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EXPERIMENTAL THERAPEUTICS

Azidothymidine Produces Synergistic Activity in Combination with Colistin against Antibiotic-Resistant *Enterobacteriaceae*

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ABSTRACT Bacterial infections remain a leading killer worldwide, which is worsened by the continuous emergence of antibiotic resistance. In particular, antibiotic-resistant *Enterobacteriaceae* are prevalent and extremely difficult to treat. Repurposing existing drugs and improving the therapeutic potential of existing antibiotics represent an attractive novel strategy. Azidothymidine (AZT) is an antiretroviral drug which is used in combination with other antivirals to prevent and to treat HIV/AIDS. AZT is also active against Gram-negative bacteria but has not been developed for that purpose. Here, we investigated the *in vitro* and *in vivo* efficacy of AZT in combination with colistin against antibiotic-resistant *Enterobacteriaceae*, including strains producing extended-spectrum beta-lactamases (ESBLs) or New Delhi metallo-beta-lactamase 1 (NDM) or carrying mobilized colistin resistance (mcr-1). The MIC was determined using the broth microdilution method. The combined effect of AZT and colistin was examined using the checkerboard method and time-kill analysis. A murine peritoneal infection model was used to test the therapeutic effect of the combination of AZT and colistin. The fractional inhibitory concentration index from the checkerboard assay demonstrated that AZT synergized with colistin against 61% and 87% of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* strains, respectively, 100% of NDM-1-producing strains, and 92% of mcr-1-producing *E. coli* strains. Time-kill analysis demonstrated significant synergistic activities when AZT was combined with colistin. In a murine peritoneal infection model, AZT in combination with colistin showed augmented activities of both drugs in the treatment of NDM-1 *K. pneumoniae* and mcr-1 *E. coli* infections. The AZT and colistin combination possesses a potential to be used coherently to treat antibiotic-resistant *Enterobacteriaceae* infections.

KEYWORDS ESBL, *Enterobacteriaceae*, NDM-1, azidothymidine, colistin, mcr-1

Bacterial infection remains a leading killer worldwide (1), and antibiotic resistance continues to plague the effective control of this pandemic health problem (2, 3). In particular, there is an urgent global threat with an increasing prevalence of multidrug-resistant *Enterobacteriaceae*, especially carbapenem-resistant *Enterobacteriaceae* (CRE) such as New Delhi metallo-beta-lactamase-1 (NMD) carriers (4–8), which are extremely resistant to almost all of our antibiotics (3, 9). As a result, our ability to treat serious community- and nosocomial-acquired infections is rapidly diminishing (10). Unfortunately, the number of new antibiotics reaching the market annually is unable to keep up with the development of bacterial antibiotic resistance (11–14). The drug discovery process itself is arduous and costly, and it is almost impossible to produce a large group of effective antibiotics within a short period of time to combat antibiotic resistance. Therefore, a different therapeutic approach is needed to replenish our antibiotic reservoir against resistant bacteria, and the most promising of such strategies is to

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TABLE 1 MIC values of antibiotics and AZT against 7 NDM-1-producing strains

Antibiotic	MIC (mg/liter)						
	K. pneumoniae strain				*Enterobacter cloacae*	*E. coli* strain	
	NCTC13443	BAA-2470	BAA-2472	BAA-2473	BAA-2468	BAA-2469	BAA-2471
Cefotaxime	>2,048	>2,048	>2,048	2,048	512	512	>2,048
Ceftazidime	>2,048	>2,048	>2,048	512	512	>2,048	>2,048
Ceftriaxone	>4,096	>4,096	>4,096	>4,096	4,096	2,048	>4,096
Aztreonam	>2,048	512	2,048	1,024	1,024	32	>2,048
Piperacillin	>2,048	1,024	>2,048	1,024	256	1,024	>2,048
Meropenem	128	128	128	16	64	32	128
Gentamicin	>256	>256	>256	>256	>256	>256	>256
Amikacin	8	>256	>256	8	>256	>256	16
Tobramycin	32	128	>1,024	16	>1,024	1,024	16
Ciprofloxacin	>64	8	32	>64	64	64	64
Levofloxacin	32	4	32	32	32	16	16
Trimethoprim	>256	>256	>256	>256	>256	>256	>256
Nitrofurantoin	256	>256	256	256	>256	32	64
Tigecycline	1	1	4	1	4	0.5	0.5
Rifampicin	1,024	256	1,024	1,024	16	4	16
Colistin	0.25	0.5	1	0.25	0.5	0.125	0.125
AZT	4	2	2	2	2	2	4

repurpose existing drugs and to restore the therapeutic potencies of existing antibiotics (15, 16).

Azidothymidine (3′-azido-3′-deoxythymidine [AZT]) is an antiretroviral drug which is used in combination with other antivirals to prevent and to treat HIV/AIDS. It inhibits viral reverse transcriptase and was the first effective treatment for HIV/AIDS (17) entering the U.S. market in 1986. AZT is also active against Gram-negative bacteria (18–22) but has not been developed or approved for that purpose. It is thought to inhibit bacterial DNA replication by chain termination. Resistance to AZT occurs in bacteria and has been attributed to two mechanisms; one of these is unknown, and the other is a deficiency of thymidine kinase which phosphorylates inactive AZT into the active triphosphate form (23).

The rapid emergence of CRE which are often resistant to many other antibiotics has left the world with colistin as the last-resort treatment option. The use of colistin has led to high rates of colistin resistance in patients with infections due to *Klebsiella pneumoniae* carbapenemase (KPC)-producing strains (24). A recent study also found that approximately 10% of NDM-1-producing CRE in the United Kingdom were colistin resistant (25), and plasmid-borne colistin resistance was also found recently in animals and humans (26). Hence, it is crucial to boost the effectiveness of colistin against colistin-resistant bacteria. However, treatment with colistin has been associated with both nephron and neurotoxic adverse effects (27). It is not known if AZT can synergistically act with colistin to treat multidrug-resistant *Enterobacteriaceae* infections, which would allow the administration of both drugs at lower doses to achieve a desired therapeutic effect while minimizing the side effects and preventing the emergence of antibiotic resistance (15, 28).

In this study, we performed the first study to retrospectively test the *in vitro* activities of AZT in combination with colistin against 74 antibiotic-resistant *Enterobacteriaceae* strains, including NDM-1-, mcr-1-, and ESBL-producing strains. In addition, the therapeutic effectiveness of AZT plus colistin was tested using a mouse peritoneal infection model.

RESULTS

***In vitro* susceptibility of AZT and colistin against 74 antibiotic-resistant *Enterobacteriaceae*.** The MICs for aztreonam, amoxicillin, piperacillin, cefotaxime, ceftriaxone, ceftazidime, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, trimethoprim, nitrofurantoin, rifampin, tigecycline, colistin, polymyxin B, and AZT were determined against the seven NDM-1-producing strains. As seen in Table 1,

TABLE 2 MIC values of antibiotics and AZT against ESBL- and mcr-1-producing *E. coli* and *K. pneumoniae*

Antibiotic	MIC for the indicated ESBL-producing organism (mg/liter)						MIC (mg/liter) for mcr-1-containing *E. coli* ($n = 13$)[a]		
	E. coli ($n = 23$)			*K. pneumoniae* ($n = 31$)					
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
Aztreonam	1–256	128	256	32–1,024	128	256			
Amoxicillin	128–2048	256	2048	256–1,024	512	1,024			
Piperacillin	1–512	16	256	16–1,024	512	1,024			
Cefotaxime	64–2048	512	1,024	32–1,024	512	1,024			
Ceftazidime	8–512	256	512	32–1,024	128	1,024			
Ceftriaxone	128–1,024	512	1,024	64–1,024	256	512			
Gentamicin	0.5–256	128	128	16–128	128	128			
Meropenem	0.03–0.25	0.125	0.25	0.03–2	0.03	1			
Imipenem	0.03–0.25	0.125	0.25	0.06–128	0.25	2			
Ciprofloxacin	0.03–256	64	256	0.06–256	128	128			
Trimethoprim	0.06–128	64	128	0.125–128	64	128			
Tigecycline	0.125–4	0.5	0.5	0.5–8	1	4			
Colistin	0.5–4	0.5	1	0.5–2	0.5	1	2–8	4	8
AZT	0.25–64	4	32	2–32	8	32	8–64	8	64

[a]Only colistin and AZT were tested.

compared with the antibiotic breakpoints (29), resistance was found in all strains for nearly all antibiotics. Only certain strains were susceptible to a number of antibiotics such as nitrofurantoin (BAA-2469), amikacin (NCTC13443, BAA2473) and tigecycline (BAA-2469, BAA-2470, NCTC13443, BAA2473, and BAA-2471). However, all NDM-1-producing strains were susceptible to colistin (29). AZT MICs ranged from 2 to 4 mg/liter.

The MICs for the antibiotics and AZT were also determined against the 54 antibiotic-resistant isolates of *E. coli* and *K. pneumoniae*. As shown in Table 2 (see also Table S3 in the supplemental material), these strains were resistant to monobactam, penicillins, and cephalosporins but were susceptible to carbapenems. Resistance was seen for gentamicin, ciprofloxacin, and trimethoprim. Ninety percent of the strains were susceptible to tigecycline and colistin. The MIC for AZT ranged from 0.25 to 64 mg/liter for *E. coli* and from 2 to 32 mg/liter for *K. pneumoniae*. The 54 multidrug-resistant *E. coli* and *K. pneumoniae* strains were tested for ESBL production using commercial ESBL-testing systems, which demonstrated that these were ESBL-producing strains (Table S3).

For colistin-resistant strains, the MICs for AZT ranged from 8 to 64 mg/liter, with the MIC_{50} at 8 mg/liter and MIC_{90} at 64 mg/liter. The range of MICs for colistin was 2 to 8 mg/liter with the MIC_{50} at 4 mg/liter and MIC_{90} at 8 mg/liter.

Checkerboard analysis of combination effects. The effects of combining AZT with colistin were determined using checkerboard assays against all 74 strains. As shown in Table 3, the combination of AZT with colistin showed synergistic activity, with a

TABLE 3 Combination activities of AZT with colistin

Strain group	Activity of the combination	FICI	Total no. (%) of strains
ESBL-producing			
E. coli	Synergy	≤ 0.5	14 (60.87)
	No interaction	0.56–1	9 (39.13)
	Antagonism	>4	0
K. pneumoniae	Synergy	≤ 0.5	27 (87.10)
	No interaction	0.56–1	4 (12.90)
	Antagonism	>4	0
NDM-1-producing	Synergy	≤ 0.5	7 (100)
	No interaction	0.56–1	0
	Antagonism	>4	0
mcr-1-containing *E. coli*	Synergy	≤ 0.5	12 (92.31)
	No interaction	0.56–1	1 (7.69)
	Antagonism	>4	0

fractional inhibitory concentration index (FICI) of ≤0.5 against 60.87% of the ESBL-producing *E. coli* strains, 87.1% of the ESBL-producing *K. pneumoniae* strains, 100% of NDM-1-producing strains, and 92.31% of colistin-resistant (mcr-1) *E. coli* strains. With the concentration of AZT ranging from 0.25 to 16 mg/liter, the MICs of colistin were significantly reduced from 32- to 256-fold against the seven NDM-1-producing strains, from 2- to 64-fold against ESBL-producing *E. coli* strains, from 2- to 512-fold against ESBL-producing *K. pneumoniae* strains, and from 4- to 256-fold against mcr-1-containing *E. coli* strains.

Time-kill analysis of AZT in combination with colistin against log-phase bacteria. The synergistic combination of AZT and colistin was performed using time-kill assays against 7 NDM-1-producing strains, 3 ESBL-producing *E. coli* strains, 3 ESBL-producing *K. pneumoniae* strains, and 3 mcr-1-containing *E. coli* strains, which showed FICIs of <0.5 for the combination. The characteristics of the 16 strains are shown in Table S4. A range of different concentrations was used starting from 2-fold of the MIC level for each of the two drugs. Data from representative strains are shown to display the combinations with synergistic activities. As shown in Fig. 1, for the NDM-1-producing *K. pneumoniae* BAA-2472 strain, colistin at 2 mg/liter was bactericidal until 7 h, which was followed by regrowth, and at 1 mg/liter (MIC) inhibited bacterial growth. AZT at 4, 2 (MIC), and 1 mg/liter was bactericidal, showing dose-dependent killing, and regrowth occurred after 8 h of drug exposure. However, when colistin at 2 mg/liter was combined with 4 and 2 mg/liter of AZT, significant killing to the limit of detection of the initial bacterial counts was achieved within 4 h, and the same killing level was seen at 8 h when the same concentration of colistin was combined with 1 mg/liter of AZT (Fig. 1A to C). When colistin at 1 mg/liter was combined with 4, 2, and 1 mg/liter of AZT, killing at the level of the limit of detection was achieved at 8 h (Fig. 1E and F). No bacterial regrowth was observed at either 24 (Fig. 1) or 48 h posttreatment (data not shown).

As shown in Fig. 2, for the mcr-1-containing *E. coli* strain Af40 (Table S1), colistin at 8 mg/liter (MIC) inhibited bacterial growth and at 4 mg/liter produced a growth pattern similar to that of the control. AZT at 4, 2, and 1 mg/liter reduced the initial counts until 4 h, and regrowth was seen. When colistin at 8 mg/liter was combined with the concentrations of 4, 2 and 1 mg/liter AZT, killing to the limit of detection was seen at 8 h (Fig. 2A to C). The same effects were seen when colistin at 4 mg/liter was combined with 4 mg/liter of AZT (Fig. 2D). Reduced effects were seen when colistin at 4 mg/liter was combined with 4 and 2 mg/liter of AZT, and killing to the limit of detection was shown at 24 h (Fig. 2E and F).

Significant synergistic activity was also observed in six other NDM-1-producing strains (Fig. S1 to S6), three ESBL-producing *E. coli strains* (Fig. S7 to S9), three ESBL-producing *K. pneumoniae* strains (Fig. S10 to S12), and two colistin-resistant mcr-1-containing *E. coli* strains (Fig. S13 and S14).

***In vivo* combination activities of AZT combined with colistin.** The *in vivo* activity of AZT in combination with colistin was studied using a murine peritoneal infection model. A dose range study of the two drugs was performed. For AZT, the minimal dose (5 mg/kg) was chosen which only inhibited bacterial growth but provided significant enhanced activities when combined with colistin methanesulfonate (CMS). For CMS, we found that 10 to 30 mg/kg showed no activity against the infected bacteria. Therefore, for the colistin-sensitive NDM-1-producing strain, we used 10 mg/kg of CMS, and for the mcr-1-containing *E. coli*, we used 20 mg/kg of CMS. The drugs were tested singly or in combination against the NDM-1-producing *K. pneumoniae* strain BAA-2472 and the mcr-1-containing *E. coli* strain Af40 (Table S4).

As shown in Fig. 3A, for the *K. pneumoniae* BAA-2472, compared with growth in the untreated control, colistin at 10 mg/kg showed no activity at both 2 and 6 h, and AZT at 5 mg/kg inhibited bacterial growth. The combination of colistin with AZT, although producing inhibition only at 2 h, exhibited 2.72-log killing of the bacterium at 6 h. The difference in the bacterial numbers between 0 h and 6 h was significant

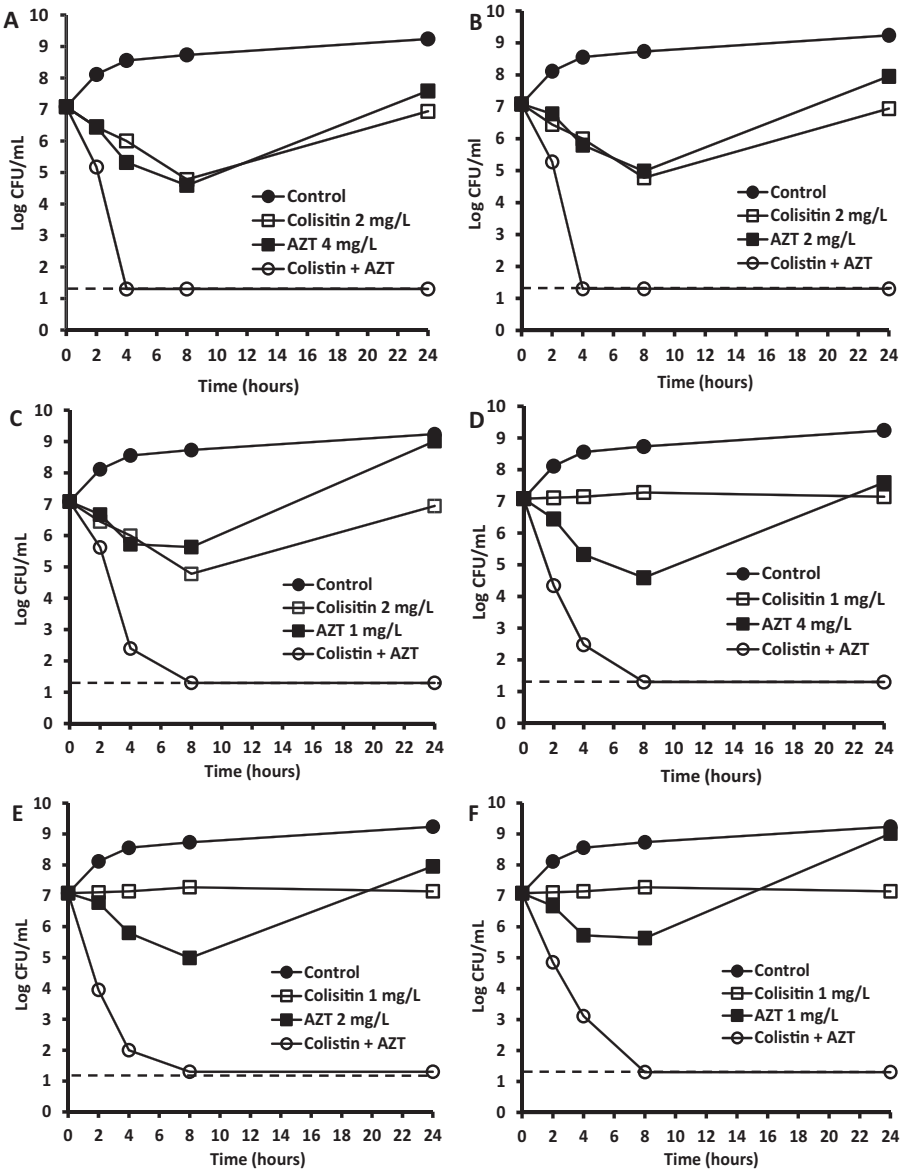

FIG 1 Time-kill analysis showing the effects of AZT in combination with colistin against NDM-1-producing *K. pneumoniae* BAA-2472. AZT and colistin alone or in combination were added to the log-phase cultures, and CFU counts were carried out at different time points. The concentrations of AZT and colistin used in combination are those shown on the respective panels for each drug alone (e.g., colistin at 2 mg/liter plus AZT at 4 mg/liter for panel A). The dashed line represents the limit of detection in the assay (30 CFU/ml).

($P < 0.001$; $n = 4$). For *E. coli* strain Af40 (Fig. 3B), colistin at 20 mg/kg showed the same growth pattern as the control, and AZT inhibited bacterial growth. The combination of colistin with AZT exhibited 1.32- and 2.96-log killing of the bacterium at 2 and 6 h, respectively. The difference in the bacterial numbers between 0 h and 2 h or 6 h was significant ($P < 0.01$ and 0.001, respectively; $n = 4$). In both untreated control groups and the colistin-treated group, all animals developed mild clinical signs, such as transiently hunched posture, at 6 h after infection. The animals in other treatment groups showed no discomfort, with normal and healthy behaviors. All animals were sacrificed at 6 h after treatment in accordance with restrictions on adverse effects in the project license.

DISCUSSION

In this study, we demonstrated for the first time that AZT synergized with colistin against 74 antibiotic-resistant *Enterobacteriaceae* strains, including NDM-1- and ESBL-

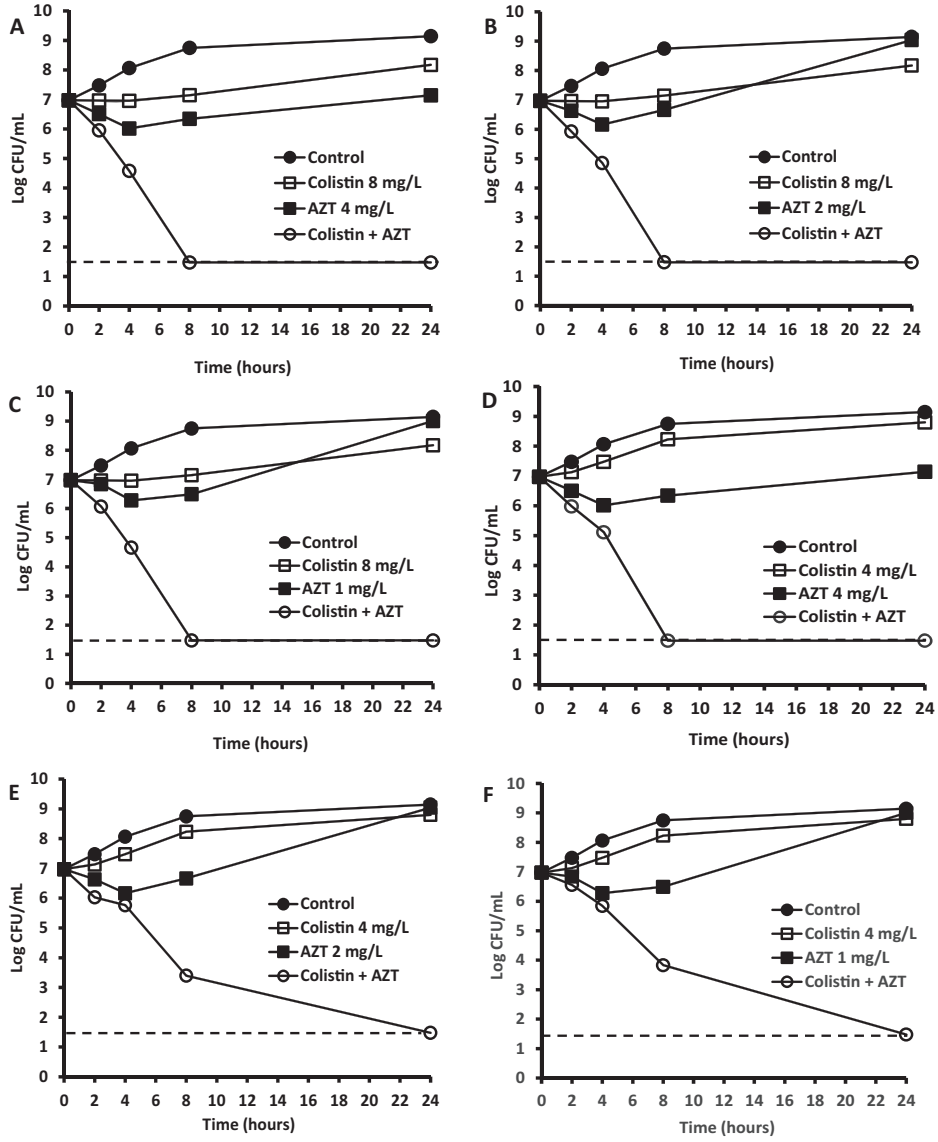

FIG 2 Time-kill analysis showing the effects of AZT in combination with colistin against mcr-1-containing colistin-resistant *E. coli* Af40. AZT and colistin alone or in combination were added to the log-phase cultures, and CFU counts were carried out at different time points. The concentrations of AZT and colistin used in combination are those shown on the respective panels for each drug alone (e.g., colistin at 8 mg/liter plus AZT at 4 mg/liter for panel A). The dashed line represents the limit of detection in the assay (20 CFU/ml).

producing and colistin-resistant strains. The antibiotic-resistant *Enterobacteriaceae* isolates used in the study covered a broad geographic distribution. The colistin-resistant strains were from some European countries and South Africa (30–32). The 7 NDM-1-producing strains represented the most resistant type of *Enterobacteriaceae*.

The clinical efficacy of AZT has been demonstrated to reduce morbidity and mortality in patients with asymptomatic or acute HIV disease (33, 34). In patients, the oral dosage is 250 to 300 mg twice daily, and the dose with intravenous (i.v.) infusion is 0.8 to 1 mg/kg every 4 h for up to 2 weeks. It has been shown that 120-mg i.v. dosing produced an area under the concentration-time curve (AUC) of 0.0014 mg · h/liter and a maximum concentration of drug in serum (C_{max}) of 0.0015 mg/liter while a 200-mg oral dosing gave rise to an AUC of 0.0017 mg · h/liter and a C_{max} of 0.0018 mg/liter (35). AZT has been shown to be active against Gram-negative bacteria (18–20), but it is not known if the concentrations used clinically are sufficient to treat bacterial infections in humans.

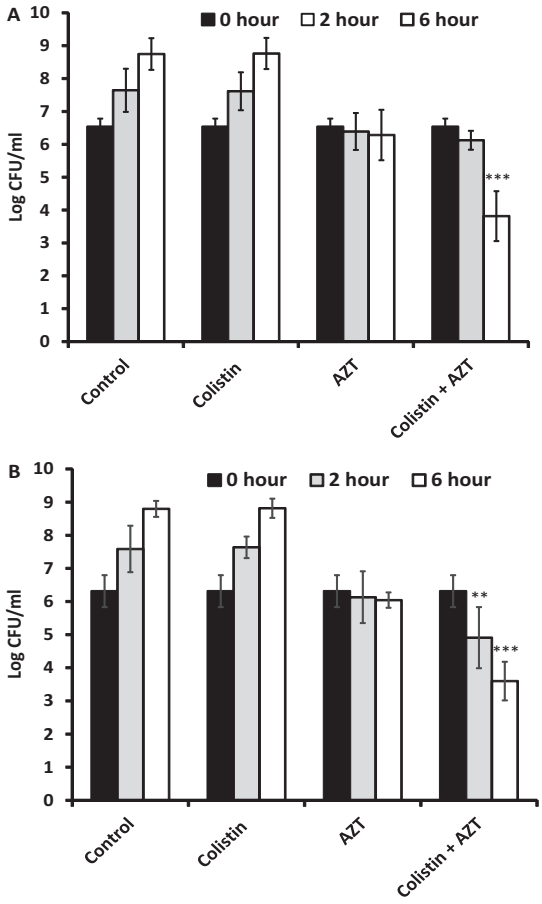

FIG 3 Effects of AZT in combination with colistin against the NDM-1-producing *K. pneumoniae* BAA-2472 and the mrc-1-containing *E. coli* strain Af40 in a mouse peritoneal infection model. (A) Mice were infected with strain BAA-2472. At 30 min after infection, treatment was initiated with AZT (5 mg/kg), CMS (10 mg/kg), and AZT plus CMS. (B) Mice were infected with strain Af40. At 30 min after infection treatment was initiated with AZT (5 mg/kg), CMS (20 mg/kg), and AZT plus CMS. Bacterial counts in the peritoneal cavity were determined from 4 mice for each group at 0 h before and at 2 and 6 h posttreatment. The experiment was repeated once. **, $P \leq 0.01$; ***, $P \leq 0.001$.

Colistin is effective against multidrug-resistant but colistin-susceptible *Pseudomonas aeruginosa*, *K. pneumoniae*, and *Acinetobacter* (36) strains and importantly against NDM-1-carrying *Enterobacteriaceae* (8). There is increasing evidence to show that colistin resistance is on the rise, especially with the discovery of plasmid-borne colistin resistance worldwide (26, 37). It is critically important to preserve and prolong the life of this last-resort antibiotic by enhanced combination therapy. Here, we have shown that in combination with AZT, the colistin MIC was significantly reduced, especially against mcr-1-containing colistin-resistant strains. The enhanced activity of colistin in combination with AZT was confirmed with time-kill assays which provided dynamic measures of bactericidal activities of the combination over time. In colistin mono-exposure, complete eradication of the NDM-1-producing *K. pneumoniae* BAA-2472 or mcr-1-containing *E. coli* Af40 strain required much higher concentrations of the drug (data not shown); however, more than 4- to 16-fold-lower concentrations of colistin in combination with AZT achieved the same effect. This is significant as the enhancement of the colistin combination with AZT will likely reduce the dose of colistin, thereby minimizing its toxic profile, but retain maximal therapeutic efficacy. These data suggest that further clinical development of the combination of colistin plus AZT may be able to achieve an effective lower-dose colistin therapy against colistin-sensitive and colistin-resistant infections.

Bacterial infections caused by carbapenem-resistant strains are life threatening, and

effective treatment is difficult to achieve. The last-resort treatment option is to use colistin (9, 39). Previous studies have shown that in bacteria AZT needs to be converted to nucleotides to inhibit bacterial DNA replication (40) and that bacterial thymidine kinase is responsible for the initiation of the activation process, i.e., phosphorylation of AZT (23, 40). Other antibiotics such as ciprofloxacin also inhibit DNA replication by blocking GyrA. The resistance profiles of ciprofloxacin and AZT are very different (Table 3). This suggests that AZT has a different mechanism of action than other antibacterial agents that are on the market. Rather, AZT is likely to act on a new target in bacteria. Further studies on how AZT acts against Gram-negative bacteria are under way in our laboratories by analysis of AZT mutants with next-generation sequencing and investigation of the AZT effect on bacteria by performing bacterial cytological profiling (BCP).

The therapeutic effectiveness of the AZT and colistin combinations was also examined using a mouse peritoneal infection model. As a potential therapeutic agent, AZT has been used to treat HIV. Its bactericidal activity has been reported *in vivo* (19). Here, we demonstrate that AZT at 5 mg/kg inhibited growth of the NDM-1-producing *K. pneumoniae* BAA-2472 and the mcr-1-containing *E. coli* Af40 strains in a mouse peritoneal infection. However, the combination of AZT with colistin improved the therapeutic activities of each single agent, with significant killing of the bacteria at 2 or 6 h in the mouse peritoneal cavity. Most importantly, while colistin methanesulfonate was completely ineffective up to 6 h of treatment, the addition of AZT was able to significantly reduce bacterial counts and attenuate the clinical signs in the animals. Here, we used colistin methanesulfonate instead of colistin sulfate. The reason was that colistin methanesulfonate is used clinically and is less toxic than colistin sulfate in mice (41). Colistin methanesulfonate is a prodrug which needs to be converted to the active form of colistin (42). The conversion normally delays the activity of the drug (42). Here, we demonstrated that with the addition of AZT, the effect of colistin methanesulfonate and AZT was significantly increased. Collectively, the data show that the application of the AZT and CMS combination therapy *in vivo* offers the potential to increase both colistin and AZT activities against antibiotic-resistant *Enterobacteriaceae*.

In conclusion, in this proof-of-principle study, we demonstrated the high therapeutic efficacy of combination therapy using AZT plus colistin against antibiotic-resistant *Enterobacteriaceae*, including mcr-1-containing and NDM-1- and ESBL-producing strains. ESBL-producing strains were confirmed using commercially accepted phenotypical methods currently used in clinical practice. The interaction between the genotypic characteristics of ESBL-producing strains and this novel combination therapy deserves further investigation. Importantly, we showed that the combination of AZT with colistin significantly reduced the bacterial burden *in vivo*. This early groundwork lays the foundation for further validation in clinical trials enabling translation of the combination therapy into clinical benefits for patients.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used were 74 antibiotic-resistant *Enterobacteriaceae* strains. These included seven strains harboring the bla_{NDM} plasmid, as follows: ATCC BAA-2468 (*E. cloacae*), ATCC BAA-2469 (*E. coli*), ATCC BAA-2470 (*K. pneumoniae*), ATCC BAA-2471 (*E. coli*), ATCC BAA-2472 (*K. pneumoniae*), ATCC BAA-2473 (*K. pneumoniae*), and NCTC13443 (*K. pneumoniae*). Also included were 13 colistin-resistant *E. coli* strains containing the mcr-1 plasmid (Table S1) (30–32, 43) and 54 antibiotic-resistant Gram-negative strains (23 *E. coli* and 31 *K. pneumoniae* strains) isolated in hospitals in Hong Kong, Taiwan, Thailand, South Korea, India, Singapore, Malaysia, and Philippines and from St. George's Hospital, London, United Kingdom. The bacterial isolates were grown in nutrient broth (Oxoid, UK), on tryptone soya agar plates (Fluka, UK), or on chrome agar orientation plates (BD, United Kingdom). AZT was obtained from Sigma-Aldrich, United Kingdom, in powder form.

Susceptibility tests of antibiotics and AZT. The MICs of antibiotics and AZT were determined using the broth microdilution method in accordance with guidelines of the Clinical and Laboratory Standards Institute (CLSI) (44). The MIC was determined using 96-well polystyrene microtiter plates (Fisher Scientific, United Kingdom). The antibiotics were diluted with 2-fold serial dilutions in triplicate, followed by addition of a standard bacterial suspension of 1×10^5 to 5×10^5 CFU/ml in cation-adjusted Mueller-Hinton broth (CA-MHB) (Sigma-Aldrich, United Kingdom). After 16 to 20 h of incubation at 37°C, the optical density (OD) readings were determined using an absorbance microplate reader (ELx800; BioTek). The lowest concentration of an antibiotic which produced an OD reading similar to that of the control (medium only) was determined as the MIC value. The MIC for each agent was identified as the lowest

concentration required to inhibit bacterial growth. The MIC_{50} and MIC_{90} values were calculated to investigate the lowest concentrations required to inhibit growth in 50% and 90% of the strains, respectively.

Detection of ESBLs in antibiotic-resistant Gram-negative isolates. Detection of the multidrug-resistant *Enterobacteriaceae* strains producing extended spectrum β-lactamases were performed according to the U.K. standard for microbiology investigations (45) using CHROMID ESBL (bioMérieux, United Kingdom) (46), a double-disc synergy test (DDST) (47), and a combination disc test (CDT) (45). Detection of ESBL-producing genes was performed by PCR using the primers listed in Table S2 in the supplemental material, followed by DNA sequencing of the PCR fragments (DNA Sequencing and Services, University of Dundee).

Checkerboard assays to determine effects of AZT in combination with antibiotics. Combinations of AZT and antibiotics were prepared using 96-well polystyrene microtiter plates with drug concentrations starting 2-fold higher than their respective MIC values and were then serially diluted in a 2-fold manner. The two drugs were mixed in a 96-well plate, followed by addition of a standard bacterial suspension at 1×10^5 to 5×10^5 CFU/ml in CA-MHB. After incubation for 16 to 20 h at 37°C, the OD values were read using an ELx800 absorbance microplate reader (BioTek). The combined effects were determined by calculating the fractional inhibitory concentration index (FICI) of the combination as follows: (MIC of drug A tested in combination)/(MIC of drug A tested alone) + (MIC of drug B tested in combination)/(MIC of drug B tested alone). Synergy was defined as an FICI of $\leq$0.5, no interaction was identified with an FICI of $>$0.5 but $<$4, and antagonism was defined as an FICI of $>$4 (48).

Time-kill analysis of antibiotics alone and in combination with AZT against log-phase bacteria. A range of different concentrations of colistin and AZT was chosen according to the checkerboard evaluation method as a synergistic combination. The drugs were prepared in 2-fold serial dilutions and added in combination or alone to log-phase bacterial culture suspensions containing 1×10^7 CFU/ml (49) in CA-MHB and incubated at 37°C. Viability expressed as the log CFU count/milliliter was determined at 0, 2, 4, 8, 24, and 48 h of incubation by plating out 100 μl of serial dilutions of the cultures onto tryptone soy agar (Oxoid) plates. The colonies on the agar plates were counted using an aCOLyte colony counter (Synbiosis) and analyzed with the counter's software. Synergistic activity was confirmed as a $\geq$ 2-$\log_{10}$ decrease in CFU counts at 24 h of the combination compared to counts with the antibiotic alone, in addition to a $\geq$ 2-$\log_{10}$ decrease compared to the count at 0 h (50).

Mouse peritoneal infection model. Female ICR mice (5 to 6 weeks old; body weight of 24 to 26 g) were used (Harlan UK Ltd.) for the mouse peritoneal infection model (51). The human medicines AZT (10 mg/ml) (Retrovir; ViiV Healthcare UK Ltd.) and colistin methanesulfonate (CMS) (Colimycin injection; Forrest) were used in the mouse study. Mice were infected intraperitoneally with 200 μl of bacterial suspension containing 10^7 CFU of the NDM-1-producing *K. pneumoniae* BAA-2472 strain and the mcr-1-containing *E. coli* strain Af40 (Table S1). After 30 min of infection, AZT (2, 5, or 10 mg/kg) and CMS (10, 20 or 30 mg/kg) singly or in combination were injected intravenously into the mice. A group of mice was treated with saline as a control group. At 30 min after infection (treatment start) and at 2 and 6 h after treatment, four mice in each group were sacrificed, and 1 ml of sterile phosphate-buffered saline (PBS) was injected intraperitoneally, followed by gentle massage of the abdomen. Peritoneal fluid was sampled aseptically. The fluid was diluted in a series of 10-fold dilutions, and 100 μl of each dilution was plated onto tryptone soy agar (Oxoid) plates. Viability was reported as log CFU count/milliliter of peritoneal fluid.

The animal husbandry guidelines and all animal experiments were performed according to the Animals Scientific Procedures Act of 1986 (Act of the Parliament of the United Kingdom 1986 c. 14) under Home Office Project license number 70/7077 with approval from St. George's, University of London ethics committee.

Statistical analysis. The significance of differences between experimental groups was determined by Student's *t* test. *P* values of $<$0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01630-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 2, PDF file, 1.6 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.01 MB.

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