae*; *P. stuartii*, *Providencia stuartii*.

was introduced in the first well of each column of the micro titer plate. After the twofold serial dilution, 20 μl of the second antibiotic, A2, was introduced at concentrations of MIC/5 or MIC/10, as determined in a preliminary assay using the MDR strain of *P. aeruginosa* PA124. Then, 80 μl of the inoculum (bacteria) was added into each well of the plate to a final concentration of 10^6 UFC/ml. The MIC obtained was that of A1 in the presence of 20 μl of A2. The fractional inhibitory concentration (FIC) was calculated as previously described [10]. The results were interpreted as follows:

$FIC \leq 0.5$ (synergy), $0.5 < FIC \leq 4$ (indifference) and $FIC > 4$ (antagonism).

Results

Antibiotic susceptibility testing using single antimicrobials and in combination with PAβN

The results of the antibacterial activity of the single antimicrobials on a panel of Gram-negative bacteria are summarized in Table 1.

These results show that the studied bacteria species were weakly sensitive to most of the antimicrobials tested, with MIC values equal to or greater than 128 mg/L. The 10 most resistant strains among the 20 tested bacteria were selected, and their susceptibilities to antimicrobials were determined in the presence of PAβN to determine whether

Table 2 The minimal inhibitory concentration of the studied antimicrobials in the presence and absence of PAβN on 10 MDR strains of bacteria.

Antimicrobials	Bacteria and MIC (mg/L)									
	E. coli		*E. aerogenes*		*K. pneumoniae*	*P. aeruginosa*	*E. cloacae*		*P. stuartii*	
	ATCC8739	AG100Atet	EA3	EA298	KP63	PA124	ECCI69	BM47	PS2636	PS299645
PAβN	>1024	>1024	1024	>1024	1024	1024	>1024	1024	>1024	>1024
AMP										
−PAβN	—	—	—	—	—	—	—	—	—	—
+PAβN	—	—	—	—	—	—	—	—	—	—
CLX										
−PAβN	—	—	—	—	128	—	512	—	128	—
+PAβN	—	—	—	—	—	—	—	—	—	—
TET										
−PAβN	128	64	—	16	16	16	32	—	—	—
+PAβN	64	8	32	64	64	16	16	128	64	16
DOX										
−PAβN	64	64	32	128	8	16	<1	16	64	16
+PAβN	8	<1	2	32	1	<0.5	<0.5	8	2	<0.5
CIP										
−PAβN	128	64	64	2	64	128	128	128	128	64
+PAβN	32	32	32	4	32	64	64	64	64	32
NOR										
−PAβN	2	8	128	<1	8	64	<1	—	128	8
+PAβN	64	4	128	64	4	32	—	—	16	64
ERY										
−PAβN	128	64	32	64	64	—	64	64	—	32
+PAβN	64	128	16	16	32	16	8	32	64	16
CHL										
−PAβN	—	64	—	—	—	128	—	—	—	—
+PAβN	128	32	128	32	32	64	64	64	128	32
KAN										
−PAβN	—	—	<1	—	—	64	—	—	64	<1
+PAβN	—	—	—	—	128	512	—	—	32	64
STR										
−PAβN	128	128	64	—	64	128	64	8	—	64
+PAβN	—	2	<1	32	32	64	32	2	—	32

'—' represents >128 mg/L; AMP,: ampicillin; CLX, cloxacillin; TET, tetracycline; DOX, doxycycline; CIP, ciprofloxacin; NOR, norfloxacin; ERY, erythromycin; CHL, chloramphenicol; KAN, kanamycin; STR, streptomycin; MDR, Multi-Drug Resistant; (+PAβN), in presence of 20 mg/L of PaβN; (−PAβN), in the absence of PAβN.

Table 3 The MICs of different antimicrobials after their association with subinhibitory concentrations of tetracycline against ten MDR bacterial strains.

Antimicrobials	Bacterial strains, MIC (mg/L) of antimicrobials and fold increase (in parenthesis) of some antimicrobials combine with TET									
	TET concentration	PA124	ATCC8739	AG100ATet	EA3	EA298	ECCI69	BM47	KP63	PS2636	PS299645
AMP	0	—	—	—	—	—	—	—	128	—	—
	MIC/10	≤0.5 (≥1024)^{S}	64 (>4)^{S}	≤0.5 (≥1024)^{S}	≤0.5 (≥1024)^{S}	>64	32 (≥16)^{S}	≤0.5 (≥1024)^{S}	64 (2)^{S}	>64	≤0.5 (≥1024)^{S}
CLX	0	—	—	—	—	—	—	128	128	128	—
	MIC/5	≤0.5 (≥1024)^{S}	>64	≤0.5 (≥1024)^{S}	≤0.5 (≥1024)^{S}	≤0.5 (≥1024)^{S}	≤0.5 (≥1024)^{S}	≤0.5 (≥256)^{S}	≤0.5 (≥256)^{S}	>64 (≥128)^{S}	≤0.5 (≥1024)^{S}
ERY	0	64	128	64	—	128	64	128	64	—	32
	MIC/5	≤0.5 (≥128)^{S}	0.5 (256)^{S}	≤0.5 (≥128)^{S}	≤0.5 (≥1024)^{S}	≤0.5 (≥256)^{S}	≤0.5 (≥128)^{S}	≤0.5 (≥256)^{S}	≤0.5 (≥128)^{S}	32 (≥16)^{S}	≤0.5 (≥64)^{S}
CHL	0	128	—	—	64	32	—	—	64	—	32
	MIC/5	≤0.5 (≥256)^{S}	32 (>16)^{S}	≤0.5 (≥1024)^{S}	≤0.5 (≥128)^{S}	≤0.5 (≥64)^{S}	≤0.5 (≥1024)^{S}	≤0.5 (≥1024)^{S}	≤0.5 (128)^{S}	64 (≥8)^{S}	≤0.5 (≥64)^{S}
KAN	0	256	—	—	—	—	—	—	—	64	≤4
	MIC/10	≤2 (≥128)^{S}	≤4 (≥128)^{S}	—	≤4 (≥128)^{S}	64 (≥8)^{S}	>6464 (≥16)^{S}	128 (≥4)^{S}	≤4 (≥128)^{S}	64 (1)^{I}	≤4
STR	0	128	128	64	—	64	64	8	64	64	128
	MIC/5	≤0.5 (≥256)^{S}	4 (32)^{S}	≤0.5 (≥128)^{S}	≤0.5 (≥1024)^{S}	≤0.5 (≥128)^{S}	≤0.5 (≥128)^{S}	≤0.5 (≥16)^{S}	≤0.5 (≥128)^{S}	>64	≤0.5 (≥256)^{S}

'—' represents ≥512r; AMP, ampicillin; CLX, cloxacillin; TET, tetracycline; DOX, doxycycline; CIP, ciprofloxacin; NOR, norfloxacin; ERY, erythromycin; CHL, chloramphenicol; KAN, kanamycin; STR, streptomycin; (S), synergy; (I), indifference.

they over-express active efflux pumps. The results summarized in Table 3 show that PAβN alone had no intrinsic activity at the concentration used (MIC $\geq$ 1024 mg/L on most of the strains). The activity of a majority of antimicrobials increased slightly in the presence of PAβN. This was observed with CIP, ERY, DOX, CHL and STR (Table 2). However, the largest increase in the activity of antimicrobials in the presence of PAβN was observed with DOX, which displayed better activity on all 10 bacterial strains (a 4- to 12-fold decrease of the MICs). These observations confirm the fact that the antimicrobials used here are expelled out of the bacterial cell in the absence of EPI. In contrast to the antimicrobials of other classes, the activity of β-lactam (in the presence of the pump inhibitor PAβN) remains unchanged. Meanwhile, the association of NOR and PAβN led to the reduction of the antibacterial activity of NOR, suggesting possible antagonistic effects.

Antibiotic susceptibility tests using combined antimicrobials

The studied antimicrobials were also combined in pairs (A1 and A2) with A2 tested at sub-inhibitory concentrations against 10 MDR bacterial strains. A preliminary study was conducted *using P. aeruginosa* PA124 (supporting information in Table S2). The lowest sub-inhibitory concentrations of A2 that resulted in the lowest MIC of A1 were MIC/5 in most cases on *P. aeruginosa* PA124. However, two cases of association at MIC/10 (AMP + TET and KAN + TET) were noted. The sub-inhibitory concentration of A2 that permitted the best activity of A1 was further extended to the other nine bacterial strains. The fractional inhibitory concentration obtained with most associations was less than or equal to 0.5 (FIC $\leq$ 0.5), representing synergistic activity between the two antimicrobials. This synergism was mainly observed in the association of tetracycline (TET and DOX as A2) to other antimicrobials. Therefore, TET was selected as antimicrobial A2 and tested in association with other antimicrobials on MDR bacteria (Table 3). The results clearly showed that TET at MIC/5 and MIC/10 significantly improved the activity of other antimicrobials. More than a 1024-fold increase was obtained when TET was combined with AMP, CLX, and CHL against the majority of the studied MDR bacteria. Additionally, no cases of antagonism were observed when the antimicrobials under study were combined with TET (Table 3).

Discussion

The antibacterial activity of different antimicrobials

A weak sensitivity of bacterial strains to the antimicrobials was observed. This is not surprising given that the strains expressed several mechanisms of resistance including active efflux [11]. Although the majority of bacterial strains were weakly sensitive, variations were observed in the activity of the antimicrobials. These variations may be due to intrinsic factors specific to each microorganism. These results corroborate previous data, indicating that the activity of the active principle can vary from one species to another or from one strain to another [12].

It was also observed that the activity of antimicrobials depends on their respective classes. It was observed that β-lactam bactericidal antimicrobials were the less active class of antimicrobials. This can be explained by the production of β-lactamases and/or the reduction of porins located at the external membrane of bacteria that are the principal resistance mechanisms of bacteria such as *Klebsiella pneumoniae* to β-lactams [1]. Conversely, the inhibitory activity of antimicrobials of the same class varies from one compound to another (Table 1). This was the case for NOR and CIP (two quinolones) on different strains of *E. coli*. This variation between antimicrobials of the same class could be explained by the structural difference between the two compounds as the ethyl group of NOR is replaced by a cyclopropyl group in CIP.

The role of efflux pumps in the susceptibility of Gram-negative bacteria to antimicrobials

PAβN is an arylpiperazine that inhibits RND (Resistance-Nodulation-cell Division) pumps by competition with antimicrobials for target sites [13,14]. To determine the role of efflux pumps in this work, the concentration of PAβused (20 μg/ml) did not exert any intrinsic effect on the bacteria, as previously demonstrated [15]. The antibacterial activity of β-lactams was not improved by the presence of PAβN. This can be explained by the non-implication of efflux pumps in the mechanism of resistance of these strains to β-lactams. Additionally, the targets of β-lactams are located in the bacterial coast; thus, the efflux provides another possibility for re-entering the bacterial cell, showing that the inhibition of the efflux pumps could

reduce this activity. These results corroborate previous data [16], which showed that the addition of efflux pumps inhibitors. Naphthylmethyl phenylalanine (NMP) and phenylalanine β-naphthylamide (PAβN) has no effect on the MIC of AMP and penicillin, which are two β-lactams. In contrast, the significant decrease in the MIC values of DOX in the presence of PAβN suggests that this antibiotic could be a substrate of the type of efflux pumps involved in the resistance of the strains used here. However, Lamers et al. [17] demonstrated last year that at concentrations equal to 25 mg/L and above, PAβN also increases the activity of antimicrobials through the permeabilization of bacterial membranes, which increases the intracellular concentration of antimicrobials. However, this could not be directly concluded from the results of this study because the concentration (20 mg/L) used is lower. The results obtained regarding the association of PAβN to NOR and CIP are contrary to previous findings [18], which showed that PAβN is the inhibitor of efflux pumps that expel quinolones. These contradictory results can be explained by the development of other mechanisms of resistance, such as the mutation of their targets, namely, DNA gyrase and topoisomerase IV [14]. In fact, the activity of CIP remains almost unchanged in the presence of PaβN, while that of DOX increased (resulting in decreased MICs) on many tested strains (Table 2). These differences could be linked to the structural differences already mentioned between the two antimicrobials.

The effects of the association of antimicrobials

The synergistic effects of antimicrobials observed on many strains could be due to the presence of preferential substrates of efflux pumps between the two combined antimicrobials, which could allow an increase in the intracellular concentration of the second antimicrobial in the bacterial cell [19]. The synergistic effect could also be due to the presence of an effector that fixes on its target site and modifies the conformation of the sites to improve the entry and interaction of the second antibiotic with its target [1]. These effects could then explain the different cases of synergisms observed in this study, specifically in the experiments using TET as antibiotic A2. It is generally admitted that tetracyclines possess bacteriostatic activity, but some previous studies revealed bactericidal activities of these drugs and their synergistic effect in combination with different antimicrobials [20,21]. The MDRGN bacteria used in this study are quite representative of the common Gram-negative resistant bacteria involved in human infectious diseases, but it would be of great importance to extend the study of the synergistic effects found here on Gram-positive resistant bacteria and their effectiveness *in vivo*. Additionally, the indifferences observed, notably with the association of KAN + TET and ERY + CIP, can be explained by the over-expression of other types of efflux pumps by these bacterial strains [22]. In contrast to these effects, the antagonism observed between NOR and CIP may be due to a negative effect of the complex formed by the two compounds, given that both antimicrobials are quinolones.

Conclusion

The overall results obtained in this study show that the association of TET to antimicrobials of other classes leads to synergistic effects. These results provide baseline information for the possible use of antimicrobial combinations, mostly between TET and other antimicrobials, to fight infections caused by multi-drug resistant bacteria.

Authors' contributions

IMK and JANK carried out the study and wrote the manuscript; VK designed the experiments; VK and JRK supervised the work; VK provided the bacterial strains; and all of the authors read and approved the final manuscript.

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Conflict of interests

The authors declare that there is no conflict of interest.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiph.2014.09.001.

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