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Clinically relevant plasma concentrations of colistin in combination with imipenem enhance pharmacodynamic activity against multidrug-resistant *P. aeruginosa* at multiple inocula

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Abstract

Use of combination antibiotic therapy may be beneficial against rapidly emerging resistance in *Pseudomonas aeruginosa*. The aim of this study was to systematically investigate *in vitro* bacterial killing and resistance emergence with colistin alone and in combination with imipenem against multidrug-resistant (MDR) *P. aeruginosa*. Time-kill studies were conducted over 48 h using 5 clinical isolates and ATCC 27853 at two inocula (~10^6 and ~10^8 cfu/mL); MDR, non-MDR, and colistin-heteroresistant and -resistant strains were included. Nine colistin/imipenem combinations were investigated. Microbiological response was examined by log changes at 6, 24 and 48 h. Colistin combined with imipenem at clinically relevant concentrations increased bacterial killing against MDR and colistin-heteroresistant isolates at both inocula. Substantial improvements in activity with combinations were observed across 48 h with all colistin concentrations at the low inoculum and with 4 and 16× MIC (or 4 and 32 mg/L) colistin at the high inoculum. Combinations were additive or synergistic at the 10^6 inoculum against imipenem-resistant isolates (MICs 16 and 32 mg/L) in 9, 11 and 12 of 18 cases (i.e., 9 combinations across 2 isolates) at 6, 24 and 48 h, respectively; the corresponding values at the 10^8 inoculum were 11, 7 and 8. Against a colistin-resistant strain (MIC 128 mg/L), 9 and 8 of 9 cases were additive or synergistic at 24 h at the 10^6 and 10^8 inocula, respectively; the corresponding values at 48 h were 5 and 7. This systematic study provides important information for optimization of colistin/imipenem combinations targeting both colistin-susceptible and -resistant subpopulations.

Introduction

The world is facing a growing threat from multidrug-resistant (MDR) Gram-negative 'superbugs' such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (19, 30, 50). This problem is compounded by a lack of novel antimicrobial agents in the drug development pipeline for Gram-negative infections (30-31, 50), in particular those caused by *P. aeruginosa* (50), and novel agents with activity against this pathogen may not be available for approximately 10 years (41). This has led to the re-evaluation of colistin (also known as polymyxin E), a multi-component cationic polypeptide antibiotic that entered clinical use in 1959 but was largely replaced in the 1970s by aminoglycosides due to concerns about the potential for nephro- and neuro-toxicity (12, 23, 26-27). Owing to its significant *in vitro* antibacterial activity against Gram-negative 'superbugs', colistin is often the only therapeutic option available to treat infections by these pathogens (1, 27, 35) and therefore its use has increased substantially over the last five years, especially in critically-ill patients (6, 27).

It is now evident that the plasma colistin concentrations achieved in critically-ill patients with the currently recommended dosage regimens are sub-optimal in a significant proportion of patients (13, 43). Unfortunately, increasing the daily dose may not be an acceptable option since nephrotoxicity is a dose-limiting adverse effect and occurs in 30 – 50% of patients (13, 16, 22). It is therefore not surprising that sub-optimal concentrations cause emergence of resistance to colistin which seriously threatens colistin therapy (45, 48). *In vivo* (21, 33) and *in vitro* (3-4) studies show the potential for the rapid emergence of colistin resistance with monotherapy. The phenomenon of colistin heteroresistance (the presence of colistin-resistant subpopulations in an isolate considered susceptible by MIC measurement) (56) has been

reported for *A. baumannii* (29, 56) and *K. pneumoniae* (34, 44, 54), but not yet for *P. aeruginosa.* Heteroresistance very likely contributes to emergence of colistin resistance. The aim of the present study was to systematically investigate the extent of *in vitro* bacterial killing and emergence of colistin resistance with colistin alone and in combination with imipenem against *P. aeruginosa*. Key aspects of this study were the use of MDR isolates with varying susceptibilities to colistin and imipenem (including colistin-heteroresistant isolates first identified in this study, and colistin- and imipenem-resistant strains), examination of combinations of clinically relevant drug concentrations at both low and high inocula, and monitoring of emergence of resistance to colistin with real-time population analysis profiles.

Materials and Methods

Bacterial isolates

Five clinical isolates and *P. aeruginosa* ATCC 27853 (American Type Culture Collection, Rockville, MD, USA) were selected to represent a mixture of colistin and imipenem susceptible and resistant strains, colistin heteroresistant and non-heteroresistant strains, and multidrug-resistant (MDR) and non-MDR strains. MDR was defined as diminished susceptibility to at least two of the following five drug classes: antipseudomonal cephalosporins, antipseudomonal carbapenems, β-lactam/β-lactamase inhibitor combinations, antipseudomonal fluoroquinolones, and aminoglycosides (40). In addition, all strains were examined by PCR for the presence of genes encoding cephalosporinases and carbapenemases, i.e. IMP, VIM, NDM, KPC, CTX-M, SHV, and CMY type β-lactamases (47, 58). Details of the isolates are contained in Table 1. All clinical isolates were collected from patients with cystic fibrosis, had different pulsed-field gel electrophoresis patterns, and were considered unrelated according to the criteria established by Tenover *et al*. (53). MICs

to colistin and imipenem were determined for each isolate in four replicates in cation-adjusted Mueller-Hinton broth (CAMHB, Ca^{2+} 23.0 mg/L, Mg^{2+} 12.2 mg/L: Oxoid, Hampshire, England) via broth microdilution (10). Storage was in tryptone soy broth (Oxoid) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) at -80ºC in cryovials (Simport Plastics, Boloeil, Quebec, Canada).

Antibiotics

Colistin sulfate was purchased from Sigma-Aldrich (Lot: 109K1574, 23,251 units/mg; St Louis, MO, USA). Colistin (sulfate) was employed in the current study since it is the active antibacterial agent formed *in vivo* after administration of its inactive prodrug, colistin methanesulfonate (CMS) (5). Imipenem was purchased from Merck Sharp and Dohme (Primaxin®, Batch: K5942; NSW, Australia). Stock solutions of each antibiotic were prepared according to the respective manufacturer's instructions immediately prior to each experiment to minimise loss from degradation, then sterilized by filtration with a 0.22-µm Millex-GP® filter (Millipore, Bedford, MA, USA).

Population analysis profiles

The possible existence of colistin-resistant subpopulations at baseline was determined via population analysis profiles (PAPs; inoculum ~10^8 cfu/mL). Colistin heteroresistance was defined as a colistin-susceptible isolate (i.e., MIC ≤ 2 mg/L) in which subpopulations were able to grow in the presence of >2 mg/L colistin in the PAPs. Samples of bacterial cell suspension (50 µL), appropriately diluted with saline, were spirally plated onto Mueller-Hinton agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) impregnated with colistin (0, 0.5, 1, 2, 3, 4, 6, 8 and 10 mg/L) using an automatic spiral plater (WASP, Don Whitley Scientific, West Yorkshire, UK). Colonies were counted using a

ProtoCOL® colony counter (Synbiosis, Cambridge, UK) after 24 h of incubation (48 h for plates with small colonies) at 35ºC; the limit of detection was 20 cfu/mL (equivalent to 1 colony per plate) and limit of quantification was 400 cfu/mL (equivalent to 20 colonies per plate) as specified in the ProtoCOL manual. Real-time PAPs for colistin were also conducted at the end of time-kill studies (see below).

Time-kill studies

To explore the antimicrobial activity of colistin and imipenem combinations, time-kill studies with each antibiotic alone or in combination were conducted on all isolates at two different starting inocula (~10^6 and ~10^8 cfu/mL). For monotherapy with colistin or imipenem, two-fold multiples of the MIC (0.25 to 64× MIC) were employed for susceptible isolates. For the colistin-resistant isolate (19147 n/m, MIC 128 mg/L), a single colistin concentration of 32 mg/L was employed. Imipenem concentrations of 1, 8 and 32 mg/L were used for imipenem-resistant isolates. In combination experiments, both antibiotics were studied at concentrations of 0.5, 4 and 16× MIC for susceptible isolates; for resistant isolates, concentrations of 1, 4 and 32 mg/L for colistin and 1, 8 and 32 mg/L for imipenem were employed. In total, nine colistin/imipenem combinations were examined for each isolate at each inoculum.

Prior to each experiment isolates were subcultured onto horse blood agar (Media Preparation Unit) and incubated at 35ºC overnight. One colony was then selected and grown overnight in 10 mL CAMHB at 37ºC from which early log-phase culture was obtained. Each antibiotic was added alone or in combination to 20 mL of a log-phase broth culture of approximately 10^6 or 10^8 cfu/mL to yield the desired concentrations. Each 20-mL culture was placed in a sterile 50-mL polypropylene tube (Greiner Bio-one) and incubated in a shaking water bath at 37ºC. Serial samples (100 µL) were collected aseptically for viable counting at 0, 0.5, 1, 2, 4, 6, 24 and 48 h, and PAPs at 48 h (see above) for all experiments involving colistin (including

combination arms), and viable counting only at 0, 1, 2, 4, 6, 24 and 48 h for experiments with imipenem alone. Immediately after sampling and serial dilution, 50 µL of bacterial cell suspension was spirally plated onto nutrient agar with enumeration after 24 h of incubation (48 h for plates with small colonies) as per PAPs above.

Pharmacodynamic (PD) analysis

Microbiological response to monotherapy and combination therapy was examined using the log change method comparing the change in $\log_{10}$ (cfu/mL) from 0 h (CFU_0) to time t (6, 24 or 48 h; CFU_t) as follows:

$$\text{Log change} = \log_{10}(CFU_t) - \log_{10}(CFU_0)$$

Single antibiotic or combination regimens causing a reduction of $\geq$1-$\log_{10}$ cfu/mL below the initial inoculum at 6, 24 or 48 h were considered active. We considered synergy to be a $\geq$2-$\log_{10}$ lower cfu/mL for the combination relative to its most active component at the specified time (42); additivity was defined as a 1 to <2-$\log_{10}$ lower cfu/mL for the combination.

Results

Microbiological response

The varying susceptibilities to colistin among the isolates are evident in the PAPs prior to colistin treatment (Figure 1). Representative time-kill profiles for colistin and imipenem monotherapy and combination therapy are shown in Figures 2 (~10^6 cfu/mL inoculum) and 3 (~10^8 cfu/mL inoculum). Log changes of viable cell counts at each inoculum with clinically relevant colistin concentrations are presented in Tables 2 and 3. Additional time-kill and log change data are presented in the electronic supplement. At the 10^6 cfu/mL inoculum, regrowth was observed to various extents at 48 h with colistin monotherapy in all susceptible

isolates with the majority of colistin concentrations. Regrowth with imipenem monotherapy was more variable and substantially less against susceptible isolates at 48 h with imipenem concentrations of ≥4 or 8× MIC, even with ESBLs present. An inoculum effect with colistin monotherapy was generally observed (Figures 2 and 3, left-hand panels). The killing by imipenem at the high inoculum was generally slightly slower than at the low inoculum, although the extent of reductions in $\log_{10}$ cfu/mL was comparable at both inocula (Figures 2 and 3).

Isolates susceptible to both colistin and imipenem. At the 10^6 inoculum, the addition of 0.5× MIC colistin to imipenem (all concentrations) resulted in additivity or synergy at 6 h in 7 of 9 cases (i.e., 3 combinations against 3 isolates), achieving ~2- to 3-$\log_{10}$ greater kill compared to the most active equivalent monotherapy and undetectable bacterial counts in many cases (Table 2 and Figure 2). By 24 or 48 h, improvements in activity with combination therapy over and above the most active monotherapy (usually imipenem) were modest, particularly when only clinically relevant concentrations of colistin (0.5 or 4× MIC) were considered. Of the 27 cases (i.e., 9 combinations against 3 isolates), 7 at 24 h and 8 at 48 h were additive or synergistic, although only one case resulted in activity (i.e., ≥1-$\log_{10}$ kill) if equivalent monotherapy with either drug was inactive. A similar pattern of activity was observed at the 10^8 inoculum. For ATCC 27853, combinations containing 4 mg/L colistin provided an additional ~2-$\log_{10}$ kill to already active monotherapy at 6 h. Against all three isolates there were 10 and 9 cases of additivity/synergy at 24 and 48 h, respectively, mostly involving colistin at 4 or 16× MIC (Table 2).

Imipenem-resistant isolates. For the two imipenem-resistant isolates (19271 n/m and 20891 n/m), there was no evidence of carbapenemase activity; most likely, an alternative resistance mechanism such as the loss of major outer membrane proteins was present. At the low inoculum, combination therapy resulted in substantial improvements in bacterial kill with all colistin concentrations across 48 h. At 6 h additivity/synergy occurred in 9 of 18 cases (i.e., 9 combinations across 2 isolates), predominantly against isolate 19271 n/m, occurred with combinations containing colistin at all concentrations, and produced additional reductions of ~2- to 6-$\log_{10}$ cfu/mL over usually active colistin monotherapy (Table 3 and Figure 2); in 5 of 6 cases involving 4 or 16× MIC colistin against 19271 n/m, bacterial counts were reduced to below the limit of detection (i.e., 20 cfu/mL). Substantial improvements in activity were also present at 24 and 48 h in both isolates at all colistin concentrations. Additivity/synergy occurred in 11 and 12 of 18 cases at 24 and 48 h, respectively, adding an additional ~1- to 4-$\log_{10}$ kill at 24 h and >2.5-$\log_{10}$ kill at 48 h compared to that of monotherapy (Table 3 and Figure 2). Interestingly, the combinations of colistin 0.5, 4 or 16× MIC plus imipenem 32 mg/L each reduced bacterial loads to below the limit of detection at 24 h against both isolates; the maximum reduction in $\log_{10}$ cfu/mL at 24 h with colistin monotherapy at 16× MIC was ~4.5. Improvements in activity with combination therapy at the high inoculum also occurred at all time points but were essentially restricted to combinations containing 4 or 16× MIC colistin. Ten of 12 cases at 6 h containing colistin 4 (Table 3) or 16× MIC (data not shown) were additive or synergistic. At 24 and 48 h, the addition of imipenem at all concentrations to 4 or 16× MIC colistin produced additivity/synergy in over half of all cases and substantially improved the activity compared with each antibiotic alone (by up to ~4-$\log_{10}$ kill).

Colistin-resistant isolate. Bacterial killing at the 10^6 inoculum was substantially enhanced at 24 h, with all tested combinations being additive or synergistic and only one combination (colistin 1 mg/L plus imipenem 0.5× MIC) inactive (Table 3). The addition of all colistin concentrations to imipenem 4 or 16× MIC produced ~3.5 to 4.5-$\log_{10}$ kill at 24 h, substantially higher than equivalent imipenem monotherapy. At 48 h all colistin concentrations in combination with imipenem 4× MIC were synergistic (~2- to 4-$\log_{10}$ kill) and substantially improved activity over equivalent monotherapy. The addition of colistin 32 mg/L to imipenem (all concentrations) was additive or synergistic at a substantially earlier time (6 h) with ~1- to 2-$\log_{10}$ greater kill than equivalent imipenem monotherapy (overall kill ~3-$\log_{10}$ cfu/mL). At the high inoculum, additivity was achieved at 6 h with all combinations containing colistin 4 mg/L (Table 3) and 32 mg/L (data not shown), and activity enhanced by ~1-$\log_{10}$ kill over imipenem monotherapy. Eight at 24 h and 7 at 48 h of 9 combinations were additive or synergistic, encompassing all colistin concentrations and in many cases resulting in additional reductions of ~1- to 4-$\log_{10}$ cfu/mL over the most active monotherapy (imipenem 16× MIC). This enhancement of activity was particularly evident with combinations containing colistin 4 or 32 mg/L, and on two occasions when combined with imipenem 16× MIC, no viable bacteria were detected at 48 h.

Emergence of colistin resistance

For the 4 colistin-heteroresistant isolates (Table 1), the proportion of resistant subpopulations at 10^8 cfu/mL ranged from 2.2×10^{-7} to 4.7×10^{-3} (Figure 1). With colistin monotherapy against the isolates susceptible to both colistin and imipenem, real-time PAPs performed at 48 h in the time-kill studies demonstrated an increase in colistin-resistant subpopulations at both the low and high inocula with clinically relevant colistin concentrations (examples in Figures 2 and 3 and the electronic supplement); no such increase was observed against isolate

19056 muc at the high inoculum. Against imipenem-resistant isolate 19271 n/m, colistin concentrations of 0.25 to 64× MIC at the low inoculum, and 1 to 64× MIC at the high inoculum resulted in nearly 100% of the remaining cells at 48 h growing in the presence of 10 mg/L colistin. In contrast, no increase in colistin-resistant subpopulations was observed for the imipenem-resistant isolate 20891 n/m at either inoculum. Combination therapy against colistin-susceptible isolates generally had little effect on the proportion of colistin-resistant subpopulations at 48 h at either inoculum, the shape of the PAPs being very similar to that obtained with equivalent colistin monotherapy (Figures 2 and 3).

Discussion

Although colistin has been commercially available for over 50 years (27), reliable PK/PD data have only recently emerged. Population PK studies have shown that plasma colistin concentrations achieved with currently recommended CMS dosage regimens are likely to be suboptimal in many patients, typically generating average steady-state plasma colistin concentrations of ~2 – 3 mg/L, with some patients achieving concentrations up to ~10 mg/L (13, 18, 24, 28, 32, 43). Increasing the daily dose of CMS in such patients may not be an option as nephrotoxicity, which occurs in ~30 – 50% of patients (16, 22), is a dose-limiting adverse effect. Given these circumstances and the current last-line status of colistin therapy, we chose to examine not only synergy but also additivity, as even a relatively small increase in activity with combination therapy may be beneficial to patient care. As colistin is almost entirely unbound in CAMHB (3), colistin concentrations of 0.5 and 4× MIC for isolates with MICs ≤1 mg/L and 16× MIC for isolates with MICs of ≤0.5 mg/L (1 and 4 mg/L for colistin-resistant isolates) used in our study are clinically relevant, even assuming plasma binding of colistin in patients is similar to that in animals (i.e., ~50% bound) (25). All imipenem

concentrations employed are readily achieved in plasma after consideration of protein binding (49).

As some data show that activity of both colistin (8) and imipenem (36) is attenuated at high compared to low inocula, experiments were conducted at both ~10^6 and ~10^8 cfu/mL. An inoculum effect was generally observed for colistin monotherapy, whereas no obvious inoculum effect was present for imipenem (Figures 2 and 3). Regrowth of all isolates was observed with colistin monotherapy even with colistin concentrations well above those which can be safely achieved clinically. Similar regrowth with colistin (or polymyxin B) monotherapy has been observed against colistin-susceptible *P. aeruginosa* both *in vitro* (4, 8, 15, 51) and *in vivo* (21). In *A. baumannii* and *K. pneumoniae*, regrowth following colistin monotherapy has been attributed to the amplification of colistin-resistant subpopulations (11, 44, 52), with colistin heteroresistance reported in both species (17, 29, 44, 54). We have reported here, for the first time, colistin heteroresistance in *P. aeruginosa.* The emergence of colistin resistance following colistin monotherapy has previously been reported in *P. aeruginosa* at both low and high inocula (4, 8), and a similar phenomenon was observed in the present study with all isolates except 20981 n/m. While *P. aeruginosa* can undergo adaptive resistance to polymyxins (14), the presence of colistin heteroresistance at baseline, and the changes in PAPs after treatment, suggest regrowth following colistin monotherapy may be due to amplification of pre-existing colistin-resistant subpopulations. This suggests care is required with colistin monotherapy against *P. aeruginosa*, even where isolates appear susceptible based on MICs.

The addition of imipenem to colistin at both inocula generally resulted in substantial improvements in bacterial killing over equivalent monotherapy against MDR *P. aeruginosa*

isolates resistant to either antibiotic, even when ESBLs were present. The improvements in activity against these isolates were observed across the 48-h duration and with all colistin concentrations at the low inoculum, and 4 and 16× MIC (or 4 and 32 mg/L) colistin at the high inoculum. Notably, the total reductions in $\log_{10}$ cfu/mL achieved with combinations containing lower colistin concentrations (0.5 and 4× MIC or 1 and 4 mg/L) were on many occasions similar in magnitude to the reductions achieved with combinations containing 16× MIC colistin, particularly at the 10^6 inoculum (Table 3). This suggests that combinations of colistin and imipenem containing clinically relevant colistin concentrations may be as effective as combinations containing higher concentrations against MDR isolates when resistance to either drug is present. This is an important result given that colistin-induced nephrotoxicity is a dose-limiting adverse effect.

The benefits in overall antibacterial activity with the addition of imipenem to colistin were less pronounced against the three isolates susceptible to both antibiotics and were generally restricted to improvements in initial kill, i.e. up to 6 h (Table 2). As a proportion of patients will achieve only low plasma colistin concentrations with currently recommended dosage regimens (13, 43), the combination of colistin and imipenem at the commencement of therapy may help to quickly reduce bacterial levels to facilitate clearance by the immune system.

Previous time-kill studies have examined colistin in combination with carbapenems against *P. aeruginosa* (2, 9, 38-39, 46). These studies examined colistin with imipenem, meropenem or doripenem at a single inoculum (~10^6 or 10^7 cfu/mL), though the emergence of colistin resistance was not examined (e.g. using PAPs). The present study is the first to investigate the

emergence of colistin resistance with colistin combination therapy. In the present investigations, in cases where the combination led to extensive killing at 48 h, meaningful interpretation of the PAPs was not possible (e.g. Figure 2, Panel B, colistin 4× MIC as monotherapy and in combination with imipenem 4× MIC). However, when bacterial numbers at 48 h were comparable, changes in PAPs with combination therapy generally mirrored those observed with equivalent exposure to colistin as monotherapy. However, in both the present study and previously reported studies (2, 9, 38-39, 46), static concentrations and instability of carbapenems in aqueous media may have contributed to the regrowth and emergence of colistin resistance at 48 h (20). Thus, it will be important to further assess the utility of these combinations against a range of isolates with varying susceptibilities (including heteroresistant strains) in dynamic *in vitro* models and *in vivo*.

Two possible reasons for an enhanced pharmacodynamic effect observed with the combination of colistin and imipenem are subpopulation synergy and mechanistic synergy as proposed previously (7). Subpopulation synergy involves one drug killing the resistant subpopulation(s) of the other drug, and *vice versa*. Four of the six isolates in the present study were colistin heteroresistant (Table 1), indicating the existence of colistin-resistant subpopulations prior to therapy. In addition, the four imipenem-susceptible isolates were imipenem heteroresistant (MIC ≤ 4 mg/L in which subpopulations grew in the presence of > 4 mg/L imipenem; data not shown). Another possibility is mechanistic synergy whereby colistin and imipenem acting on different cellular pathways increase the rate or extent of killing of the other drug. In Gram-negative bacteria carbapenems must first gain entry into the periplasmic space in order to bind to critical penicillin-binding proteins located on the cytoplasmic membrane (37, 55). A number of resistance mechanisms may operate to limit the concentration of carbapenems in the periplasm including the presence of carbapenem-

hydrolyzing enzymes and loss of outer membrane proteins (55). Polymyxins cause considerable permeabilization of the outer membrane (57). It is possible that the effect of colistin on membrane permeability results in substantially increased concentrations of imipenem in the periplasm and improved bactericidal activity. Subpopulation and mechanistic synergy are not mutually exclusive and both may operate simultaneously. Further studies, including mechanism-based mathematical modelling, are ongoing to investigate the mechanism(s) underpinning the enhanced pharmacodynamic activity observed.

In the battle against rapidly emerging bacterial resistance in Gram-negative 'superbugs', rational approaches to the use of combinations of existing antibiotics may be greatly beneficial. To the best of our knowledge, this is the first systematic study on the PD of colistin in combination with imipenem against *P. aeruginosa*, including MDR and colistin heteroresistant strains, at both low and high inocula. Clinically relevant concentrations of colistin in combination with imipenem substantially increased bacterial killing against MDR *P. aeruginosa* at both inocula when isolates were resistant to either antibiotic. Further investigations in *in vitro* pharmacodynamic systems, animal infection models and clinical studies are warranted to optimize colistin/imipenem combinations targeting both colistin-susceptible and -resistant subpopulations.

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557 **Table 1:** Minimum inhibitory concentrations (MICs) of the *P. aeruginosa* isolates used in
558 this study
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Isolate	MIC (mg/L)[a]		cephalosporinase and carbapenemase typing	MDR[b]
	Colistin	Imipenem		
ATCC 27853[c]	1	2	negative	No
19147 n/m	128	4	IMP & CTX-M positive[d]	Yes
19056 muc	0.5	4	negative	Yes
20509 n/m[c]	0.5	1	negative	No
19271 n/m[c]	2	32	negative	Yes
20891 n/m[c]	1	16	negative	Yes

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[a] CLSI breakpoints (S, susceptible; I, intermediate; R, resistant): Colistin, S≤2 mg/L, I=4 mg/L, R≥8 mg/L; Imipenem, S≤4 mg/L, I=8 mg/L, R≥16 mg/L (10).
[b] Multidrug-resistance (MDR) was defined as diminished susceptibility to ≥2 of the following five drug classes: antipseudomonal cephalosporins, antipseudomonal carbapenems, β-lactam/β-lactamase inhibitor combinations, antipseudomonal fluoroquinolones, and aminoglycosides (40).
[c] Colistin heteroresistant. Heteroresistance to colistin was defined as an isolate with colistin MIC ≤2 mg/L in which subpopulations were able to grow in the presence of >2 mg/L colistin (55).
[d] Contains genes encoding IMP type carbapenemase and CTX-M type extended-spectrum β-lactamase (ESBL).

562 **Table 2:** Log changes at 6, 24 or 48 h at two inocula with various clinically relevant concentrations of colistin (Col) and imipenem (Imi) against 3 isolates of
563 *P. aeruginosa* susceptible to both antibiotics. Gray background indicates activity (a reduction of ≥1-$\log_{10}$ cfu/mL below the initial inoculum); green
564 background indicates synergy (a ≥2-$\log_{10}$ decrease in the number of cfu/mL between the combination and its most active component); red background
565 indicates additivity (a 1.0 to <2-$\log_{10}$ decrease in the number of cfu/mL between the combination and its most active component).

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(CFU_t)$ - $\log_{10}(CFU_0)$)										
			Col 0.5× MIC	Col 4× MIC	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 0.5× MIC + Imi 0.5× MIC	Col 0.5× MIC + Imi 4× MIC	Col 0.5× MIC + Imi 16× MIC	Col 4× MIC + Imi 0.5× MIC	Col 4× MIC + Imi 4× MIC	Col 4× MIC + Imi 16× MIC
ATCC 27853	~10^6	6	-0.41	-5.93	-0.03	-2.77	-2.83	**-2.90**	**-4.39**	**-5.95**	-4.69	-5.95	-5.99
		24	+3.20	+0.06	+2.91	-3.14	-3.66	**+1.71**	-3.58	**-5.95**	**-2.20**	-2.68	-3.34
		48	+3.80	+1.35	+3.60	-1.06	-1.81	+3.49	**-2.39**	**-2.90**	**+0.04**	-1.82	-2.27
	~10^8	6	+0.33	-2.48	-0.06	-2.04	-2.08	-1.00	-2.79	-2.73	**-5.62**	**-4.94**	**-4.69**
		24	+1.55	+0.05	+1.67	+0.14	-3.73	+1.50	-0.48	-3.42	-0.01	**-3.51**	-4.54
		48	+2.05	+1.65	+2.02	+1.96	-2.89	+2.04	+1.79	-3.30	+0.92	**-2.25**	-3.60
19056 muc	~10^6	6	-2.34	-5.19	+0.45	-3.81	-5.49	**-5.66**	**-5.69**	-5.79	-5.88	-5.92	-5.75
		24	+1.63	-2.64	+2.66	-3.41	-5.49	+1.69	-4.39	-5.79	-2.62	**-5.92**	-5.75
		48	+3.13	+0.08	+3.27	+1.93	-5.49	+2.53	**-0.27**	-5.79	-0.44	**-1.06**	-5.75
	~10^8	6	+0.11	-7.51	-0.83	-3.76	-4.22	-1.68	-4.00	-4.05	-7.96	-8.22	-7.95
		24	+0.79	-3.49	-0.01	-3.22	-5.02	+0.19	-3.46	-4.60	-2.50	**-6.92**	**-7.95**
		48	+1.42	-0.15	+0.47	+0.24	-6.08	+0.39	+0.27	-5.91	+0.08	**-3.18**	**-7.95**
20509 n/m	~10^6	6	+1.47	-3.18	-1.71	-3.08	-3.83	-2.29	**-5.97**	**-6.14**	**-4.34**	**-6.10**	**-5.90**
		24	+3.18	+2.39	+3.10	-2.30	-3.33	+3.26	-3.04	-3.93	+1.86	**-4.41**	-4.30
		48	+3.61	+3.07	+3.46	-1.08	-1.43	+3.48	-1.00	-1.68	+3.21	-1.40	-1.93
	~10^8	6	+0.82	-0.01	-0.81	-2.65	-2.65	-0.52	-2.86	-2.55	-1.03	-2.75	-2.53
		24	+1.62	+2.04	+1.02	-0.45	-1.48	+1.40	-0.59	**-2.60**	+0.81	**-1.88**	**-3.63**
		48	+1.92	+2.19	+1.25	+0.96	-1.28	+1.70	+1.58	-0.98	+1.75	+1.03	**-2.37**

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567

568 **Table 3:** Log changes at 6, 24, or 48 h at two inocula with various clinically relevant concentrations of colistin (Col) and imipenem (Imi) against 1 colistin-
569 resistant, imipenem-susceptible isolate and 2 colistin-susceptible, imipenem-resistant isolates of *P. aeruginosa*. Gray background indicates activity (a
570 reduction of ≥1-$\log_{10}$ cfu/mL below the initial inoculum); green background indicates synergy (a ≥2-$\log_{10}$ decrease in the number of cfu/mL between the
571 combination and its most active component); red background indicates additivity (a 1.0 to <2-$\log_{10}$ decrease in the number of cfu/mL between the
572 combination and its most active component). For colistin-resistant isolate 19147 n/m, synergy or additivity were compared with imipenem monotherapy only.

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(CFU_t)$ - $\log_{10}(CFU_0)$)									
Col resistant, Imi susceptible			Col 32 mg/L		Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 1.0 mg/L + Imi 0.5× MIC	Col 1.0 mg/L + Imi 4× MIC	Col 1.0 mg/L + Imi 16× MIC	Col 4.0 mg/L + Imi 0.5× MIC	Col 4.0 mg/L + Imi 4× MIC	Col 4.0 mg/L + Imi 16× MIC
19147 n/m	~10^6	6	-0.08		-0.35	-1.44	-1.28	-1.28	-1.46	-1.77	**-1.77**	-1.60	-1.83
		24	+2.16		+2.27	-0.58	-2.57	**-0.71**	**-3.28**	**-4.57**	**-2.01**	**-3.89**	**-4.22**
		48	+2.49		+3.02	+2.50	-4.55	+2.57	**-3.03**	-4.46	**+1.74**	**-1.99**	-3.54
	~10^8	6	+0.04		-1.04	-1.56	-1.36	-1.83	-1.82	-1.85	**-2.40**	**-2.70**	**-2.54**
		24	+0.55		+1.31	-0.33	-2.67	**+0.14**	**-3.24**	-3.38	**-1.10**	**-4.60**	**-4.61**
		48	+0.86		+1.79	+1.37	-3.21	+1.18	**-0.10**	-3.19	**+0.42**	**-3.82**	**-7.69**
Col susceptible, Imi resistant			Col 0.5× MIC	Col 4× MIC	Imi 1.0 mg/L	Imi 8.0 mg/L	Imi 32 mg/L	Col 0.5× MIC + Imi 1.0 mg/L	Col 0.5× MIC + Imi 8.0 mg/L	Col 0.5× MIC + Imi 32 mg/L	Col 4× MIC + Imi 1.0 mg/L	Col 4× MIC + Imi 8.0 mg/L	Col 4× MIC + Imi 32 mg/L
19271 n/m	~10^6	6	-1.89	-3.32	+1.68	+1.17	-0.54	-1.32	-2.00	**-4.45**	-3.43	**-5.77**	**-5.71**
		24	+0.49	-2.19	+2.95	+2.89	-1.27	+0.71	+0.24	**-5.75**	-3.00	**-5.77**	**-5.71**
		48	+2.87	+1.83	+3.01	+2.93	+1.56	+2.88	+2.81	**-2.61**	**+0.15**	**-1.52**	**-5.71**
	~10^8	6	-0.05	-2.22	+0.56	+0.26	-0.95	**-1.05**	-0.94	-1.57	-3.13	**-3.48**	**-4.90**
		24	+0.67	-1.13	+0.84	+0.39	-1.70	**-0.37**	-0.08	-2.47	-1.86	-1.76	**-3.88**
		48	+0.83	-1.26	+0.83	+0.46	+0.15	+0.94	+0.98	+0.25	-1.09	+0.22	**-2.30**
20891 n/m	~10^6	6	+1.38	-5.95	+1.68	-0.26	-2.61	**+0.35**	**-6.02**	**-5.90**	-4.62	-5.90	-5.83
		24	+2.28	-3.43	+2.53	+2.27	-3.82	+2.35	**-2.90**	**-5.90**	-3.85	-3.64	**-5.83**
		48	+2.48	-1.84	+2.42	+2.28	+0.01	+2.43	+2.44	**-3.82**	-0.42	**-3.34**	**-5.83**
	~10^8	6	+0.21	-4.67	+0.13	-0.77	-2.50	-0.33	-1.32	-2.54	**-5.76**	**-7.39**	**-7.52**
		24	+0.60	-1.12	+0.32	-0.31	-3.64	+0.54	+0.47	-2.38	**-3.39**	**-3.47**	-4.27
		48	+0.77	-0.69	+0.55	+0.21	+0.07	+0.91	+0.84	+1.00	**-3.08**	**-2.44**	**-2.89**

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574

575 **Figure Captions**

576 **Figure 1**. Baseline PAPs of the reference strain and all clinical isolates using an initial
577 inoculum of ~10^8 cfu/mL. The Y-axis starts from the limit of detection and the limit of
578 quantification (LOQ) is indicated by the horizontal broken line.

579 **Figure 2**. Representative time-kill curves (left-hand panels) with various clinically relevant
580 concentrations of colistin and imipenem alone and in combination at an inoculum of ~10^6
581 cfu/mL against (A) 19147 n/m (colistin-resistant, imipenem-susceptible MDR), (B) 20509
582 n/m (colistin- and imipenem-susceptible non-MDR) and (C) 20891 n/m (colistin-susceptible,
583 imipenem-resistant MDR). Right-hand panels show the respective PAPs at baseline (0 h) and
584 after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy or
585 neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of
586 quantification (LOQ) is indicated by the horizontal broken line.

587 **Figure 3**. Representative time-kill curves (left-hand panels) with various clinically relevant
588 concentrations of colistin and imipenem alone and in combination at an inoculum of ~10^8
589 cfu/mL against (A) 19147 n/m (colistin-resistant, imipenem-susceptible MDR), (B) 20509
590 n/m (colistin- and imipenem-susceptible nonMDR) and (C) 20891 n/m (colistin-susceptible,
591 imipenem-resistant MDR). Right-hand panels show the respective PAPs at baseline (0 h) and
592 after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy or
593 neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of
594 quantification (LOQ) is indicated by the horizontal broken line.

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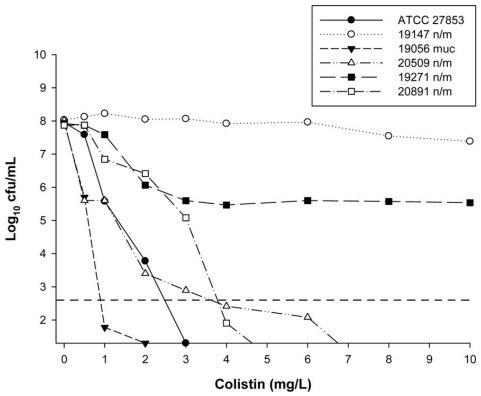

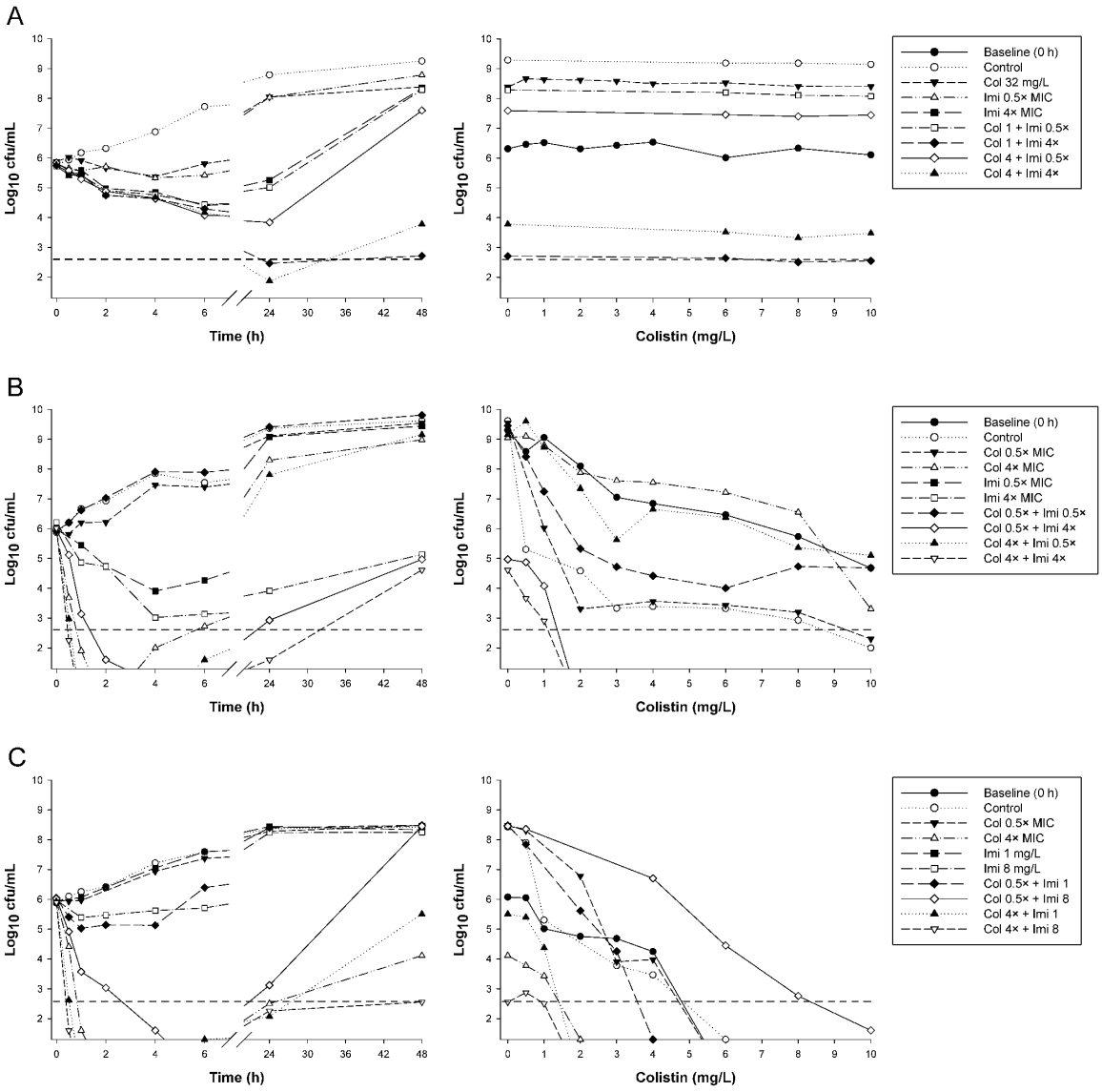

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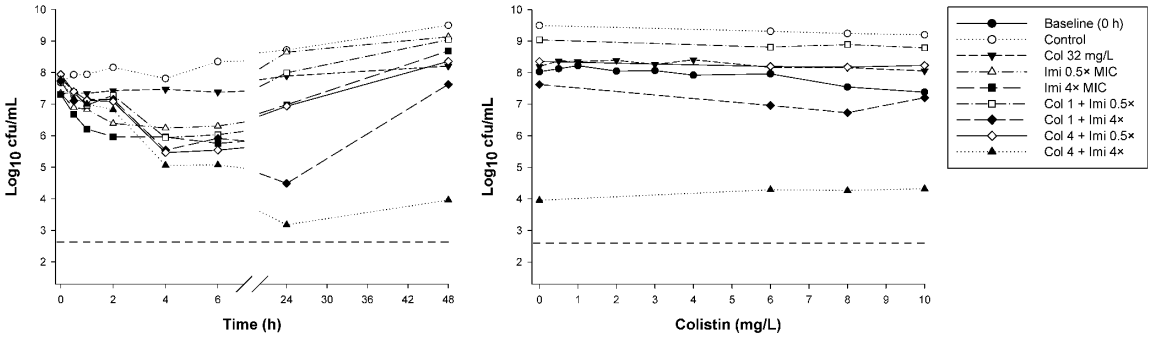

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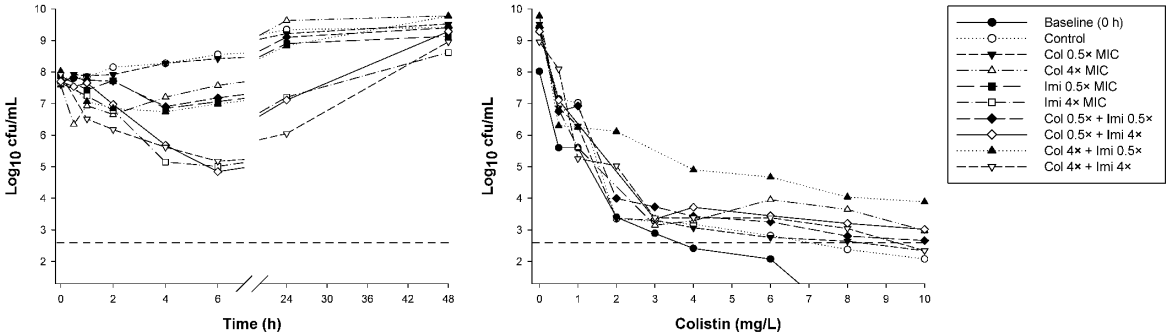

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