**In vitro activity of minocycline alone or in combination in multidrug-resistant Acinetobacter baumannii isolates**

Carlos Hernan Rodríguez, Marcela Nastro, Carlos Vay and Angela Famiglietti

Laboratorio de Bacteriología, Departamento de Bioquímica Clínica, Hospital de Clínicas José de San Martín, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

Minocycline (MIN) usually shows good activity against Acinetobacter baumannii strains. The reintroduction to the market of intravenous MIN provides an additional agent to the limited options for the treatment of A. baumannii infections. The activity of MIN as a single agent and in combination with rifampicin (RIF), colistin (COL) or imipenem (IMI) was evaluated by means of killing curves and 24 h-time-kill curves in five A. baumannii isolates which were selected on the basis of different antimicrobial resistance profiles. MIN showed bacteriostatic activity in three isolates (2 × or 16 × MIC) and bactericidal activity in the other isolates (64 × MIC). In isolates harbouring the tetB gene, the associations studied were always indifferent. However, in isolates not harbouring tetB, the use of MIN in combination showed a rapid synergistic effect (at 4 h) in four out of nine combinations (two with RIF and one each with IMI and COL). At 24 h, this effect was observed in six out of nine combinations (two in each association). MIN in combination with RIF, IMI and COL showed bactericidal synergy in most of the isolates which did not harbour the tetB gene, but the combinations were not synergistic in tetB-positive isolates.

**INTRODUCTION**

Acinetobacter baumannii is commonly associated with nosocomial infections. This organism is usually multi-drug-resistant (MDR) and the therapeutic options to treat A. baumannii infections are very limited (Peleg et al., 2008). Unfortunately, no new antibiotics will be available for MDR Gram-negative pathogens in the near future. Old antibiotics, such as colistin (COL) and minocycline (MIN), are generally the most active against MDR A. baumannii strains (Peleg et al., 2008). MIN resistance is widespread, mainly due to the presence of the tetB gene (Huys et al., 2005). However, isolates that produce it present a wide range of MIC values (Huys et al., 2005). The reintroduction of intravenous MIN to the market provides an additional agent to the limited options for the treatment of infections by MDR A. baumannii (Goff & Kaye 2014). Experience with intravenous MIN to treat infections by A. baumannii, particularly nosocomial pneumonia, is scarce and the majority of the patients reported in the literature received more than one antibiotic (Wood et al., 2003; Ritchie & Garavaglia-Wilson, 2014). Only a few reports have described interactions between MIN and other antibiotics in A. baumannii, none of them involving strains harbouring the tetB gene; the renewed clinical interest in MIN requires a re-evaluation of these associations (Tan et al., 2007; Liang et al., 2011).

The aim of this study was to assess the *in vitro* antibacterial activity of MIN alone or in combination with rifampicin (RIF), imipenem (IMI) and COL against five A. baumannii clinical isolates, selected on the basis of their resistance profiles.

**METHODS**

Five clinical isolates of MDR A. baumannii were selected on the basis of different antimicrobial resistance profiles from routine clinical samples of patients admitted to the intensive care unit of a university hospital of Buenos Aires city during the period August–December 2014. Strains were identified using matrix-assisted laser desorption ionization-time-of-flight MS Biotype software version 3.1 (Bruker Daltonik). Susceptibility was determined using a VITEK 2 system (bioMérieux) and interpreted according to the clinical breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2012). PFGE was performed with ApaI as described previously (Seifert et al., 2005). The detection of OXA-type carbapenemases and \\textit{bla}_{\text{OXA}} genes was performed using PCR amplification followed by sequencing, as described previously (Woodford et al., 2006; Poirel et al., 2011).

MICs of A. baumannii to MIN, COL, RIF and IMI were determined by the agar dilution method, and interpreted according to the CLSI and Societe Française de Microbiologie for RIF (Members of the SFM...
Antibiogram Committee, 2003; CLSI, 2012). Antibiotic powders were obtained from their respective manufacturers (COL, RIF, MIN: Sigma; IMI: Merck, Sharp & Dohme). The activities of COL, RIF, IMI and their combinations with MIN were evaluated by killing curves. These antimicrobials are either the most active or the most utilized ones, alone or in combination, against A. baumannii infections. The following concentrations were used, COL, 2 μg ml\(^{-1}\); RIF, 4 μg ml\(^{-1}\); IMI, 8 μg ml\(^{-1}\); MIN, 4 μg ml\(^{-1}\); Briefly, tubes containing cation-supplemented Mueller–Hinton broth (Laboratorios Britania) with and without antibiotic were inoculated with A. baumannii strains in an exponential-phase inoculum of ~5 × 10\(^{8}\) c.f.u. ml\(^{-1}\). Tubes were incubated in an ambient atmosphere at 35 \(^{\circ}\)C. At time 0, 4 and 24 h after inoculation, serial 10-fold dilutions were performed and aliquots were plated onto nutrient agar; the number of c.f.u. were counted after 24 h of incubation at 37 \(^{\circ}\)C. To avoid the in vitro carry-over effect, the sample was allowed to be absorbed by the agar until the plate surface appeared dry and then it was spread over the plate. The time-kill studies were performed twice and thus results were analysed by using mean c.f.u. values from the duplicate plates for each isolate.

The 24 h-time-kill curves were produced in exponential-growth phase with two of the clinical isolates (3AAb and 543Ab). Exponential-growth phase studies were performed using a tube macrodilution method in Mueller–Hinton broth with 10\(^{5}\) c.f.u. ml\(^{-1}\) inoculum and multiple antibiotic concentrations. The final antibiotic concentrations were: MIN (0.125–64 μg ml\(^{-1}\)) with fixed subinhibitory concentrations of COL (0.25 μg ml\(^{-1}\)), RIF (4 μg ml\(^{-1}\)) and IMI (8 μg ml\(^{-1}\)). Each tube was incubated for 24 h at 37 \(^{\circ}\)C, and then 0.1 ml from each tube was diluted and plated onto brain heart infusion agar; finally, the number of c.f.u. was counted after 24 h of incubation at 37 \(^{\circ}\)C. The studies were performed twice and analysed using mean c.f.u. values. The lower limit of detection of this in vitro method was 20 c.f.u. ml\(^{-1}\).

The bactericidal activity of single antibiotics or combinations was defined as a ≥3 \(\log_{10}\) c.f.u. ml\(^{-1}\) decrease in the viable count compared with the initial inoculum, whereas bacteriostatic activity was defined as a <3 \(\log_{10}\) c.f.u. ml\(^{-1}\) decrease. Synergism and antagonism were respectively defined as ≥2 \(\log_{10}\) c.f.u. ml\(^{-1}\) decrease or increase in the viable count with the combination compared with the most active agent alone at different time points (Rodriguez et al. 2010).

The time required to decrease the viable count to half the initial inoculum (\(t_{50}\)) for each antimicrobial agent and their combinations was assessed by the time-kill curves.

The change in \(\log_{10}\) c.f.u. ml\(^{-1}\) over the 24 h incubation (\(\Delta\log\) c.f.u.24 h) for each association was calculated according to the equation: \(\Delta\log\) c.f.u.24 h = 24 h \(\log_{10}\) c.f.u. ml\(^{-1}\) – 0 h \(\log_{10}\) c.f.u. ml\(^{-1}\), where c.f.u. counts were mean values for duplicate experiments in two isolates (with and without the tetB gene). In addition, the \(\Delta\log\) c.f.u.24 h for each association was converted to an effect by the equation: effect = \(\Delta\log\) c.f.u.24 h (max) – \(\Delta\log\) c.f.u.24 h (for each MIN concentration), where \(\Delta\log\) c.f.u.24 h (max) refers to the MIN concentration with the largest positive \(\Delta\log\) c.f.u.24 h.

**RESULTS**

All isolates were resistant to ampicillin/sulbactam, piperacillin/tazobactam, ceftriaxone, ceftazidime, imipenem, meropenem, amikacin, gentamicin, trimetroprime/sulphamethoxazole and ciprofloxacin. All the strains harboured \(\text{bla}_{XXA.23}\) Carbapenemase, and 3AAb and 1416Ab also carried \(\text{bla}_{\text{tetB}}\). Strain 543Ab was resistant to COL and RIF by undefined mechanisms, and strain 971Ab showed COL heteroresistance, which was previously defined by Li et al.
(2006) (Table 1). Five different PFGE types were delineated in the A. baumannii isolates analysed. Epidemiological characteristics, MIC values to MIN, RIF, IMI and COL for the five A. baumannii strains, and the in vitro effects of MIN, COL, RIF and IMI alone and in combination with MIN are shown in Table 1.

In time-kill experiments, MIN showed bacteriostatic activity in three isolates (2 × or 16 × MIC) and bactericidal activity in the other two isolates (64 × MIC). The kill curve for the isolate (1416Ab) studied with subinhibitory concentrations was similar to the growth control curve. In isolates harbouring tetB, the associations studied were always indifferent, whereas in isolates which did not carry the tetB gene, the use of MIN in combination showed a rapid synergistic effect (at 4 h) in four out of nine combinations (two with RIF and one each with IMI and COL). At 24 h this effect was observed in six out of nine combinations (two in each association) (Table 2).

In the 24 h-time-kill curves obtained for strains 3AAb and 543Ab, MIN alone exhibited bactERICidal activity at concentrations up to 16 µg ml⁻¹. However, no synergy was observed in isolate 3AAb, which was tetB-positive. In the isolate not harbouring tetB, the MIN combinations showed a synergistic activity, and the bactERICidal effect was extended to MIN concentrations in the range 2–8 µg ml⁻¹ in the case of COL and 0.5–8 µg ml⁻¹ for RIF and IMI (Fig. 1).

The tₜ₀ values of MIN in combination with COL, IMI or RIF were lower than for MIN alone against tetB-negative isolates, whereas they were similar in isolates harbouring tetB (Table 2).

The relationship between MIN concentration and effect in tetB-negative isolates showed a maximum effect at MIN concentrations of ~4 µg ml⁻¹ for MIN/COL and 16 µg ml⁻¹ for MIN/RIF and MIN/IMI (Fig. 2).

**DISCUSSION**

Tetracyclines show time-dependent killing and have an increased post-antibiotic effect. Their activity is mainly bacteriostatic. Free drug area under the concentration-time curve/MIC appears to be the parameter which was most closely linked to the antibacterial effect. Pharmacokinetic studies have indicated that peak serum concentrations following a 200 mg intravenous dose of MIN range from 2.52 to 6.63 µg ml⁻¹ (mean 4.18 µg ml⁻¹) (Castanheira et al., 2014).

Unlike other previously published studies, we included in the analysis two strains harbouring the tetB gene and the synergistic activity of associations with different concentrations of MIN was evaluated (Tan et al., 2007; Liang et al., 2011).

Although the clinical relevance of this in vitro synergy is difficult to determine, it is logical to think that reaching and maintaining bactERICidal MIN concentrations at the infection site would lead to successful therapeutic results.
As was mentioned earlier, MIN (and tetracyclines in general) typically have a bacteriostatic activity; however, some authors have reported a bactericidal effect in *A. baumannii* isolates (Bantar et al., 2008). In our study, MIN showed bactericidal activity only at concentrations ≥ 32 µg ml⁻¹ or when the related concentration/MIC in killing curves was ≥ 64 µg ml⁻¹. However, these concentrations cannot be achieved with therapeutic doses (Fig. 1).

The associations studied in this work have modified the bacteriostatic activity of MIN alone for a rapid initial bactericidal effect in the majority of the isolates without *tetB* and this synergy occurred at various concentrations of MIN, including the peak concentration and trough at habitual doses. The major effect (> Δlog c.f.u.24 h) was detected at concentrations similar to or slightly higher than the peak level (Table 2, Figs 1 and 2).

In the literature, the MIN/IMI association showed a synergistic effect in isolates both susceptible and resistant to carbapenems (Rodríguez-Hernández et al., 2000). Similar to a report by Liang et al. (2011), we detected synergy in IMI-resistant isolates working with subinhibitory concentrations of carbapenems (4–8 µg ml⁻¹) (Liang et al., 2011). In 24 h-time-kill curves, we confirmed the synergy observed in time-kill curves with one concentration of MIN (4 µg ml⁻¹) in isolates without *tetB* and this synergy was determined using similar concentrations to those achieved therapeutically (peak 6.6 µg ml⁻¹; trough 1.4 µg ml⁻¹) (Fig. 1). In addition, the *t₅₀* values showed a quicker decrease (at 12 h) (Table 2, Figs 1 and 2).

### Fig. 1. Relationship between drug effect (log₁₀c.f.u. of clinical *A. baumannii* strains) and MIN concentration (alone or in combination) in the 24 h-time-kill curve study. (a) 3AAb. (b) 543Ab. Concentrations used were: MIN (0.125–64 µg ml⁻¹) with fixed subinhibitory concentrations of COL (0.25 µg ml⁻¹), RIF (4 µg ml⁻¹) and IMI (8 µg ml⁻¹).

### Fig. 2. Relationship between drug effect [effect = Δlog c.f.u.24 h (max)−Δlog c.f.u.24 h; see Methods] for each MIN concentration and MIN concentration for each association in the 24 h-time-kill curve study. (a) 3AAb. (b) 543Ab.
The bactericidal activity of MIN/RIF was observed irrespective of the RIF MIC value (4 or 128 µg ml−1). One study reported the activity of RIF combined with MIN not including isolates with high RIF MIC values (Liang et al., 2011). The features of this synergy are similar to the that obtained with IMI, except for the value of t50, which in the isolate 543Ab (MIC RIF 128 µg ml−1) is slightly lower than the t50 corresponding to MIN alone (Table 2, Figs 1 and 2).

MIN/COL synergy was observed in heteroresistant COL strains by suppression of regrowth of the resistant subpopulations. In the COL-resistant strain we observed bactericidal synergy at 24 h despite the high MIC value to both antimicrobials. The COL/RIF synergy in COL-resistant A. baumannii isolates has been described previously (Rodriguez et al., 2010), but to the best of our knowledge, this is the first study reporting the MIN/COL synergistic activity in COL-resistant A. baumannii isolates. The mechanism remains unknown, but the instability of COL-resistant isolates could play an important role in the synergistic activity obtained. Surprisingly, the COL t50 value of the COL-resistant strain is lower than that obtained with the heteroresistant isolate, presumably due to the regrowth of the resistant subpopulations; nevertheless, in the associations with MIN the t50 value is close to 2 h irrespective of the susceptibility to COL (Table 2, Figs 1 and 2).

The activity of MIN and combinations in isolates with tetB were indifferent, irrespective of the MIN MIC value and the concentrations of MIN utilized in the experiments (1/8 × or 8 × MIC). Moreover, the higher Δlog c.f.u.24 h was detected at concentrations not achieved under therapeutic conditions (Table 2, Figs 1 and 2).

Only Tan et al. (2007) have previously reported the performance of MIN-resistant strains. Contrary to our results, they reported synergy in three of four isolates studied, by inhibition of regrowth of the COL-resistant subpopulations.

CONCLUSIONS

MIN has proved to be one of the best antimicrobial agents against MDR A. baumannii; however, the fact that MIN usually presents bacteriostatic activity hinders its use for the treatment of severe infections. The combinations with COL, RIF or IMI showed bactericidal activity in both susceptible and resistant A. baumannii isolates. However, the lack of synergy in isolates with tetB should prevent its use as an empiric treatment in hospitals with a high presence of these strains.

ACKNOWLEDGEMENTS

This work was supported by grants from the Secretaría de Ciencia y Técnica de la Universidad de Buenos Aires (UBACyT 20020130100 167BA) to A. F.

REFERENCES


