Azidothymidine produces synergistic activity in combination with colistin against antibiotic-resistant Enterobacteriaceae

Yanmin Hu[1,2*], Yingjun Liu[1] and Anthony Coates[1,2]

[1]Institute for Infection and Immunity, St George's University of London, London. [2]Helperby Therapeutics Group plc, London, UK.

Running title: Azidothymidine combination with colistin

*Corresponding author. Tel: +44-2087255706; Fax: +44-2087250137. E-mail: ymhu@sgul.ac.uk

Abstract

Bacterial infections remain the leading killer worldwide which is worsened by the continuous emergence of antibiotic resistance. In particular, antibiotic-resistant Enterobacteriaceae is prevalent and extremely difficult to treat. Reusing existing drugs and rejuvenating 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 *in vitro* and *in vivo* efficacy of AZT in combination with colistin against antibiotic-resistant Enterobacteriaceae including extended-spectrum beta-lactamase (ESBL), New Delhi metallo-beta-lactamase 1 (NDM) or the mobilized colistin resistance (*mcr*-1) producing strains. Minimum inhibitory concentration was determined using the broth microdilution method. The combinatory 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. Fractional inhibitory concentration index from checkerboard assay demonstrated that AZT synergized with colistin against 61% and 87% of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*, respectively, 100% of NDM-1-producing strains and 92% of *mcr*-1 producing *E. coli*. Time-kill analysis demonstrated significant synergistic activities when AZT was combined with colistin. In the 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. AZT and colistin combination poses a potential to be used coherently to treat antibiotic-resistant Enterobacteriaceae infections.

Keywords: Enterobacteriaceae, azidothymidine, colistin, ESBL, NDM-1, *mcr*-1

INTRODUCTION

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 reuse 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 US 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 which 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 *K. pneumoniae* carbapenemases (KPC) - producing strains (24). A recent study also found that approximately 10% of NDM-1 producing CRE were colistin resistant in the UK (25) and plasmid-born 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 allows the administration of both drugs at lower doses to achieve a desired therapeutic effect while minimising the side effects and to prevent 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 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.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used were 74 antibiotic-resistant Enterobacteriaceae strains including 7 strains harboring the bla_{NDM} plasmid which were ATCC BAA-2468 (*Enterobacter cloacae*), ATCC BAA-2469 (*E. coli*), ATCC BAA-2470 (*K. pneumoniae*), ATCC BAA-2471 (*E. coli*), ATCC BAA-2472 (*K. pneumoniae*) and ATCC BAA-2473 (*K. pneumoniae*) and NCTC13443 (*K. pneumoniae*), 13 colistin resistant *E. coli* containing *mcr*-1 plasmid (Table S1) (29-32), 54 antibiotic-resistant Gram-negative strains (23 *E. coli* and 31 *K. pneumoniae*) isolated in the hospitals in Hong Kong, Taiwan, Thailand, Korea, India, Singapore, Malaysia, Philippines and St George's Hospital, London. The bacterial isolates were grown in nutrient broth (Oxoid, UK), on tryptone soya agar plates

(Fluka, UK) or on Chrome agar Orientation plates (BD, UK). AZT was obtained from Sigma-Aldrich, UK as powder form.

Susceptibility tests of antibiotics and AZT. The minimum inhibitory concentrations (MIC) of antibiotics and AZT were determined using the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (33). MIC was performed using 96-well polystyrene micro-titre plates (Fisher Scientific, UK). The antibiotics were diluted with two-fold serial dilutions in triplicate followed by addition of a standard bacterial suspension of 1-5 × 10^5 CFU/mL in cation adjusted Mueller Hinton Broth (CA-MHB, Sigma-Aldrich, UK). After 16 - 20 hours 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 a similar OD reading as the control (medium only) was determined as 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 the antibiotic-resistant Gram-negative isolates. Detection of the multidrug-resistant Enterobacteriaceae producing extended spectrum β-lactamases were performed according to the UK standard for microbiology investigations (34) using CHROMID ESBL (bioMérieux, UK) (35), double-disc synergy test (DDST) (36) and combination disc test (CDT) (34). Detection of ESBL genes were performed by polymerase chain reaction (PCR) using the primers (Table S2) followed by DNA sequencing of the PCR fragments (DNA Sequencing & Services, University of Dundee).

Checkerboard assays to determine combination effects of AZT with antibiotics. Combination of AZT and antibiotic was prepared using 96 well polystyrene micro-titre plates with drug concentrations starting two-fold higher than their MIC values, and were then

Antimicrobial Agents and Chemotherapy

serially diluted in a two-fold manner. The two drugs were mixed in a 96 well plate followed by addition of a standard bacterial suspension at 1-5 x 10^5 CFU/mL in CA-MHB. After incubation for 16 - 20 hours at 37°C, the OD values were read using the ELx800 absorbance microplate reader (BioTek). The combinatory 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 a FICI ≤0.5, no interaction was identified with an FICI >0.5 but <4 and antagonism if the FICI was >4 (37).

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 as a synergistic combination. The drugs was prepared in a two-fold serial dilution and was added in combination or alone to log phase bacterial cultures suspension containing 1 x 10^7 CFU/mL (38) in CA-MHB, and incubated at 37°C. Viability expressed as log CFU/mL was determined at 0, 2, 4, 8, 24 and 48 hours 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 analysed with the counter's software. Synergistic activity was confirmed as a≥2-$\log_{10}$ decrease in CFU counts at 24 hours of the combination compared to the antibiotic alone, in addition to a ≥2-$\log_{10}$ decrease compared to the zero hour count (39).

Mouse peritoneal infection model. Female ICR mice (five to six weeks old, body weight 24 - 26 g) were used (Harlan UK Ltd) for the mouse peritoneal infection model (40). Human medicines of AZT (Retrovir® 10 mg/ml, ViiV Healthcare UK Ltd) and colistin methanesulfonate (CMS) (Colomycin® injection, Forrest) were used in the mouse study.

Mice were infected intraperitoneally with two hundred microliter bacterial suspension containing 10^7 CFU of the NDM-1 *K. pneumoniae* BAA2472 and the *mcr*-1 *E. coli* strain Af40 (Table S1). After 30 minutes of infection, AZT (2, 5 or 10 mg/kg) and CMS (10, 20 or 30 mg/kg) singly or in combination was injected intravenously into the mice. A group of mice was treated with saline as a control group. At 30 minutes after infection (treatment starting), 2 and 6 hours after treatment, 4 mice in each group were sacrificed and 1 ml sterile PBS was injected intraperitoneally followed by gently massaging of the abdomen. Peritoneal fluid was sampled aseptically. The fluid was diluted in a serial of 10-fold dilutions and 100 µl of each dilution were plated onto tryptone soy agar (Oxoid) plates. Viability was defined as Log CFU/ml of peritoneal fluid.

The animal husbandry guidelines and all animal experiments were performed according to the Animals Scientific Procedures Act, 1986 (an Act of the Parliament of the United Kingdom 1986 c. 14) (Home Office Project licence 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 <0.05 were considered significant.

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, rifampicin, tigecycline, colistin, polymyxin B, and AZT were determined against the 7 NDM-1 strains. As seen in Table 1, compared with the antibiotic breakpoints (41) 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 (BAA-2471) and tigecycline (BAA-2469, BAA-2470 and BAA-2471).

However, all NDM-1 strains were susceptible to colistin (41). AZT MIC ranged from 2 to 4 mg/L.

The MICs for the antibiotics and AZT were also determined against the 54 antibiotic-resistant isolates, *E. coli* and *K. pneumoniae*. As shown in Table 2 and Table S3, these strains were resistant to monobactam, penicillins and cephalosporins but were susceptible to carbapenems. Resistance was seen for gentamicin, ciprofloxacin and trimethoprim. 90% of the strains were susceptible to tigecycline and colistin. The MIC for AZT ranged from 0.25 to 64 for *E. coli* and 2 to 32 for *K. pneumoniae*. The 54 multidrug-resistant *E. coli* and *K. pneumoniae* were tested for ESBL production using commercial ESBL-testing systems and demonstrated that these were ESBL producing strains (Table S3).

For colistin resistant strains, the MIC for AZT ranged from 8 to 64 with MIC50 at 8 mg/L and MIC90 at 64 mg/L. The range of MIC for colistin was 2 to 8 mg/L with MIC50 at 4 mg/L and MIC90 at 8 mg/L.

Checkerboard analysis of combination effects. The effects of combining AZT with colistin were determined using checkerboard assays against all the 74 strains. As shown in Table 3, the combination of AZT with colistin showed synergistic activity with FIC index ≤0.5 against 60.87% of the ESBL *E. coli*, 87.1% of the ESBL *K. pneumoniae,* 100% of NDM-1 strains and 92.31% of colistin resistant (*mcr*-1) *E. coli*. With the concentration of AZT range from 0.25 to 16 mg/L, the MICs of colistin were significantly reduced from 32 to 256-fold against the seven NDM-1 strains, 2 to 64-fold against ESBL *E. coli*, 2 to 512 fold against ESBL *K. pneumoniae* and 4 to 256 fold against *mcr*-1 containing *E. coli*.

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, 3 ESBL *E. coli* and 3 ESBL *K. pneumoniae* and 3 *mcr*-1 *E. coli* which showed an FICI <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 or MIC level for each of the two drugs. Data from representative strains are shown to display combinations with the synergistic activities. As shown in Figure 1 for the NDM-1 *K. pneumoniae* BAA2472, colistin at 2 mg/L was bactericidal until 7 hours followed by a regrowth and at 1 mg/L (MIC) inhibited bacterial growth. AZT at 4, 2 (MIC) and 1 mg/L was bactericidal showing dose-dependent kill and regrowth occurred after 8 hours of drug exposure. However when colistin at 2 mg/L combined with 4 and 2 mg/L of AZT, significant killing to the limit of detection of initial bacterial counts was achieved within 4 hours, and the same kill was seen at 8 hours when the same concentration of colistin combined with 1 mg/L of AZT (Figure 1A-1C). When colistin at 1 mg/L was combined with 4, 2 and 1 mg/L of AZT, kill at the level of limit of detection was achieved at 8 hours (Figure 1E-1F). No bacterial regrowth was observed in both 24 (Figure 1) and 48 hours of post-treatment (data not shown).

As shown in Figure 2, for the *mcr*-1 *E. coli* strain Af40 (Table S1), colistin at 8 mg/L (MIC) inhibited bacterial growth and at 4 mg/L showed the similar growth pattern as the control. AZT at 4, 2 and 1 mg/L reduced the initial counts till 4 hours and regrowth was seen. When colistin at 8 mg/L was combined with the concentrations of 4, 2 and 1 mg/L AZT, kill to the limit of detection was seen at 8 hours (Figure 2A – 2C). The same effects were seen when colistin at 4 mg/L was combined with 4 mg/L of AZT (Figure 2D). Reduced effects were seen when colistin at 4 mg/L with 4 and 2 mg/L of AZT and kill to the limit of detection was shown at 24 hours (Figure 2E and 2F).

Significant synergistic activity was also observed in other 6 NDM-1 strains (Figure S1-S6), 3 ESBL *E. coli* (Figure S7-S9) and 3 ESBL *K. pneumoniae* (Figure S10-S12) and two colistin resistant *mcr*-1 *E. coli* (Figure S13-S14).

***In vivo* combination activities of AZT combined with colistin.** The *in vivo* activity of AZT 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 dosages (5 mg/kg) was chosen which only inhibited bacterial growth but provide significant enhanced activities when combined with CMS. For CMS, we found that 10 to 30 mg/kg showed no activities against the infected bacteria. Therefore, for the colistin sensitive NDM-1 strain, we used 10 mg/kg of CMS and for the *mcr*-1 *E. coli*, we used 20 mg/kg of CMS. The drugs were tested singly or in combination against the NDM-1 *K. pneumoniae* BAA2472 and the *mcr*-1 *E. coli* strain Af40 (Table S4).

As shown in Figure 3A, for strain *K. pneumoniae* BAA2472, compared with the untreated control, colistin at 10 mg/kg showed no activities at both 2 and 6 hours and AZT at 5 mg/kg inhibited bacterial growth. Combination of colistin with AZT, although only showing inhibition at 2 hours, exhibited 2.72 log kill of the bacterium at 6 hours. The difference of the bacterial numbers between zero hour and 6 hours was significant ($P < 0.001$, n=4). For *E. coli* strain Af40 (Figure 3B), colistin at 20 mg/kg showed the same growth pattern as the control and AZT inhibited bacterial growth. Combination of colistin with AZT exhibited 1.32 and 2.96 log kill of the bacterium at 2 and 6 hours, respectively. The difference of the bacterial numbers between zero hour and 2 hours or 6 hours 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 hours after infection. The animals in other treatment groups showed no discomfort with normal and heathy behaviors. All animals were sacrificed at 6 hours after treatment according to the restriction of adverse effects in the project licence.

DISCUSSION

In this study, we demonstrated for the first time that AZT synergized with colistin against 74 antibiotic-resistant Enterobacteriaceae including NDM-1, ESBL 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 (29-31). The 7 NDM-1 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 human immunodeficiency virus (HIV) disease (43, 44). In patients, the oral dosage is 250 – 300 mg twice daily and intravenous infusion is 0.8 – 1 mg/kg every 4 hours for up to 2 weeks. It has been shown that 120 mg iv dosing produced an AUC of 0.0014 mg.h/L and a Cmax of 0.0015 mg/L while 200 mg oral dosing gave rise to an AUC of 0.0017 mg.h/L and a Cmax of 0.0018 mg/L (45). AZT has been shown to be active against Gram-negative bacteria (18-20), it is not known if the concentrations used clinically are sufficient to treat bacterial infections in humans.

Colistin is effective against multidrug-resistant but colistin susceptible *Pseudomonas aeruginosa*, *K. pneumoniae, Acinetobacter* (46) and importantly NDM-1 carrying Enterobacteriaceae (8). There is increasing evidence to show that colistin resistance is on the rise, especially the discovery of plasmid born colistin resistance worldwide (26, 47, 48). It is critically important to preserve and prolong the life of the last resort of antibiotic by enhanced combination therapy. Here we have shown that in combination with AZT, 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 *K. pneumoniae* BAA2472 or *mcr*-1 *E. coli* Af40 strains required much higher concentrations of the drug (data not shown), however, more than 4 to 16-fold lower concentrations of colistin when combined with AZT achieved the same effect. This is significant as enhancement of colistin

combination with AZT will likely reduce the dose of colistin but retain maximal therapeutic efficacy hence minimize its toxic profile. These data suggest that further clinical development of a colistin plus AZT combination 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, 49). Previous studies have shown that in bacteria AZT needs to be converted to the nucleotide to inhibit bacterial DNA replication (50) and that bacterial thymidine kinase is responsible for the initiation of the activation process - phosphorylation of AZT (23, 50). Other antibiotics such as ciprofloxacin also inhibit DNA replication by blocking GyrA. But comparison of the resistance profiles of ciprofloxacin and AZT are very different (see Table 3). This suggests that AZT has a different mechanism of action to other antibacterial agents which are in 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 underway 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 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 the NDM-1 *K. pneumoniae* BAA2472 and the *mcr*-1 *E. coli* strain Af40 growth in the mouse peritoneal infection. However, the combination of AZT with colistin improved the therapeutic activities of each single agent with significant kill of the bacteria at 2 or 6 hours in mouse peritoneal cavity. Most importantly, when colistin methanesulfonate was completely ineffective up to 6 hours 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 (51). Colistin methanesulfonate is a prodrug which needs to convert to the active form of colistin (52). The conversion normally delays the activity of the drug (52). 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 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 AZT-plus-colistin combination therapy against antibiotic-resistant Enterobacteriaceae, including *mcr*-1, NDM-1 and ESBL strains. ESBL strains were confirmed using commercially-accepted phenotypical methods currently using in clinical practice. The interaction between the genotypic characteristics of ESBL 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.

ACKNOWLEDGMENTS

We are grateful for financial support from Helperby Therapeutics Group Ltd. We thank Alexander Liu for critical discussion. We would like to thank Professor Jae-Hoon Song and Professor So Hyun Kim from Asian Network for Surveillance of Resistant Pathogens and Asia Pacific Foundation for Infectious Diseases for kindly providing the *E. coli* and *K. pneumoniae* strains. We are also grateful for kindly providing of the *mcr*-1 *E. coli* strains by Professor Patrice Nordmann from University of Fribourg. We would like to thank Selina Le for technical help.

REFERENCE

1. **Toone EJ.** 2011. Bacterial infection remains a leading cause of death in both Western and developing world. Preface. Advances in enzymology and related areas of molecular biology **77:**xi-xiii.
2. **Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, Vlieghe E, Hara GL, Gould IM, Goossens H, Greko C, So AD, Bigdeli M, Tomson G, Woodhouse W, Ombaka E, Peralta AQ, Qamar FN, Mir F, Kariuki S, Bhutta ZA, Coates A, Bergstrom R, Wright GD, Brown ED, Cars O.** 2013. Antibiotic resistance-the need for global solutions. The Lancet infectious diseases **13:**1057-1098.
3. **Peleg AY, Hooper DC.** 2010. Hospital-acquired infections due to gram-negative bacteria. N Engl J Med **362:**1804-1813.
4. **Zhu J, Sun L, Ding B, Yang Y, Xu X, Liu W, Zhu D, Yang F, Zhang H, Hu F.** 2016. Outbreak of NDM-1-producing *Klebsiella pneumoniae* ST76 and ST37 isolates in neonates. Eur J Clin Microbiol Infect Dis **35:**611-618.
5. **Ahmed-Bentley J, Chandran AU, Joffe AM, French D, Peirano G, Pitout JD.** 2013. Gram-negative bacteria that produce carbapenemases causing death attributed to recent foreign hospitalization. Antimicrobial agents and chemotherapy **57:**3085-3091.
6. **Bogaerts P, Bouchahrouf W, de Castro RR, Deplano A, Berhin C, Pierard D, Denis O, Glupczynski Y.** 2011. Emergence of NDM-1-producing Enterobacteriaceae in Belgium. Antimicrobial agents and chemotherapy **55:**3036-3038.
7. **Shaheen BW, Nayak R, Boothe DM.** 2013. Emergence of a New Delhi metallo-beta-lactamase (NDM-1)-encoding gene in clinical *Escherichia coli* isolates recovered from companion animals in the United States. Antimicrobial agents and chemotherapy **57:**2902-2903.
8. **Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N.** 2010. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. The Lancet infectious diseases **10:**597-602.
9. **Livermore DM, Warner M, Mushtaq S, Doumith M, Zhang J, Woodford N.** 2011. What remains against carbapenem-resistant Enterobacteriaceae? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline, nitrofurantoin, temocillin and tigecycline. International journal of antimicrobial agents **37:**415-419.
10. **Paterson DL.** 2006. Resistance in gram-negative bacteria: Enterobacteriaceae. Am J Infect Control **34:**S20-28; discussion S64-73.
11. **Coates A, Hu Y, Bax R, Page C.** 2002. The future challenges facing the development of new antimicrobial drugs. Nature reviews. Drug discovery **1:**895-910.
12. **Coates AR, Halls G, Hu Y.** 2011. Novel classes of antibiotics or more of the same? British journal of pharmacology **163:**184-194.
13. **Coates AR, Hu Y.** 2006. New strategies for antibacterial drug design: targeting non-multiplying latent bacteria. Drugs in R&D **7:**133-151.
14. **Coates AR, Hu Y.** 2007. Novel approaches to developing new antibiotics for bacterial infections. British journal of pharmacology **152:**1147-1154.
15. **Kalan L, Wright GD.** 2011. Antibiotic adjuvants: multicomponent anti-infective strategies. Expert Rev Mol Med **13:**e5.

16. **Aligholi M, Emaneini M, Taherikalani M, Shahsavan S, Jabalameli F, Asadollahi P, Khoramian B, Eslampour MA.** 2011. Time-kill study and synergistic activity of cell-wall inhibitor antibiotics in combination with gentamicin against *Enterococcus faecalis* and Enterococcus faecium. Acta Microbiol Immunol Hung **58:**219-226.
17. **Ezzell C.** 1987. AZT given the green light for clinical treatment of AIDS. Nature **326:**430.
18. **Herrmann JL, Lagrange PH.** 1992. Intracellular activity of zidovudine (3'-azido-3'-deoxythymidine, AZT) against *Salmonella typhimurium* in the macrophage cell line J774-2. Antimicrobial agents and chemotherapy **36:**1081-1085.
19. **Keith BR, White G, Wilson HR.** 1989. In vivo efficacy of zidovudine (3'-azido-3'-deoxythymidine) in experimental gram-negative-bacterial infections. Antimicrobial agents and chemotherapy **33:**479-483.
20. **Monno R, Marcuccio L, Valenza MA, Leone E, Bitetto C, Larocca A, Maggi P, Quarto M.** 1997. In vitro antimicrobial properties of azidothymidine (AZT). Acta Microbiol Immunol Hung **44:**165-171.
21. **Smith KP, Kirby JE.** 2016. Validation of a High-Throughput Screening Assay for Identification of Adjunctive and Directly Acting Antimicrobials Targeting Carbapenem-Resistant Enterobacteriaceae. Assay and drug development technologies **14:**194-206.
22. **Peyclit L, Baron SA, Yousfi H, Rolain JM.** 2018. Zidovudine: A salvage therapy for mcr-1 plasmid-mediated colistin-resistant bacterial infections? International journal of antimicrobial agents **52:**11-13.
23. **Lewin CS, Allen RA, Amyes SG.** 1990. Mechanisms of zidovudine resistance in bacteria. J Med Microbiol **33:**235-238.
24. **Capone A, Giannella M, Fortini D, Giordano A, Meledandri M, Ballardini M, Venditti M, Bordi E, Capozzi D, Balice MP, Tarasi A, Parisi G, Lappa A, Carattoli A, Petrosillo N, network S-G.** 2013. High rate of colistin resistance among patients with carbapenem-resistant *Klebsiella pneumoniae* infection accounts for an excess of mortality. Clin Microbiol Infect **19:**E23-30.
25. **Jain A, Hopkins KL, Turton J, Doumith M, Hill R, Loy R, Meunier D, Pike R, Livermore DM, Woodford N.** 2014. NDM carbapenemases in the United Kingdom: an analysis of the first 250 cases. The Journal of antimicrobial chemotherapy **69:**1777-1784.
26. **Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J.** 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. The Lancet infectious diseases **16:**161-168.
27. **Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, Paterson DL.** 2006. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. The Lancet infectious diseases **6:**589-601.
28. **Hu Y, Liu A, Vaudrey J, Vaiciunaite B, Moigboi C, McTavish SM, Kearns A, Coates A.** 2015. Combinations of beta-Lactam or Aminoglycoside Antibiotics with Plectasin Are Synergistic against Methicillin-Sensitive and Methicillin-Resistant *Staphylococcus aureus*. PLoS ONE **10:**e0117664.
29. **Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P.** 2016. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of Escherichia coli. The Lancet infectious diseases **16:**281.
30. **Nordmann P, Lienhard R, Kieffer N, Clerc O, Poirel L.** 2016. Plasmid-Mediated Colistin-Resistant *Escherichia coli* in Bacteremia in Switzerland. Clin Infect Dis **62:**1322-1323.
31. **Poirel L, Kieffer N, Brink A, Coetze J, Jayol A, Nordmann P.** 2016. Genetic Features of MCR-1-Producing Colistin-Resistant *Escherichia coli* Isolates in South Africa. Antimicrobial agents and chemotherapy **60:**4394-4397.

32. **Kieffer N, Aires-de-Sousa M, Nordmann P, Poirel L.** 2017. High Rate of MCR-1-Producing *Escherichia coli* and *Klebsiella pneumoniae* among Pigs, Portugal. Emerging infectious diseases **23:**2023-2029.
33. **The Clinical & Laboratory Standards Institute.** 2015. Methods for dilution antimicrobial susceptibility testing for bacteria that grew aerobically: approved standard: Tenth edition. CLSI document M07-A10. Wayne, PA, USA: CLSI,.
34. **England PH.** 2016. Detection of Enterobacteriaceae producing extended spectrum β-lactamase, UK Standards for Microbiology Investigations. The Standards Unit, Microbiology Services, PHE.
35. **Reglier-Poupet H, Naas T, Carrer A, Cady A, Adam JM, Fortineau N, Poyart C, Nordmann P.** 2008. Performance of chromID ESBL, a chromogenic medium for detection of Enterobacteriaceae producing extended-spectrum beta-lactamases. J Med Microbiol **57:**310-315.
36. **The Clinical & Laboratory Standards Institute.** 2015. Performance standards for antimicrobial susceptibility testing; Twenty-Fifth Informational Supplement. CLSI document M100-S25. Wayne, PA, USA: CLSI.
37. **Odds FC.** 2003. Synergy, antagonism, and what the chequerboard puts between them. The Journal of antimicrobial chemotherapy **52:**1.
38. **Soren O, Brinch KS, Patel D, Liu Y, Liu A, Coates A, Hu Y.** 2015. Antimicrobial Peptide Novicidin Synergizes with Rifampin, Ceftriaxone, and Ceftazidime against Antibiotic-Resistant Enterobacteriaceae In Vitro. Antimicrobial agents and chemotherapy **59:**6233-6240.
39. **White RL, Burgess DS, Manduru M, Bosso JA.** 1996. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrobial agents and chemotherapy **40:**1914-1918.
40. **Mygind PH, Fischer RL, Schnorr KM, Hansen MT, Sonksen CP, Ludvigsen S, Raventos D, Buskov S, Christensen B, De Maria L, Taboureau O, Yaver D, Elvig-Jorgensen SG, Sorensen MV, Christensen BE, Kjaerulff S, Frimodt-Moller N, Lehrer RI, Zasloff M, Kristensen HH.** 2005. Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature **437:**975-980.
41. **European Committee on Antimicrobial Susceptibility Testing.** 2018. Breakpoint tables for interpretation of MICs and zone diameters, version 1.3. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_8.1_Breakpoint_Tables.pdf.
42. **Wiegand I, Geiss HK, Mack D, Sturenburg E, Seifert H.** 2007. Detection of extended-spectrum beta-lactamases among Enterobacteriaceae by use of semiautomated microbiology systems and manual detection procedures. Journal of clinical microbiology **45:**1167-1174.
43. **Cooper DA, Gatell JM, Kroon S, Clumeck N, Millard J, Goebel FD, Bruun JN, Stingl G, Melville RL, Gonzalez-Lahoz J, et al.** 1993. Zidovudine in persons with asymptomatic HIV infection and CD4+ cell counts greater than 400 per cubic millimeter. The European-Australian Collaborative Group. N Engl J Med **329:**297-303.
44. **Cooper DA, Gatell JM, Kroon S, Clumeck N, Millard J, Goebel FD, Bruun JN, Stingl G, Melville RL, Gonzalez-Lahoz J, Stevens JW, Fiddian P, the European-Australian Collaborative Group.** 1987. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. N Engl J Med **317:**185-191.
45. **Blum MR, Liao SH, Good SS, de Miranda P.** 1988. Pharmacokinetics and bioavailability of zidovudine in humans. Am J Med **85:**189-194.

46. **Falagas ME, Grammatikos AP, Michalopoulos A.** 2008. Potential of old-generation antibiotics to address current need for new antibiotics. Expert review of anti-infective therapy **6:**593-600.
47. **Zurfuh K, Poirel L, Nordmann P, Nuesch-Inderbinen M, Hachler H, Stephan R.** 2016. Occurrence of the Plasmid-Borne mcr-1 Colistin Resistance Gene in Extended-Spectrum-beta-Lactamase-Producing Enterobacteriaceae in River Water and Imported Vegetable Samples in Switzerland. Antimicrobial agents and chemotherapy **60:**2594-2595.
48. **MacVane SH, Crandon JL, Nichols WW, Nicolau DP.** 2014. Unexpected in vivo activity of ceftazidime alone and in combination with avibactam against New Delhi metallo-beta-lactamase-producing Enterobacteriaceae in a murine thigh infection model. Antimicrobial agents and chemotherapy **58:**7007-7009.
49. **Stone NR, Woodford N, Livermore DM, Howard J, Pike R, Mushtaq S, Perry C, Hopkins S.** 2011. Breakthrough bacteraemia due to tigecycline-resistant Escherichia coli with New Delhi metallo-beta-lactamase (NDM)-1 successfully treated with colistin in a patient with calciphylaxis. The Journal of antimicrobial chemotherapy **66:**2677-2678.
50. **Elwell LP, Ferone R, Freeman GA, Fyfe JA, Hill JA, Ray PH, Richards CA, Singer SC, Knick VB, Rideout JL, Zimmerman TP.** 1987. Antibacterial activity and mechanism of action of 3'-azido-3'-deoxythymidine (BW A509U). Antimicrobial agents and chemotherapy **31:**274-280.
51. **Chiang SR, Chuang YC, Tang HJ, Chen CC, Chen CH, Lee NY, Chou CH, Ko WC.** 2009. Intratracheal colistin sulfate for BALB/c mice with early pneumonia caused by carbapenem-resistant Acinetobacter baumannii. Crit Care Med **37:**2590-2595.
52. **Bergen PJ, Li J, Rayner CR, Nation RL.** 2006. Colistin methanesulfonate is an inactive prodrug of colistin against Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy **50:**1953-1958.

Figure legends

Figure 1. Time Kill analysis showing the effects of AZT in combination with colistin against NDM-1 *K. pneumoniae* BAA2472. AZT and colistin alone or in combination were added to the log phase cultures and CFU counts were carried out at different time points. Combination concentrations of AZT and colistin are colistin 2 mg/L + AZT 4 mg/L (A), colistin 2 mg/L + AZT 2 mg/L (B), colistin 2 mg/L + AZT 1 mg/L (C), colistin 1 mg/L + AZT 4 mg/L (D), colistin 1 mg/L + AZT 2 mg/L (E) and colistin 1 mg/L + AZT 1 mg/L (F). The dash line is the limit of detection in the assay (30 CFU/ml).

Figure 2. Time Kill analysis showing the effects of AZT in combination with colistin against *mcr*-1 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.

Combination concentrations of AZT and colistin are colistin 8 mg/L + AZT 4 mg/L (A), colistin 8 mg/L + AZT 2 mg/L (B), colistin 8 mg/L + AZT 1 mg/L (C), colistin 4 mg/L + AZT 4 mg/L (D), colistin 4 mg/L + AZT 2 mg/L (E) and colistin 4 mg/L + AZT 1 mg/L (F). The dash line is the limit of detection in the assay (20 CFU/ml).

Figure 3. Effects of AZT in combination with colistin against the NDM-1 *K. pneumoniae* BAA2472 and the *mrc*-1 *E. coli* strain Af40 in a mouse peritoneal infection model. A. Mice were infected with strain BAA2472. Treatment was initiated 30 minutes after infection with AZT (5 mg/kg), CMS (10 mg/kg) and AZT plus CMS. B. Mice were infected with strain Af40. Treatment was initiated 30 minutes after infection 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 hour before and 2 and 6 hours post-treatment. The data has been repeated once. ** indicates p≤0.01. *** indicates p≤0.001.

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

	MIC (mg/L)						
Antibiotics	NCTC13443 *K. pneumoniae*	BAA – 2468 *E. cloacae*	BAA – 2469 *E. coli*	BAA – 2470 *K. pneumoniae*	BAA – 2471 *E. coli*	BAA – 2472 *K. pneumoniae*	BAA – 2473 *K. pneumoniae*
Cefotaxime	>2048	512	512	>2048	>2048	>2048	2048
Ceftazidime	>2048	512	>2048	>2048	>2048	>2048	512
Ceftriaxone	>4096	4096	2048	>4096	>4096	>4096	>4096
Aztreonam	>2048	1024	32	512	>2048	2048	1024
Piperacillin	>2048	256	1024	1024	>2048	>2048	1024
Meropenem	128	64	32	128	128	128	16
Gentamicin	>256	>256	>256	>256	>256	>256	>256
Amikacin	8	>256	>256	>256	16	>256	8
Tobramycin	32	>1024	1024	128	16	>1024	16
Ciprofloxacin	>64	64	64	8	64	32	>64
Levofloxacin	32	32	16	4	16	32	32
Trimethoprim	>256	>256	>256	>256	>256	>256	>256
Nitrofuratoin	256	>256	32	>256	64	256	256
Tigecycline	1	4	0.5	1	0.5	4	1
Rifampicin	1024	16	4	256	16	1024	1024
Colistin	0.25	0.5	0.125	0.5	0.125	1	0.25
AZT	4	2	2	2	4	2	2

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

	E. coli (23)			*K. pneumoniae* (31)			*mcr*-1 *E. coli* (13)		
	MIC range (mg/L)	MIC50	MIC90	MIC range (mg/L)	MIC50	MIC90	MIC range (mg/L)	MIC50	MIC90
Aztreonam	1 - 256	128	256	32 - 1024	128	256	-	-	-
Amoxicillin	128 - 2048	256	2048	256 - 1024	512	1024	-	-	-
Piperacillin	1 - 512	16	256	16 - 1024	512	1024	-	-	-
Cefotaxime	64 - 2048	512	1024	32 - 1024	512	1024	-	-	-
Ceftazidime	8 - 512	256	512	32 - 1024	128	1024	-	-	-
Ceftriaxone	128 - 1024	512	1024	64 - 1024	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

-, not tested

Table 3. Combination activities of AZT with colistin

			Total numbers (%) of strains
Strains	Combination activity	FICI	AZT + colistin
ESBL *E. coli*	synergy	≤ 0.5	14 (60.87%)
	no interaction	0.56 -1	9 (39.13%)
	antagonism	>4	0
ESBL *K. pneumoniae*	synergy	≤ 0.5	27 (87.10%)
	no interaction	0.56 -1	4 (12.90%)
	antagonism	>4	0
NDM-1 Strains	synergy	≤ 0.5	7 (100%)
	no interaction	0.56 -1	0
	antagonism	>4	0
mcr-1 *E. coli*	synergy	≤ 0.5	12 (92.31%)
	no interaction	0.56 -1	1 (7.69%)
	antagonism	>4	0