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Short Communication

The anthelmintic oxyclozanide restores the activity of colistin against colistin-resistant Gram-negative bacilli

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ABSTRACT

Due to the significant increase in antimicrobial resistance in Gram-negative bacilli (GNB), development of non-antimicrobial therapeutic alternatives, which can be used together with the few and non-optimal available antimicrobial agents such as colistin, has become an urgent need. In this context, dysregulation of the bacterial cell wall could be a therapeutic adjuvant to the activity of colistin. The aim of this study was to analyse the activity of oxyclozanide, an anthelmintic drug, in combination with colistin against colistin-susceptible (Col-S) and colistin-resistant (Col-R) GNB. Three Col-S reference strains and 13 clinical isolates (1 Col-S, 12 Col-R) of *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were studied. Microdilution assays and time–kill curves were performed to examine the activity of oxyclozanide in combination with colistin. The outer membrane protein (OMP) profile, membrane permeability and cell wall structure of Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae* in the presence of oxyclozanide were assessed by SDS-PAGE, fluorescence microscopy and transmission electron microscopy, respectively. Oxyclozanide in combination with colistin increased the activity of colistin against Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae*. Time–kill curves showed synergistic activity between oxyclozanide and colistin against these bacterial isolates. Moreover, Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae* in the presence of oxyclozanide presented greater permeability and disruption of their cell wall than Col-S strains, without modification of their OMP profile. These data suggest that combination of oxyclozanide and colistin may be a new alternative for the treatment of Col-R GNB infections.

1. Introduction

There are few options available for the treatment of multidrug-resistant (MDR) Gram-negative bacilli (GNB), and last-resort treatments such as colistin are no longer effective in an increasing number of cases, leading to a 28-day mortality rate of 43% in hospitalised patients with bacteraemia, ventilator-associated or hospital-acquired pneumonia, or urosepsis caused by GNB [1].

Although colistin currently retains significant in vitro activity against most GNB, resistance to colistin is being increasingly reported among clinical isolates [2]. The SENTRY Antimicrobial Surveillance Program performed worldwide surveys in 2006–2009 and 2009–2011 and reported that colistin resistance among GNB (*Acinetobacter* spp., *Pseudomonas aeruginosa* and *Klebsiella* spp.) increased from 0.1–1.5% to 0.7–3.6% [3,4], probably due to extensive and/or inadequate use of colistin worldwide for treating infections caused by MDR-GNB.

Therefore, the development of new strategic antimicrobial therapeutic approaches for use alone or in combination with one of the scarce but clinically relevant antibiotics has become urgent. In this environment, 'repurposing' (defined as investigating new uses for existing drugs) has gained renewed interest, as reflected by several recent studies [5].

Oxyclozanide, which is used in veterinary medicine for treating fluke infections in ruminants [6], exhibits activity against

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Staphylococcus aureus, Clostridioides (*Clostridium*) *difficile* and *Helicobacter pylori*, probably due to disruption of their cell envelope [5,7]. Niclosamide, the lead compound of oxyclozanide, has also been identified as a potent antibacterial drug against *H. pylori* and *P. aeruginosa* owing to inhibition of quorum sensing and various virulence genes as well as reduction of elastase and pyocyanin levels [5].

Currently, there is no study regarding the combination of oxyclozanide and colistin to restore the activity of colistin against GNB. The aim of this study was therefore to determine the in vitro activity of oxyclozanide in combination with colistin against colistin-susceptible (Col-S) and colistin-resistant (Col-R) *Acinetobacter baumannii, P. aeruginosa* and *Klebsiella pneumoniae* isolates.

2. Materials and methods

2.1. Bacterial isolates

Three Col-S reference strains were used in this study, including *A. baumannii* ATCC 17978, *P. aeruginosa* PAO1 and *K. pneumoniae* CECT 997. In addition, 13 clinical isolates were studied, including 5 Col-R *A. baumannii* isolated from a hospital outbreak in 2000 in Spain, 5 clinical Col-R *P. aeruginosa* isolated from patients with cystic fibrosis, and 1 Col-S and 2 Col-R clinical *K. pneumoniae* isolates.

2.2. Amplification and sequencing of pmrA, pmrB, mgrB, phoP and phoQ genes

To investigate the possible contribution of *pmrAB, mgrB* and *phoPQ* to colistin resistance in *A. baumannii, P. aeruginosa* and *K. pneumoniae*, these genes were analysed to detect any genetic alterations, as previously described (Supplementary Table S1) [8].

2.3. In vitro susceptibility testing

Minimum inhibitory concentrations (MICs) of colistin (Sigma, Madrid, Spain), oxyclozanide (Sigma) and colistin + 2 mg/L oxyclozanide against Col-S and Col-R isolates were determined in two independent experiments by broth microdilution assay as previously described [8], and according to Clinical and Laboratory Standards Institute (CLSI) (*A. baumannii* and *P. aeruginosa*) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) (*K. pneumoniae*) recommendations [9,10]. A concentration of 2 mg/L oxyclozanide was used in these experiments as chequerboard assays showed the greatest synergy with colistin (data not shown).

2.4. Chequerboard assay

The chequerboard assay was performed in 96-well plates. Expanded details of this assay are given in the Supplementary material.

2.5. Time–kill kinetic assays

Time–kill curves of *A. baumannii* ATCC 17978 and Col-R #14, *P. aeruginosa* PAO1 and Col-R M4, and *K. pneumoniae* CECT 997 and Col-R Kp21 were performed with a starting inoculum of 1×10^6 CFU/mL in cation-adjusted Mueller–Hinton broth in the presence of 2 mg/L oxyclozanide and colistin (sub-MIC), alone or in combination, in two independent experiments as previously described [8]. Oxyclozanide was considered synergistic when the combination with colistin reduced the bacterial concentration ≥2 log CFU/mL compared with colistin alone.

2.6. Analysis of outer membrane proteins (OMPs) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Bacterial cells were grown in Luria–Bertani (LB) broth to log phase, were incubated with 2 mg/L oxyclozanide for 4 h or 24 h, and were lysed by sonication. OMP profiles were determined by SDS-PAGE as previously described [11].

2.7. Transmission electron microscopy

Bacterial cells were grown in LB broth and were incubated in the absence or presence of 2 mg/L oxyclozanide for 24 h. The cell pellet was harvested by ultracentrifugation at 4600 × *g* for 15 min and was resuspended in 2.5% glutaraldehyde containing 0.1 M cacodylate buffer to fix the samples. Following three rounds of centrifugation in the same conditions and washing with 0.1 M cacodylate buffer, the pellet was included in agarose resin using an automatic processor and then ultrathin sections were obtained by ultramicrotome. These sections were placed into a grid for visualisation using a Zeiss Libra 120 transmission electron microscope (Zeiss, Oberkochen, Germany).

2.8. Membrane permeability assay

Bacterial cells were grown in LB broth and were incubated in the absence or presence of 2 mg/L oxyclozanide for 24 h. The cell pellet was harvested by ultracentrifugation at 4600 × *g* for 15 min. Bacterial cells were washed with 1 × phosphate-buffered saline (PBS) and after centrifugation in the same conditions as described before, the pellet was resuspended in 100 µL of 1 × PBS containing 10 µL of ethidium homodimer-1 (EthD-1) (Invitrogen, Waltham, MA, USA). After 10 min of incubation, (i) 10 µL of pellet was placed onto a slide for visualisation using a Leica DM-6000 fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) and (ii) 100 µL was placed into a 96-well plate to measure fluorescence at 0, 10, 15, 20, 30, 40, 70, 100 and 130 min using a Typhoon™ FLA 9000 laser scanner (GE Healthcare Life Sciences, Piscataway, NJ, USA) and quantification using ImageQuant TL software (GE Healthcare Life Sciences).

3. Results

3.1. Colistin minimum inhibitory concentrations and resistance mechanisms

MICs for the Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae* isolates ranged from 4 mg/L to > 512 mg/L, whilst those for the Col-S reference strains were 0.5–1 mg/L. Analysis of the *pmrA, pmrB, mgrB, phoP* and *phoQ* gene sequences showed that Col-R *A. baumannii* isolates presented different amino acids substitutions only in PmrB [8]; Col-R *P. aeruginosa* isolates presented different amino acid substitutions in PmrA, PmrB, PhoP and PhoQ, a nucleotide deletion in *pmrB* or nonstop codon insertion in *phoQ*; and Col-R *K. pneumoniae* isolates presented IS*1* transposase insertion in *mgrB* or different amino acids substitutions in PmrA and PmrB, without amino acid substitutions in PhoP and PhoQ (Supplementary Table S2).

3.2. In vitro activity of oxyclozanide in combination with colistin against colistin-susceptible and colistin-resistant Acinetobacter baumannii, Pseudomonas aeruginosa and Klebsiella pneumoniae

Oxyclozanide, alone or in combination with colistin, was tested against reference strains and clinical isolates of Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae*. The MICs are shown in Table 1. Oxyclozanide alone showed high MICs of 128–512 mg/L

Table 1
Minimum inhibitory concentrations (MICs) of oxyclozanide (Oxz) and colistin (Col), alone or in combination, against Col-susceptible and Col-resistant *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

Species	Isolate	MIC (mg/L)			Fold change in MIC of Col
		Col alone	Oxz alone	Col in combination[a]	
A. baumannii	ATCC 17978	0.5 (S)	256	0.06	8
	#11	256 (R)	256	1	256
	#14	4 (R)	128	<0.125	>32
	#17	4 (R)	128	0.25	16
	#21	128 (R)	128	16	8
	#113	16 (R)	256	<2	>8
P. aeruginosa	PAO1	1 (S)	512	0.25	4
	M1	>512 (R)	256	32	>16
	M2	>512 (R)	128	64	>8
	M3	64 (R)	128	≤4	≤16
	M4	16 (R)	128	<0.25	>64
	M5	>512 (R)	128	8	>64
K. pneumoniae	CECT 997	0.5 (S)	256	0.25	2
	Kp07	2 (S)	512	1	2
	Kp21	32 (R)	512	4	8
	Kp29	16 (R)	512	4	4

S, susceptible; R, resistant.
[a] MIC of Col in combination with 2 mg/L Oxz.

for Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae*. Oxyclozanide at 2 mg/L in combination with colistin significantly increased the activity of colistin against all Col-S and Col-R isolates, being more active against Col-R isolates. This concentration of oxyclozanide was chosen based on data from chequerboard assays in which 2 mg/L oxyclozanide was the most common synergistic concentration with colistin against Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumonia*e.

3.3. Time–kill curves

The ability of oxyclozanide in combination with colistin to kill Col-S (ATCC 17978) and Col-R (#14) *A. baumannii*, Col-S (PAO1) and Col-R (M4) *P. aeruginosa*, and Col-S (CECT 997) and Col-R (Kp21) *K. pneumoniae* was examined by time–kill assay. Oxyclozanide at 2 mg/L in combination with colistin at 0.25 mg/L, a sub-MIC of colistin for *A. baumannii* ATCC 17978, showed no synergistic activity compared with colistin alone in the first 8 h, followed by later synergistic activity decreasing the bacterial cell count by 4.91 log CFU/mL compared with colistin alone at 24 h. In the case of *P. aeruginosa* PAO1 and *K. pneumoniae* CECT 997 strains, 2 mg/L oxyclozanide in combination with 0.5 mg/L and 0.25 mg/L colistin, a colistin sub-MIC of PAO1 and CECT 997 strains, respectively, no showed synergistic activity compared with colistin alone during 24 h (Table 2).

Regarding the Col-R isolates, 2 mg/L oxyclozanide in combination with 2, 4 and 16 mg/L colistin, a colistin sub-MIC of #14, M4 and PAO1 and Kp21 isolates, respectively, showed higher synergistic activity, decreasing the bacterial cell count compared with colistin alone after 24 h by 6.43, 3.70 and 3.50 log CFU/mL, respectively (Table 2). In a control experiment, 2 mg/L oxyclozanide had no effect on the growth of Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae* (Table 2).

3.4. Effect of oxyclozanide on the outer membrane protein profile

Supplementary Fig. S1 illustrates the OMP profile of *A. baumannii* (ATCC 17978 and #14), *P. aeruginosa* (PAO1 and M4) and *K. pneumoniae* (CECT 997 and Kp21) in the absence or presence of 2 mg/L oxyclozanide incubated for 4 h or 24 h. No changes on the OMP profile were observed in all conditions, suggesting that oxyclozanide does not alter the expression of OMPs.

3.5. Effect of oxyclozanide on bacterial permeability

To study the effect of oxyclozanide on bacterial membrane permeability, Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae* isolates were treated with 2 mg/L oxyclozanide and were incubated with EthD-1. Fluorescence microscopy analysis showed a large increase in cellular fluorescence in Col-S and Col-R *A. baumannii* and *P. aeruginosa* isolates in the presence of oxyclozanide compared with untreated cells. This increase was greater in Col-R compared with Col-S isolates. The results were similar with *K. pneumoniae*, although in this case the fluorescence increase was lower in Col-S and Col-R isolates (Fig. 1A). Quantitatively, 2 h of fluorescence monitoring using Typhoon™ laser scanner confirmed the results of fluorescence microscopy that oxyclozanide increases the permeability of Col-S and Col-R *A. baumannii* and *P. aeruginosa* isolates, with Col-R isolates being more permeable. In contrast, a small increase was observed in Col-S and Col-R *K. pneumoniae* isolates. No fluorescence was observed in the control condition. These data suggest that oxyclozanide affects bacterial permeability.

3.6. Effect of oxyclozanide on the integrity of the bacterial cell envelope

Based on the observed effect of oxyclozanide on membrane permeability, we hypothesised that oxyclozanide may disrupt the integrity of the cell envelope of Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae*. For this reason, we analysed the structure of the cell envelope of these pathogens by transmission electron microscopy. It was observed that oxyclozanide disrupts to different degrees the morphology of the bacterial membrane in all studied isolates compared with isolates without oxyclozanide treatment. This disorder appears to be partial and not around the entire bacterial membrane (Supplementary Fig. S2).

4. Discussion

This study showed that oxyclozanide increases the activity of colistin against Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae* isolates, being more active against Col-R isolates. It is noteworthy to mention that the use of oxyclozanide alone did not affect the growth of Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae* isolates, which is consistent with previously published data in which oxyclozanide did not present bactericidal

Table 2
Analysis of time–kill curves of colistin-susceptible (Col-S) and colistin-resistant (Col-R) *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in the absence or presence of colistin (Col), oxyclozanide (Oxz), or Col + 2 mg/L Oxz during 24 h

Species	Col susceptibility profile	Condition	ΔLog CFU/mL at[a,b]:		
			4 h	8 h	24 h
A. baumannii	Col-S	ATCC 17978	1.87	2.33	3.21
		ATCC 17978 + Oxz	+1.85	2.18	2.12
		ATCC 17978 + Col	−3.69	−3.1	2.55
		ATCC 17978 + Col + Oxz	−1.53	−2.46	**−2.36**
	Col-R	#14	1.5	2.67	2.79
		#14 + Oxz	1.3	2.72	2.66
		#14 + Col	−1.62	−3.32	0.93
		#14 + Col + Oxz	−2.29	−3.82	**−5.5**
P. aeruginosa	Col-S	PAO1	0.88	2.42	3.15
		PAO1 + Oxz	1.14	2.47	3.01
		PAO1 + Col	−4.45	−2.35	0.04
		PAO1 + Col + Oxz	−2.74	−3.11	0.65
	Col-R	M4	−0.32	1.16	2.7
		M4 + Oxz	−0.42	−0.42	2.17
		M4 + Col	−3.96	−2.4	−0.07
		M4 + Col + Oxz	−5.26	**−6.04**	**−3.77**
K. pneumoniae	Col-S	CECT 997	1.6	3.08	3.55
		CECT 997 + Oxz	1.29	2.9	3.44
		CECT 997 + Col	−2.73	−3.66	0.36
		CECT 997 + Col + Oxz	−1.81	−4.12	0.64
	Col-R	Kp21	1.48	2.87	3.12
		Kp21 + Oxz	1.44	3.03	3.11
		Kp21 + Col	−0.8	0.51	2.36
		Kp21 + Col + Oxz	−1.81	**−1.94**	**−1.14**

[a] Compared with log CFU/mL at 0 h.
[b] Values in bold indicate the presence of synergy when the combination of Col + Oxz reduced the bacterial concentration ≥2 log CFU/mL compared with Col alone.

or bacteriostatic effects [7]. Previous independent work showed that the lead compound niclosamide can potentiate the activity of colistin against Col-S and Col-R *A. baumannii* and *K. pneumoniae* clinical isolates [8]. However, to date the question remains of how oxyclozanide acts on these pathogens.

Oxyclozanide showed an MIC range against Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae* isolates ranging from 128–512 mg/L. Due to its use as an anthelminthic agent [6], no susceptibility breakpoints for oxyclozanide are defined. However, the MICs obtained are in accordance to those reported analysing the in vitro activity of oxyclozanide and its lead compound niclosamide against *A. baumannii, P. aeruginosa* and *K. pneumoniae* [7,8].

Besides the fact that oxyclozanide alone did not present bactericidal and bacteriostatic effects, more important is that its combination with colistin potentiates the effect of colistin alone against clinical Col-S and Col-R isolates as determined by time–kill assay. The in vitro activity observed with oxyclozanide in combination with colistin suggests there is a strong possibility to repurpose oxyclozanide for antibacterial use against MDR-GNB. These results are in accordance with those reported in previous studies in which the combination of oxyclozanide and other anthelmintic drugs such as niclosamide and closantel, with colistin reversed the colistin resistance and enhanced the activity of colistin against *A. baumannii, P. aeruginosa* and *K. pneumoniae* [8,12–14]. Also noteworthy is that no synergistic effects were observed with oxyclozanide in combination with other antimicrobial agents such as imipenem, ciprofloxacin and vancomycin (data not shown). The combination of colistin with other antimicrobials agents has been proved to be useful, both in vitro and in vivo, against MDR-GNB [2], as other examples of combinations.

Despite the fact that oxyclozanide does not appear to inhibit the growth of *A. baumannii, P. aeruginosa* and *K. pneumoniae*, we showed for the first time that oxyclozanide partly disrupts the cell envelope of the studied bacterial pathogens. These data are consistent with previously published reports showing that oxyclozanide causes significant bacterial cell envelope damage of *S. aureus* and *H. pylori* [5]. It is well known that colistin targets the bacterial outer membrane [2] and we suggest that disruption of the integrity of the bacterial cell envelope allows colistin to bind more to its target independently of the colistin resistance level. Evidence supports this hypothetical model in which we demonstrated that oxyclozanide did not modify the OMP profile or expression.

It should be noted that Col-R *K. pneumoniae* was less permeable than *A. baumannii* and *P. aeruginosa* following oxyclozanide treatment. This difference might explain the lower fold change of colistin MIC following incubation with oxyclozanide observed for strain Kp21 (8-fold change). For strains #14 and M4, the fold change was >32 and >64, respectively.

On the other hand, oxyclozanide is known for its poor absorption and bioavailability [15]. This characteristic is shared by other salicylanilide drugs [15]. Two previous studies have reported that a single oral dose of 15 mg/kg oxyclozanide in goats and sheep to treat trematode infections resulted in median plasma oxyclozanide concentrations of 7 mg/L and 19 mg/L, respectively [16,17]. These values exceed by 3.5-fold and 9.5-fold, respectively, the concentration of oxyclozanide used in the current study. Unfortunately, extensive binding to plasma proteins has been reported for oxyclozanide and other salicylanilides, which currently limits their systemic and intravenous application [15]. A possible requirement for new formulations to increase their bioavailability should be take into account in the pre-clinical development of this approach. Of note, positive results have been seen with the *O*-alkylamino-tethered niclosamide derivative, for which a potent antibacterial effect against carbapenemase-producing and colistin-resistant Enterobacteriaceae isolates has been observed [18]. Moreover, an inhalable nanosuspension and salt form of

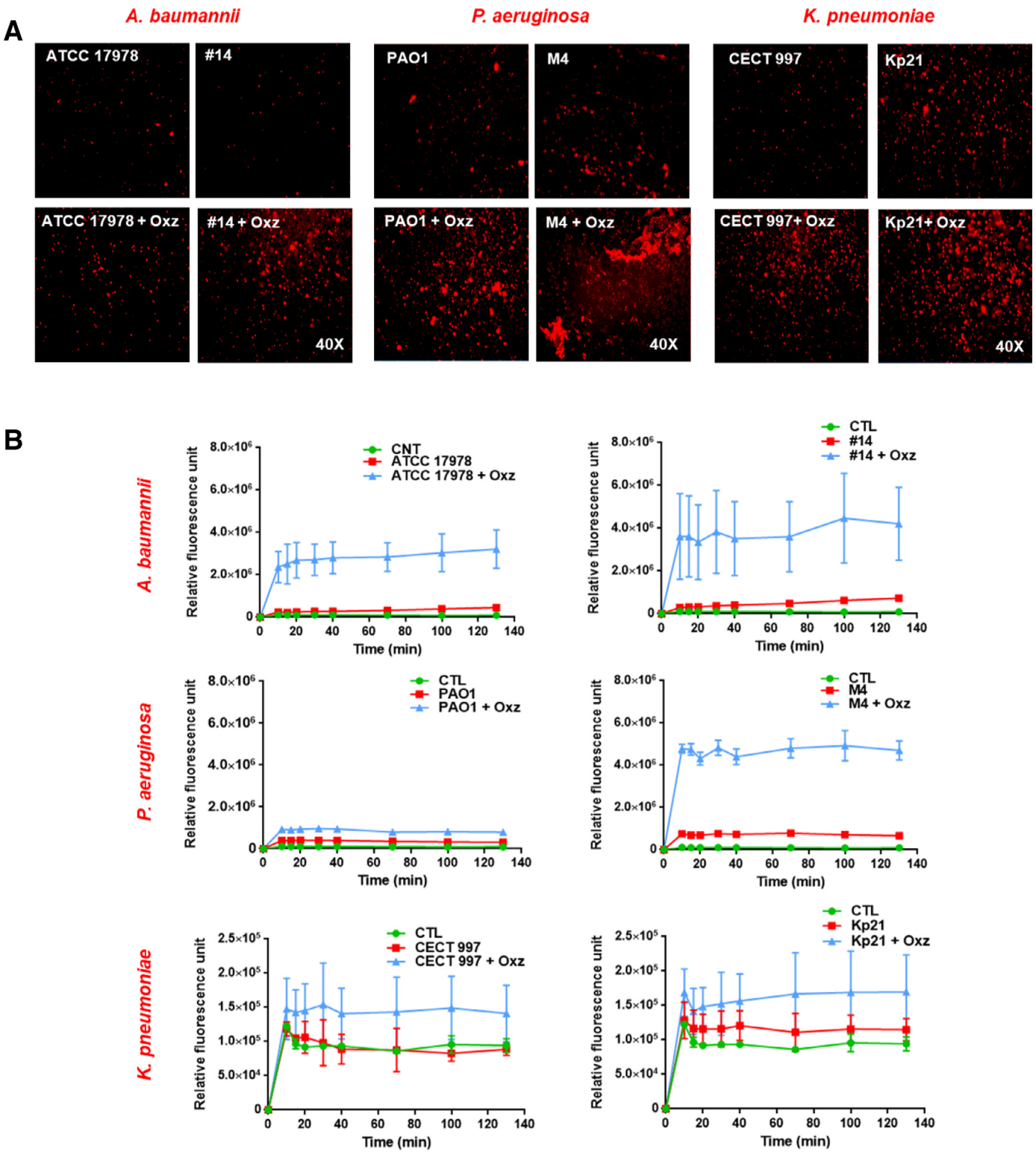

Fig. 1. Effect of oxyclozanide (Oxz) on bacterial permeability. Membrane permeabilisation by Oxz in colistin-susceptible (Col-S) and colistin-resistant (Col-R) isolates of *Acinetobacter baumannii* (Col-R ATCC 17978 and Col-S #14), *Pseudomonas aeruginosa* (Col-R PAO1 and Col-S M4), and *Klebsiella pneumoniae* (Col-R CECT 997 and Col-S Kp21) in the absence and presence of 2 mg/L Oxz incubated for 24 h: (A) visualised by fluorescence microscopy; and (B) quantified by Typhoon™ laser scanning. CTL, control.

niclosamide (niclosamide ethanolamine) presented a better solubility profile and inhibited *P. aeruginosa* quorum sensing [19,20].

5. Conclusions

Oxyclozanide potentiated the effect of colistin against Col-S and Col-R *A. baumannii, P. aeruginosa* and *K. pneumoniae* isolates. This effect might be due to disruption of the bacterial cell envelope. These results provide new insights into the use of oxyclozanide in combination with colistin as a new alternative for the treatment of infections caused by GNB. Further data regarding pharmacokinetics/pharmacodynamics and in vivo therapeutic efficacy are required to investigate the usefulness of this alternative therapy.

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Declaration of Competing Interest

None declared.

Ethical approval

Not required.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.07.006.

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