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Urinary bactericidal activity of colistin and azidothymidine combinations against *mcr-1*-positive colistin-resistant *Escherichia coli*

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ABSTRACT

A phase 1 clinical study was performed to assess the pharmacokinetics and safety of intravenous (i.v.) administration of colistin methanesulfonate (CMS) and azidothymidine (AZT) alone and in combination. Seven healthy subjects received three (every 12 h) 1-h i.v. infusions of 4, 2 and 2 million international units (MIU) of CMS co-administered with 200, 100 and 100 mg of AZT, respectively. In an ex vivo study, urinary bactericidal titres (UBTs) and time–kill curve determinations were performed in artificial urine spiked with colistin sulfate and AZT according to median and minimum peak concentrations in urine measured after the first and third dose using four *mcr-1*-positive colistin-resistant and five colistin-susceptible Gram-negative isolates. Reciprocal UBTs for the different colistin concentrations obtained in urine ranged from 1–128 and 0–2 for colistin-susceptible and colistin-resistant isolates, respectively. Combination with AZT could increase UBTs up to two dilution steps each for the Enterobacteriaceae and *Acinetobacter* strains tested. In contrast, the combination had no activity against *Pseudomonas* strains. In time–kill curves, the combination showed bactericidal activity against colistin-resistant strains even when the substances alone were not bactericidal. Thus, combination of CMS with AZT shows promising synergistic activity against Gram-negative uropathogens, including colistin-resistant Enterobacteriaceae. According to the urinary bactericidal activity, a maintenance dosage of 2 MIU of CMS combined with 100 mg of AZT twice daily may be sufficient for the treatment of urinary tract infections (UTIs) caused by colistin-susceptible strains. However, the dosage requires optimisation for efficient treatment of UTIs caused by colistin-resistant strains.

1. Introduction

Gram-negative bacteria, especially Enterobacteriaceae, are a major cause both of community- and hospital-acquired urinary tract infections (UTIs). There has been a continuous emergence of antimicrobial resistance in UTI pathogens as well as a shortage of newly approved antibiotics for common but clinically important indications [1]. The number of antimicrobial agents to treat infections caused by antimicrobial-resistant Enterobacteriaceae has declined rapidly and the polymyxins, such as colistin, are considered the last-resort treatment [2].

Recently, studies illustrated that the combination of azidothymidine (AZT) and colistin showed synergistic antibacterial activity against Enterobacteriaceae strains in vitro and in mice [3–5]. Previously, we used serum concentrations obtained from a phase 1 study with healthy volunteers receiving a combination of colistin methanesulfonate (CMS) and AZT [6] for in vitro determination of serum bactericidal activities against colistin-resistant *Escherichia coli* strains compared with colistin-susceptible Gram-negative strains [7]. The results provided evidence that a combination of colistin and AZT potentiates the serum bactericidal effect of colistin against colistin-resistant *E. coli*. The aim of the current study was to determine, in a second part, the urinary bactericidal activity of colistin and AZT combinations against four *mcr-1*-positive colistin-resistant *E. coli* strains compared with colistin-susceptible *E. coli, Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains using urinary concentrations of both drugs obtained from the same phase 1 study [6].

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Table 1
Urinary concentrations of colistin and azidothymidine (AZT) obtained 0–3 h after administration

Dose	Colistin (mg/L)		AZT (mg/L)
	CB	≙CS	
First dose			
Minimum	55	66.6	41.5
Median	106	128.3	70.4
Maximum	776	939	893
Second dose			
Minimum	37.8	45.7	24.9
Median	54.9	66.4	46.4
Maximum	107	129.5	99.2
Third dose			
Minimum	14.9	18	11.8
Median	46.9	56.7	39
Maximum	217	262.6	444

CB, colistin base; CS, colistin sulfate.

2. Materials and methods

2.1. Ethics

The ethics for the phase 1 study have been described elsewhere [6,7].

2.2. Study design and urinary concentrations

The study design, sample collection and analysis as well as detailed safety information, laboratory test results and pharmacokinetic evaluations of this phase 1 study have been presented elsewhere [6,7]. Maximum, median and minimum urinary concentrations of colistin and AZT obtained in the collection period 0–3 h after start of the first, second and third dose are presented in Table 1. Concentrations of colistin are expressed in colistin base (CB), whose molecular weight differs slightly from colistin sulfate (CS) (CB, 1155.5; CS, 1400.7; corresponding to colistin B [8,9]). Therefore, the obtained CB concentrations were converted to CS concentrations (factor 1.212), which were then used for spiking the artificial urine (Table 1).

2.3. Bacterial strains

Four clinical *E. coli* isolates harbouring the *mcr-1* colistin resistance gene were used (Table 2). In addition, three carbapenem-resistant strains (*K. pneumoniae* BAA-2470, *P. aeruginosa* 1640801 and *A. baumannii* CHD102) as well as two reference strains (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) were included in the study.

2.4. Determination of minimum bactericidal concentrations (MBCs)

MBCs were determined as described elsewhere [7] according to Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards [11–13]. Cation-adjusted Mueller–Hinton broth (CA-MHB) (Sigma-Aldrich, Darmstadt, Germany) and artificial urine (containing (in g/L): $CaCl_2$, 0.49; $MgCl_2{*}6H_2O$, 0.65; NaCl, 4.6; Na_2SO_4, 2.3; Na_2 citrate · $2H_2O$, 0.65; $Na_2C_2O_4$, 0.02; KH_2PO_4, 2.8; KCl, 1.6; NH_4Cl, 1.0; urea, 25.0; gelatin, 5.0; and tryptone soya broth, 10.0; pH 6.1 [14]) were used as media. The final inoculum, which was confirmed by actual plating, ranged from $1–5 \times 10^6$ CFU/mL. All determinations were repeated at least three times.

2.5. Determination of urinary bactericidal titres (UBTs)

UBTs corresponding to the maximum dilution titre of urine allowing bactericidal activity were determined as described previously [15]. A two-fold serial dilution (range 1:0, 1:1 to 1:1024) of artificial urine spiked with CS and AZT (Sigma-Aldrich) was prepared in a 96-well polycarbonate plate (BioRad Laboratories, Munich, Germany). Previous studies have shown that binding of colistin to polycarbonate is much lower than to polystyrene (data not shown). Agent-free artificial urine was used as diluent. The final inoculum, which was confirmed by actual counting, ranged from $1.4–3.3 \times 10^6$ CFU/mL. Plates were incubated at 37°C for 20 ± 2 h in ambient air. Then, 3 µL of the cultured artificial urine was transferred onto Iso-Sensitest agar supplemented with 5% blood using a one-time inoculator and the plates were incubated overnight at 37°C. The number of colonies subsequently grown was used to determine the bactericidal endpoint. Urinary bactericidal activity was defined as a >99.9% (>3 log) reduction in the initially inoculated colony count. A UBT of 0 was defined as no bactericidal activity, whereas a UBT of 1 was used when only the undiluted artificial urine displayed bactericidal activity. UBT data were represented by reciprocals of the factor of the highest dilution showing bactericidal action. All determinations were repeated at least twice.

2.6. Time–kill curves

Time–kill curve analyses were performed as described previously [7] by culturing the test strains (inoculum ca. 1×10^6 CFU/mL) in artificial urine in the presence of CS and AZT alone and in combination. Time–kill curves with colistin-susceptible strains using minimum third dose concentrations were performed once. All determinations for the colistin-resistant strains were repeated three times.

3. Results

3.1. Minimal bactericidal concentrations and urinary bactericidal titres

The MBCs of CS in CA-MHB and artificial urine were 8–32 mg/L and >64 mg/L, respectively, for all tested colistin-resistant *E. coli* strains (Table 3). For the tested colistin-susceptible Enterobacteriaceae strains, median MBCs of CS in CA-MHB/artificial urine ranged from 2–4/2–4 mg/L, whereas median MBCs were between 1–4/8–32 mg/L for the tested *Pseudomonas* and *Acinetobacter* strains. Median MBCs of AZT in CA-MHB and artificial urine for *E. coli* ATCC 25922, *E. coli* Af48 and *K. pneumoniae* BAA-2470 ranged between 0.5–16 mg/L and were between 64 mg/L and >64 mg/L for all other strains tested (Table 3).

To determine the urinary bactericidal activity following intravenous (i.v.) infusion, artificial urine was spiked with CS and AZT according to the measured median and minimum urinary concentrations of the collection period 0–3 h after start of the first and third dose (Table 1) alone or in combination. Since the minimum concentrations of the first dose corresponded closely to the median third dose concentrations, only the first dose median as well as the third dose median and minimum concentrations were tested.

UBTs for CS were positive (range 1–2) for 4/4 tested colistin-resistant *E. coli* strains using the median first dose concentration, for 3/4 strains using the median third dose concentration and for 0/4 strains using the minimum third dose concentration. The approach with AZT yielded reproducible results for only two of the four strains. Combination of CS with AZT showed similar or 1–2 dilution steps better results compared with colistin alone for median first and third dose concentrations. For colistin-susceptible strains,

Table 2
Features of *mcr-1*-positive *Escherichia coli* isolates used in this study

E. coli strain	*mcr-1*-positive plasmid type/size (ca. kb)	Sequence type (ST)	Source	Co-resistance	Co-resistance gene	Country of isolation	Reference
Af23	IncI2/70	ST10	Human blood	AMX/CIP/SXT/TET	bla_{TEM-1}	South Africa	[10]
Af24	IncI2/65	ST1007	Human pus	AMX/CIP/SXT/TET/CHL	bla_{TEM-1}	South Africa	[10]
Af48	IncX4/30	ST624	Human urine	AMX/CEF/CIP/SXT/TET/KAN	bla_{CMY-2}	South Africa	[10]
CDF6	IncHI2/150	ST446	Human urine	AMX/CTX/CIP/SXT/TET	bla_{CTX-M}	Switzerland	–

AMX, amoxicillin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol; CEF, cefalotin; KAN, kanamycin; CTX, cefotaxime.

Table 3
Minimum bactericidal concentrations (MBCs) of colistin sulfate (CS) and azidothymidine (AZT) in CA-MHB and artificial urine

	Median (range) MBC (in mg/L)								
	Ec Af23	Ec Af24	Ec Af48	Ec CDF6	Ec ATCC 25922	Kp BAA-2470	Pa ATCC 27853	Pa 1640801	Ab CHD102
CA-MHB									
CS	8 (4–64)	16 (16–32)	12 (8–32)	32 (8–32)	2 (1–2)	4 (2–8)	4 (2–8)	2 (1–4)	1 (1)
AZT	>64 (>64)	>64 (>64)	16 (16–128)	>64 (32–>64)	2 (1–4)	1 (1–2)	>64 (>64)	>64 (>64)	>64 (>64)
Artificial urine									
CS	>64 (>64)	>64 (>64)	>64 (>64)	>64 (>64)	2 (0.5–4)	4 (2–8)	32 (16–32)	16 (16–32)	8 (8)
AZT	>64 (>64)	>64 (>64)	4 (1–32)	64 (2 to >64)	2 (1–2)	0.5 (0.5–2)	>64 (>64)	>64 (>64)	>64 (>64)

CA-MHB, cation-adjusted Mueller–Hinton broth; Ec, *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Pa, *Pseudomonas aeruginosa*; Ab, *Acinetobacter baumannii*.

Table 4
Reciprocal urinary bactericidal titres (UBTs) at median and/or minimum urinary concentrations (UCs) after the first dose (0–3 h urine collection period) and third dose (24–27 h urine collection period)

	Reciprocal UBT								
	Ec Af23	Ec Af24	Ec Af48	Ec CDF6	Ec ATCC 25922	Kp BAA-2470	Pa ATCC 27853	Pa 1640801	Ab CHD102
Median UC first dose									
CS	1	1	2	2	128	64	8	8	8
AZT	1	0	*	*	32	256	0	0	0
CS+AZT	4	1	*	*	256	256	8	8	16
Median UC third dose									
CS	1	0	1	1	16	32	4	4	4
AZT	0	0	*	*	32	32	0	0	0
CS+AZT	4	1	*	*	128	128	4	2	8
Minimum UC third dose									
CS	0	0	0	0	4	16	1	2	2
AZT	0	0	*	*	16	32	0	0	0
CS+AZT	0	0	*	*	16	32	1	1	2

CS, colistin sulfate; AZT, azidothymidine; Ec, *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Pa, *Pseudomonas aeruginosa*; Ab, *Acinetobacter baumannii*.
* Interpretation of AZT alone and in combination was not possible due to incoherent data.

UBTs for median first/median third/minimum third dose concentrations were 64–128/32–16/4–16 for Enterobacteriaceae, 8/4/1–2 for *P. aeruginosa* and 8/4/2 for *A. baumannii*. When CS was combined with AZT, UBTs were 1–2 dilution steps higher for colistin-susceptible *E. coli* and *A. baumannii* for median first and third dose concentrations as well as for *K. pneumoniae* for median third dose concentrations. Combination of minimum third dose concentrations of CS and AZT showed no improvement compared with CS alone for all strains tested. Furthermore, none of the tested combinations had any benefit in killing the *P. aeruginosa* strains tested (Table 4).

3.2. Time–kill curves

Artificial urine spiked with concentrations of CS and AZT alone or in combination according to the obtained phase 1 median and median/minimum urinary concentrations 0–3 h after start of the first and third dose, respectively (Table 1), were also used to perform time–kill curve analysis of the *mcr-1*-positive strains.

The median first dose CS concentration killed >99.9% of the inoculated bacteria after 30–60 min for all *mcr-1*-positive strains. Furthermore, no detectable colonies were found after at the latest 4 h. Using the median third dose concentration, CS decreased bacterial numbers for all *mcr-1*-positive strains tested. However, a reduction below the detection limit after 24 h of incubation occurred only partially (1/3 repetitions for *E. coli* Af23 and CDF6, and 2/3 repetitions for *E. coli* Af48). The minimum third dose concentration of CS reduced not more than initial growth of the bacteria, but after 24 h of incubation control levels were reached (Figs 1–3).

AZT alone decreased bacterial numbers within the first 2–4 h for all concentrations tested. Re-growth to control levels occurred after 6–24 h for all strains using the minimum third dose concentration, for all strains except 2/3 repetitions of *E. coli* Af48 using the median third dose concentration, and for *E. coli* Af24, *E. coli* CDF6 as well as one repetition of *E. coli* Af23 using the median first dose concentration (Figs 1–3).

Combination of median first dose concentrations of CS and AZT was bactericidal within 6 h, while it accelerated killing of 3/4 tested strains compared with CS alone (Fig. 1). Using median third dose concentrations, combination of CS and AZT was bactericidal in all repetitions for all strains except *E. coli* CDF6 (Fig. 2). Combination of minimum third dose concentrations of CS and AZT showed bactericidal activities after 24 h in triplicate only for one strain (*E. coli* Af23). For the three remaining strains, the three repetitions showed at least once a reduction of the bacterial counts below the detection limit, but also at least once a re-growth of the bacteria after 24 h (Fig. 3).

For the five colistin-susceptible strains, the minimum third dose CS concentration was sufficient to kill all bacteria in at least 6 h

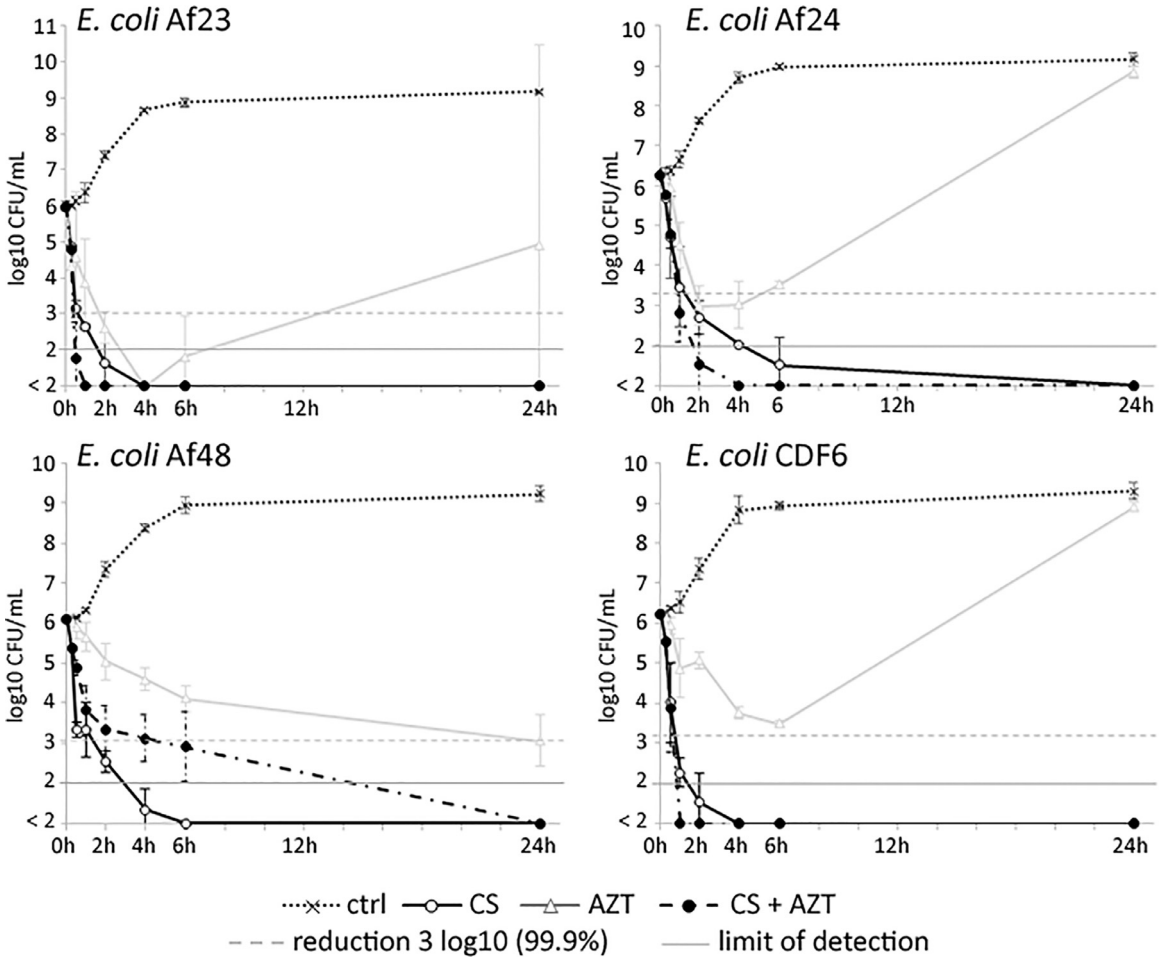

Fig. 1. Time–kill curves using median urinary concentrations after the first dose (0–3 h urine collection period). Bacteria were inoculated with ca. 1×10^6 CFU/mL in artificial urine. After adding colistin sulfate (CS) and azidothymidine (AZT), alone or in combination, according to median urinary concentrations after the first dose, bacteria were incubated at 37°C and 180 rpm. Ctrl, control without any addition. Error bars indicate the standard deviation.

(Fig. 4). Therefore, the higher concentrations were not tested. The minimum AZT concentration reduced bacterial numbers of tested Enterobacteriaceae in the first hours with a subsequent re-growth to control amounts, but did not affect *P. aeruginosa* and *A. baumannii* (Fig. 4). Combination of CS with AZT slightly accelerated killing of the *A. baumannii* strain tested but was not in addition beneficial for the tested concentrations on killing of the colistin-susceptible *E. coli, K. pneumoniae* and *P. aeruginosa* strains.

All samples spiked with CS and AZT showing visible growth by turbidity after 24 h of incubation were used for subsequent minimum inhibitory concentration (MIC) determination of CS as well as AZT. Growth in the presence of CS or AZT did not affect the susceptibility against CS; median fold changes over the MIC of control bacteria growing without any substances added were both 1. Furthermore, bacteria grown with CS showed no difference in AZT MIC (median fold change over control = 1.0). In contrast, 24 h of growth in AZT-containing media increased the AZT MIC by approximately 8-fold.

4. Discussion

This annex ex vivo study was carried out as part 2 of a phase 1 trial in healthy volunteers [6] and evaluated the bactericidal activity of peak urinary concentrations obtained during i.v. treatment with CMS in combination with AZT.

It was observed that the MBCs of CS in CA-MHB were several dilution steps lower than in artificial urine for *mcr-1*-positive *E. coli* and *P. aeruginosa* strains. The lower pH and an increased amount of divalent cations in artificial urine compared with CA-MHB contribute to this phenomenon [16–18]. For this reason, the results of pharmacodynamic studies obtained in serum cannot simply be extrapolated to a possible outcome in urine only by taking into account the different concentrations measured in serum and urine. Specific urodynamic studies in urine are therefore justified.

Determination of bactericidal titres in artificial urine was performed, which show only the effect after 24 h of incubation, and of time–kill curves in artificial urine, showing dynamic changes of bacterial counts during 24 h of incubation, using constant drug concentrations. The results showed that the minimum urinary CS concentration after 2 million international units (MIU) of CMS infusion was sufficient to eliminate colistin-susceptible strains. This is similar to our previous results on the bactericidal activity of the concentrations obtained in plasma [7]. However, bactericidal concentrations of CS against all tested colistin-resistant strains could only be obtained with median urinary concentrations after 4 MIU of CMS infusion. For the combination of CS with AZT, the data showed that AZT potentiates the bactericidal effect of colistin against Enterobacteriaceae, even that of some colistin-resistant *E. coli*, and *A. baumannii* in artificial urine. With respect to the tested *Pseudomonas* strains, however, the combination with AZT had no advantage. These results are similar to our previous results for serum bactericidal activity [7].

For the colistin-susceptible strains, the beneficial effect of the combination was mote clearly visible with UBT determination

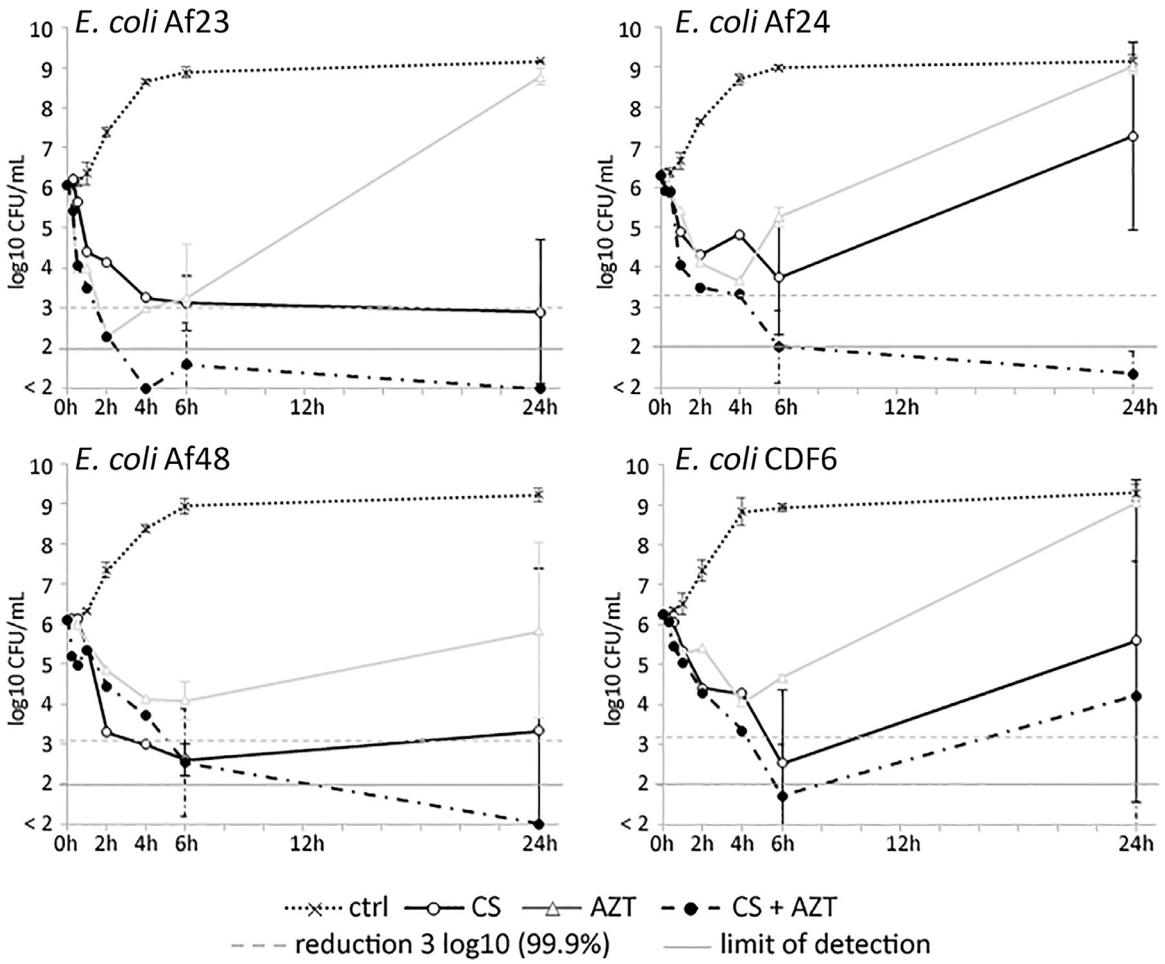

Fig. 2. Time–kill curves with median urinary concentrations after the third dose (24–27 h urine collection period). Bacteria were inoculated with ca. 1×10^6 CFU/mL in artificial urine. After adding colistin sulfate (CS) and azidothymidine (AZT), alone or in combination, according to median urinary concentrations after the third dose, bacteria were incubated at 37°C and 180 rpm. Ctrl, control without any addition. Error bars indicate the standard deviation.

compared with time–kill curves. The minimum third dose CS concentration alone, with a concentration of 4–8-fold higher than the urinary MBC of the colistin-susceptible Enterobacteriaceae, reduced the bacterial numbers by almost 3 log within 15 min. Therefore, no additional effect of AZT could be detected. The dilution methodology of the UBT determination allows a better presentation of positive effects.

In contrast, for the colistin-resistant strains, the time–kill curve determinations gave better results compared with UBT determinations. Since UBT determination is a one-timepoint method after 24 h of incubation, acceleration of the killing as seen in the time–kill curves could not be demonstrated. On the other hand, especially the colistin-resistant strains sometimes showed heteroresistance against AZT. Heteroresistance describes a phenomenon where subpopulations of seemingly isogenic bacteria exhibit a range of susceptibilities to a particular substance [19]. This heteroresistance was noticeable by 'skip wells' (wells that exhibit no growth although growth occurs at higher concentrations) in serial dilutions even in multiple repetitions. This resulted in uninterpretable UBTs for *E. coli* CDF6 and *E. coli* Af48.

Membrane permeabilisation by colistin [20] may increase the entry of AZT into bacterial cells. The antibacterial activity of AZT can be traced back to inhibition of replicative DNA synthesis following incorporation of AZT triphosphate [3,4]. This effect is often only temporary. Mutations resulting in a lack of thymidine kinase lead to increased resistance against AZT [3,4]. Increased AZT MICs of the *mcr-1*-positive *E. coli* strains after growing in the presence of the deoxyribonucleoside analogue during the time–curve experiments led to the assumption that mutations are selected which enhanced resistance against AZT. However, no cross-resistance to CS was seen. Rather, the combination of CS and AZT potentiated killing of *mcr-1*-positive *E. coli*, especially at lower concentrations after the third dosage compared with CS alone. Compared with Enterobacteriaceae, AZT alone did not affect the growth of the *Pseudomonas* and *Acinetobacter* strains tested as these bacteria often naturally lack a thymidine kinase [21,22]. Nevertheless, the current data suggest a beneficial effect of AZT on colistin activity against the *A. baumannii* strain tested.

These results revealed that a low dose of 2 MIU of CMS infusion combined with 100 mg of AZT twice daily appears to be sufficient for the treatment of UTIs caused by colistin-susceptible strains. The combination also showed improved killing of some colistin-resistant *E. coli* strains in urine compared with 2 MIU of CMS infusion alone. However, especially at the minimum concentrations, the killing effect could not be reproduced for all strains tested. Therefore, the dosage for treatment of complicated UTIs caused by colistin-resistant *E. coli* probably needs to be increased. Due to a possible elevated risk of nephrotoxicity [23], CMS should be increased carefully. No nephrotoxicity has been described so far for AZT [24].

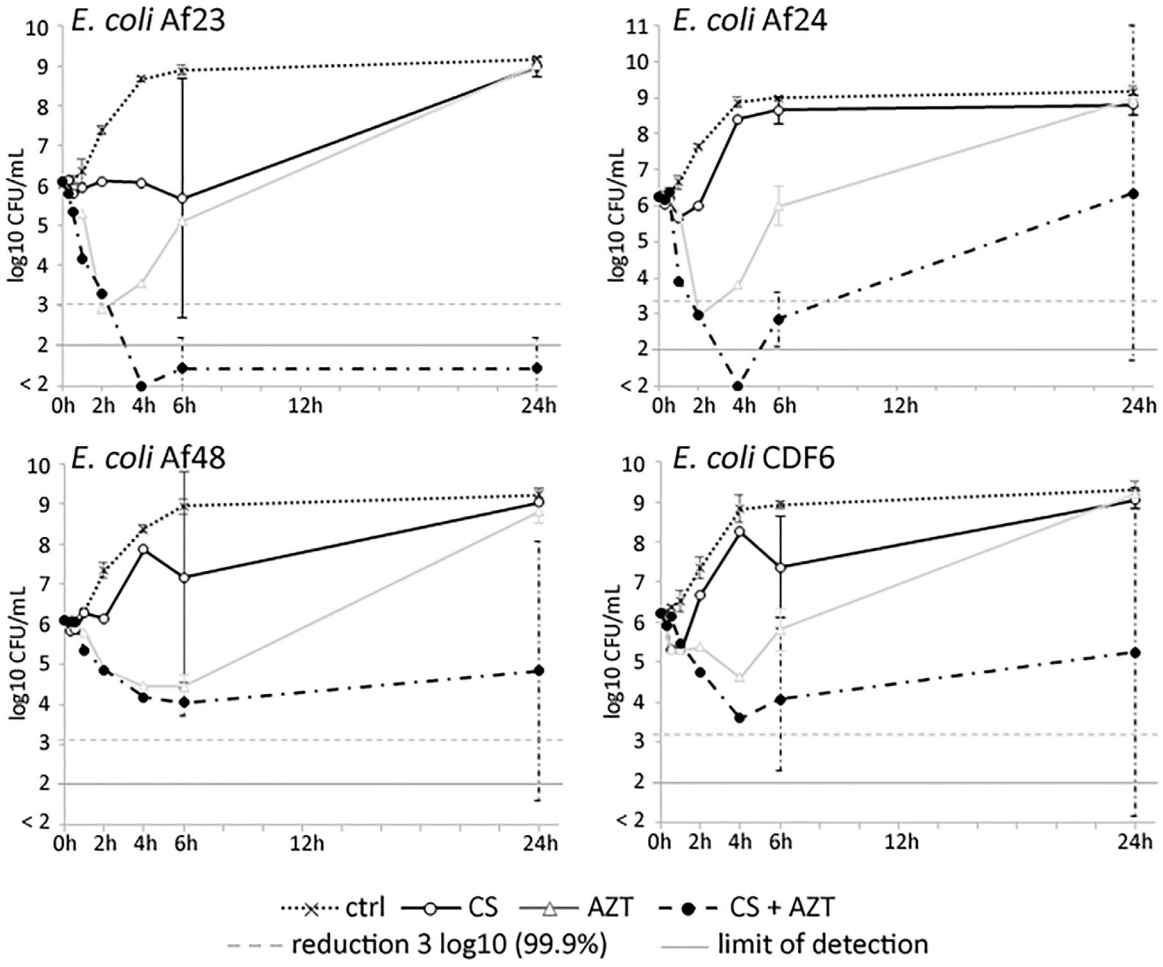

Fig. 3. Time–kill curves with minimum urinary concentrations after the third dose (24–27 h urine collection period). Bacteria were inoculated with ca. 1×10^6 CFU/mL in artificial urine. After adding colistin sulfate (CS) and azidothymidine (AZT), alone or in combination, according to minimum urinary concentrations after the third dose, bacteria were incubated at 37°C and 180 rpm. Ctrl, control without any addition. Error bars indicate the standard deviation.

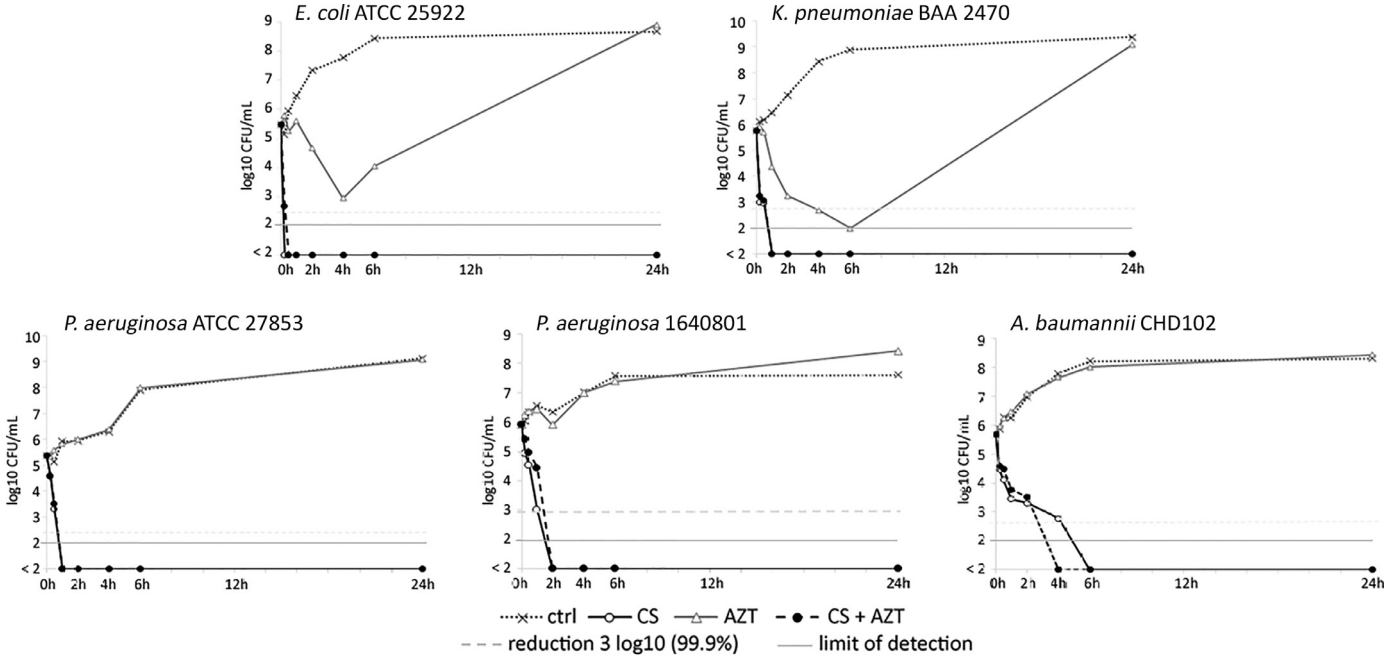

Fig. 4. Time–kill curves of colistin-susceptible strains with minimum urinary concentrations after the third dose (24–27 h urine collection period). Bacteria were inoculated with ca. 1×10^6 CFU/mL in artificial urine. After adding colistin sulfate (CS) and azidothymidine (AZT), alone or in combination, according to minimum urinary concentrations after the third dose bacteria, were incubated at 37°C and 180 rpm. Ctrl, control without any addition.

5. Conclusions

In this part 2 ex vivo study, combination of CMS with AZT showed promising activity against Gram-negative uropathogens, including colistin-resistant *E. coli*. According to the urinary bactericidal activity, a dosage of 2 MIU of CMS plus 100 mg of AZT is sufficient for the treatment of UTIs with colistin-susceptible strains. In contrast, for UTIs caused by colistin-resistant *E. coli*, the combination of 2 MIU of CMS with 100 mg of AZT showed good indications that AZT potentiates the bactericidal effect of colistin. However, a higher dosage should be tested in well-designed clinical studies.

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Competing interests

ML is a Collaborator for Helperby Therapeutics; KGN is an Investigator for Enteris Biopharma, a Scientific Advisor (Review Panel or Advisory Committee) for Bionorica, Enteris Biopharma, Helperby Therapeutics, Leo Pharma, MerLion, MSD Sharp & Dohme, OM Pharma, Paratek, Rosen Pharma, Roche and Zambon, and has served on speaker's bureaux for Bionorica, Daiichi Sankyo, Leo Pharma, OM Pharma, Rosen Pharma and Zambon; YH is a Grant Investigator for Helperby Therapeutics; AC is a Shareholder (excluding diversified mutual funds) of Helperby Therapeutics; FMEW is a Collaborator for Helperby Therapeutics, an Investigator for Enteris BioPharma, and a Scientific Advisor (Review Panel or Advisory Committee) for Bionorica, Enteris BioPharma, Helperby Therapeutics, Leo Pharma, MerLion, OM Pharma, Rosen Pharma, Achaogen, AstraZeneca, Janssen, MSD, Shionogi and Pfizer.

Ethical approval

Not required.

Author contribution statement

ML performed all experiments and wrote the manuscript, with close cooperation and supervision of FMEW and considering critical comments from all coauthors. KGN, YM and AC made also substantial contributions to the conception and design and all authors contributed to analysis and interpretation of the data. YM and AC are the co-inventors of the antibiotic resistance breaker technology, and in particular of the combination of AZT and colistin/CMS (patents pending). They were the first to test this combination against highly resistant Enterobacteriaceae (ECCMID 2018 #O0574). They originated the concept and performed the background work upon which this work is based. YH and AC revised the manuscript critically for important intellectual content and gave final approval of the revised version.

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