

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

3 Yanmin Hu^{1,2*}, Yingjun Liu¹ and Anthony Coates^{1,2}

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

6 Running title: Azidothymidine combination with colistin

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24 *Corresponding author. Tel: +44-2087255706; Fax: +44-2087250137. E-mail:

25 ymhu@sgul.ac.uk

26 Abstract

27 Bacterial infections remain the leading killer worldwide which is worsened by the continuous
28 emergence of antibiotic resistance. In particular, antibiotic-resistant Enterobacteriaceae is
29 prevalent and extremely difficult to treat. Reusing existing drugs and rejuvenating the
30 therapeutic potential of existing antibiotics represent an attractive novel strategy.
31 Azidothymidine (AZT) is an antiretroviral drug which is used in combination with other
32 antivirals to prevent and to treat HIV/AIDS. AZT is also active against Gram-negative
33 bacteria but has not been developed for that purpose. Here we investigated *in vitro* and *in*
34 *vivo* efficacy of AZT in combination with colistin against antibiotic-resistant
35 Enterobacteriaceae including extended-spectrum beta-lactamase (ESBL), New Delhi
36 metallo-beta-lactamase 1 (NDM) or the mobilized colistin resistance (*mcr-1*) producing
37 strains. Minimum inhibitory concentration was determined using the broth microdilution
38 method. The combinatory effect of AZT and colistin was examined using the checkerboard
39 method and time-kill analysis. A murine peritoneal infection model was used to test the
40 therapeutic effect of the combination of AZT and colistin. Fractional inhibitory concentration
41 index from checkerboard assay demonstrated that AZT synergized with colistin against
42 61% and 87% of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*, respectively,
43 100% of NDM-1-producing strains and 92% of *mcr-1* producing *E. coli*. Time-kill analysis
44 demonstrated significant synergistic activities when AZT was combined with colistin. In the
45 murine peritoneal infection model, AZT in combination with colistin showed augmented
46 activities of both drugs in the treatment of NDM-1 *K. pneumoniae* and *mcr-1 E. coli*
47 infections. AZT and colistin combination poses a potential to be used coherently to treat
48 antibiotic-resistant Enterobacteriaceae infections.

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

50

51 INTRODUCTION

52 Bacterial infection remains a leading killer worldwide (1) and antibiotic resistance continues
53 to plague the effective control of this pandemic health problem (2, 3). In particular, there is
54 an urgent global threat with an increasing prevalence of multidrug-resistant
55 Enterobacteriaceae, especially carbapenem-resistant Enterobacteriaceae (CRE) such as
56 New Delhi Metallo-beta-lactamase-1 (NMD) carriers (4-8) which are extremely resistant to
57 almost all of our antibiotics (3, 9). As a result, our ability to treat serious community and
58 nosocomial acquired infections is rapidly diminishing (10). Unfortunately, the number of new
59 antibiotics reaching the market annually is unable to keep up with the development of
60 bacterial antibiotic resistance (11-14). The drug discovery process itself is arduous and
61 costly and it is almost impossible to produce a large group of effective antibiotics within a
62 short period of time to combat antibiotic resistance. Therefore, a different therapeutic
63 approach is needed to replenish our antibiotic reservoir against resistant bacteria and the
64 most promising of such strategies is to reuse existing drugs and to restore the therapeutic
65 potencies of existing antibiotics (15, 16).

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

74 The rapid emergence of CRE which are often resistant to many other antibiotics, has left
75 the world with colistin as the last resort treatment option. The use of colistin has led to high

76 rates of colistin resistance in patients with infections due to *K. pneumoniae*
77 carbapenemases (KPC) - producing strains (24). A recent study also found that
78 approximately 10% of NDM-1 producing CRE were colistin resistant in the UK (25) and
79 plasmid-born colistin resistance was also found recently in animals and humans (26).
80 Hence it is crucial to boost the effectiveness of colistin against colistin resistant bacteria.
81 However, treatment with colistin has been associated with both nephron- and neurotoxic
82 adverse effects (27). It is not known if AZT can synergistically act with colistin to treat
83 multidrug-resistant Enterobacteriaceae infections which allows the administration of both
84 drugs at lower doses to achieve a desired therapeutic effect while minimising the side
85 effects and to prevent emergence of antibiotic resistance (15, 28).

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

90 MATERIALS AND METHODS

91 **Bacterial strains and growth conditions.** The bacterial strains used were 74 antibiotic-
92 resistant Enterobacteriaceae strains including 7 strains harboring the bla_{NDM} plasmid which
93 were ATCC BAA-2468 (*Enterobacter cloacae*), ATCC BAA-2469 (*E. coli*), ATCC BAA-2470
94 (*K. pneumoniae*), ATCC BAA-2471 (*E. coli*), ATCC BAA-2472 (*K. pneumoniae*) and ATCC
95 BAA-2473 (*K. pneumoniae*) and NCTC13443 (*K. pneumoniae*), 13 colistin resistant *E. coli*
96 containing *mcr-1* plasmid (Table S1) (29-32), 54 antibiotic-resistant Gram-negative strains
97 (23 *E. coli* and 31 *K. pneumoniae*) isolated in the hospitals in Hong Kong, Taiwan, Thailand,
98 Korea, India, Singapore, Malaysia, Philippines and St George's Hospital, London. The
99 bacterial isolates were grown in nutrient broth (Oxoid, UK), on tryptone soya agar plates

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

102 **Susceptibility tests of antibiotics and AZT.** The minimum inhibitory concentrations (MIC)
103 of antibiotics and AZT were determined using the broth microdilution method in accordance
104 with the Clinical and Laboratory Standards Institute (CLSI) guidelines (33). MIC was
105 performed using 96-well polystyrene micro-titre plates (Fisher Scientific, UK). The
106 antibiotics were diluted with two-fold serial dilutions in triplicate followed by addition of a
107 standard bacterial suspension of $1-5 \times 10^5$ CFU/mL in cation adjusted Mueller Hinton Broth
108 (CA-MHB, Sigma-Aldrich, UK). After 16 - 20 hours of incubation at 37°C, the optical density
109 (OD) readings were determined using an absorbance microplate reader (ELx800, BioTek).
110 The lowest concentration of an antibiotic which produced a similar OD reading as the
111 control (medium only) was determined as MIC value. The MIC for each agent was identified
112 as the lowest concentration required to inhibit bacterial growth. The MIC₅₀ and MIC₉₀ values
113 were calculated to investigate the lowest concentrations required to inhibit growth in 50%
114 and 90% of the strains, respectively.

115 **Detection of ESBLs in the antibiotic-resistant Gram-negative isolates.** Detection of the
116 multidrug-resistant Enterobacteriaceae producing extended spectrum β -lactamases were
117 performed according to the UK standard for microbiology investigations (34) using
118 CHROMID ESBL (bioMérieux, UK) (35), double-disc synergy test (DDST) (36) and
119 combination disc test (CDT) (34). Detection of ESBL genes were performed by polymerase
120 chain reaction (PCR) using the primers (Table S2) followed by DNA sequencing of the PCR
121 fragments (DNA Sequencing & Services, University of Dundee).

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

125 serially diluted in a two-fold manner. The two drugs were mixed in a 96 well plate followed
126 by addition of a standard bacterial suspension at $1-5 \times 10^5$ CFU/mL in CA-MHB. After
127 incubation for 16 - 20 hours at 37°C, the OD values were read using the ELx800
128 absorbance microplate reader (BioTek). The combinatory effects were determined by
129 calculating the fractional inhibitory concentration index (FICI) of the combination as follows:
130 (MIC of Drug A, tested in combination) / (MIC of Drug A, tested alone) + (MIC of Drug B,
131 tested in combination) / (MIC of Drug B, tested alone). Synergy was defined as a FICI ≤ 0.5 ,
132 no interaction was identified with an FICI >0.5 but <4 and antagonism if the FICI was >4
133 (37).

134 **Time-Kill analysis of antibiotics alone and in combination with AZT against log-phase**

135 **bacteria.** A range of different concentrations of colistin and AZT was chosen according to
136 the checkerboard evaluation as a synergistic combination. The drugs was prepared in a
137 two-fold serial dilution and was added in combination or alone to log phase bacterial
138 cultures suspension containing 1×10^7 CFU/mL (38) in CA-MHB, and incubated at 37°C.
139 Viability expressed as log CFU/mL was determined at 0, 2, 4, 8, 24 and 48 hours of
140 incubation by plating out 100 μ L of serial dilutions of the cultures onto tryptone soy agar
141 (Oxoid) plates. The colonies on the agar plates were counted using an aCOLyte colony
142 counter (Synbiosis) and analysed with the counter's software. Synergistic activity was
143 confirmed as ≥ 2 -log₁₀ decrease in CFU counts at 24 hours of the combination compared
144 to the antibiotic alone, in addition to a ≥ 2 -log₁₀ decrease compared to the zero hour count
145 (39).

146 **Mouse peritoneal infection model.** Female ICR mice (five to six weeks old, body weight
147 24 - 26 g) were used (Harlan UK Ltd) for the mouse peritoneal infection model (40). Human
148 medicines of AZT (Retrovir[®] 10 mg/ml, ViiV Healthcare UK Ltd) and colistin
149 methanesulfonate (CMS) (Colomycin[®] injection, Forrest) were used in the mouse study.

150 Mice were infected intraperitoneally with two hundred microliter bacterial suspension
151 containing 10^7 CFU of the NDM-1 *K. pneumoniae* BAA2472 and the *mcr-1 E. coli* strain
152 Af40 (Table S1). After 30 minutes of infection, AZT (2, 5 or 10 mg/kg) and CMS (10, 20 or
153 30 mg/kg) singly or in combination was injected intravenously into the mice. A group of
154 mice was treated with saline as a control group. At 30 minutes after infection (treatment
155 starting), 2 and 6 hours after treatment, 4 mice in each group were sacrificed and 1 ml
156 sterile PBS was injected intraperitoneally followed by gently massaging of the abdomen.
157 Peritoneal fluid was sampled aseptically. The fluid was diluted in a serial of 10-fold dilutions
158 and 100 μ l of each dilution were plated onto tryptone soy agar (Oxoid) plates. Viability was
159 defined as Log CFU/ml of peritoneal fluid.

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

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

166 RESULTS

167 ***In vitro* susceptibility of AZT and colistin against 74 antibiotic-resistant**
168 **Enterobacteriaceae.** The MICs for aztreonam, amoxicillin, piperacillin, cefotaxime,
169 ceftriaxone, ceftazidime, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin,
170 levofloxacin, trimethoprim, nitrofurantoin, rifampicin, tigecycline, colistin, polymyxin B, and
171 AZT were determined against the 7 NDM-1 strains. As seen in Table 1, compared with the
172 antibiotic breakpoints (41) resistance was found in all strains for nearly all antibiotics. Only
173 certain strains were susceptible to a number of antibiotics such as nitrofurantoin (BAA-
174 2469), amikacin (BAA-2471) and tigecycline (BAA-2469, BAA-2470 and BAA-2471).

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

177 The MICs for the antibiotics and AZT were also determined against the 54 antibiotic-
178 resistant isolates, *E. coli* and *K. pneumoniae*. As shown in Table 2 and Table S3, these
179 strains were resistant to monobactam, penicillins and cephalosporins but were susceptible
180 to carbapenems. Resistance was seen for gentamicin, ciprofloxacin and trimethoprim. 90%
181 of the strains were susceptible to tigecycline and colistin. The MIC for AZT ranged from
182 0.25 to 64 for *E. coli* and 2 to 32 for *K. pneumoniae*. The 54 multidrug-resistant *E. coli* and
183 *K. pneumoniae* were tested for ESBL production using commercial ESBL-testing systems
184 and demonstrated that these were ESBL producing strains (Table S3).

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

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

196 **Time kill analysis of AZT in combination with colistin against log-phase bacteria.** The
197 synergistic combination of AZT and colistin was performed using time kill assays against 7
198 NDM-1, 3 ESBL *E. coli* and 3 ESBL *K. pneumoniae* and 3 *mcr-1 E. coli* which showed an
199 FICI < 0.5 for the combination. The characteristics of the 16 strains are shown in Table S4.

200 A range of different concentrations was used starting from 2 fold or MIC level for each of
201 the two drugs. Data from representative strains are shown to display combinations with the
202 synergistic activities. As shown in Figure 1 for the NDM-1 *K. pneumoniae* BAA2472, colistin
203 at 2 mg/L was bactericidal until 7 hours followed by a regrowth and at 1 mg/L (MIC)
204 inhibited bacterial growth. AZT at 4, 2 (MIC) and 1 mg/L was bactericidal showing dose-
205 dependent kill and regrowth occurred after 8 hours of drug exposure. However when
206 colistin at 2 mg/L combined with 4 and 2 mg/L of AZT, significant killing to the limit of
207 detection of initial bacterial counts was achieved within 4 hours, and the same kill was seen
208 at 8 hours when the same concentration of colistin combined with 1 mg/L of AZT (Figure
209 1A-1C). When colistin at 1 mg/L was combined with 4, 2 and 1 mg/L of AZT, kill at the level
210 of limit of detection was achieved at 8 hours (Figure 1E-1F). No bacterial regrowth was
211 observed in both 24 (Figure 1) and 48 hours of post-treatment (data not shown).

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

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

223 ***In vivo* combination activities of AZT combined with colistin.** The *in vivo* activity of AZT
224 combination with colistin was studied using a murine peritoneal infection model. A dose

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

232 As shown in Figure 3A, for strain *K. pneumoniae* BAA2472, compared with the untreated
233 control, colistin at 10 mg/kg showed no activities at both 2 and 6 hours and AZT at 5 mg/kg
234 inhibited bacterial growth. Combination of colistin with AZT, although only showing inhibition
235 at 2 hours, exhibited 2.72 log kill of the bacterium at 6 hours. The difference of the bacterial
236 numbers between zero hour and 6 hours was significant ($P < 0.001$, $n=4$). For *E. coli* strain
237 Af40 (Figure 3B), colistin at 20 mg/kg showed the same growth pattern as the control and
238 AZT inhibited bacterial growth. Combination of colistin with AZT exhibited 1.32 and 2.96
239 log kill of the bacterium at 2 and 6 hours, respectively. The difference of the bacterial
240 numbers between zero hour and 2 hours or 6 hours was significant ($P < 0.01$ and 0.001 ,
241 respectively, $n=4$). In both untreated control groups and the colistin treated group, all
242 animals developed mild clinical signs such as transiently hunched posture at 6 hours after
243 infection. The animals in other treatment groups showed no discomfort with normal and
244 heathy behaviors. All animals were sacrificed at 6 hours after treatment according to the
245 restriction of adverse effects in the project licence.

246

247 DISCUSSION

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

250 strains. The antibiotic-resistant Enterobacteriaceae isolates used in the study covered a
251 broad geographic distribution. The colistin resistant strains were from some European
252 countries and South Africa (29-31). The 7 NDM-1 strains represented the most resistant
253 type of Enterobacteriaceae.

254 The clinical efficacy of AZT has been demonstrated to reduce morbidity and mortality in
255 patients with asymptomatic or acute human immunodeficiency virus (HIV) disease (43, 44).
256 In patients, the oral dosage is 250 – 300 mg twice daily and intravenous infusion is 0.8 – 1
257 mg/kg every 4 hours for up to 2 weeks. It has been shown that 120 mg iv dosing produced
258 an AUC of 0.0014 mg.h/L and a Cmax of 0.0015 mg/L while 200 mg oral dosing gave rise
259 to an AUC of 0.0017 mg.h/L and a Cmax of 0.0018 mg/L (45). AZT has been shown to be
260 active against Gram-negative bacteria (18-20), it is not known if the concentrations used
261 clinically are sufficient to treat bacterial infections in humans.

262 Colistin is effective against multidrug-resistant but colistin susceptible *Pseudomonas*
263 *aeruginosa*, *K. pneumoniae*, *Acinetobacter* (46) and importantly NDM-1 carrying
264 Enterobacteriaceae (8). There is increasing evidence to show that colistin resistance is on
265 the rise, especially the discovery of plasmid born colistin resistance worldwide (26, 47, 48).
266 It is critically important to preserve and prolong the life of the last resort of antibiotic by
267 enhanced combination therapy. Here we have shown that in combination with AZT, colistin
268 MIC was significantly reduced, especially against *mcr-1* containing colistin resistant strains.
269 The enhanced activity of colistin in combination with AZT was confirmed with time kill
270 assays which provided dynamic measures of bactericidal activities of the combination over
271 time. In colistin mono exposure, complete eradication of the NDM-1 *K. pneumoniae*
272 BAA2472 or *mcr-1 E. coli* Af40 strains required much higher concentrations of the drug
273 (data not shown), however, more than 4 to 16-fold lower concentrations of colistin when
274 combined with AZT achieved the same effect. This is significant as enhancement of colistin

275 combination with AZT will likely reduce the dose of colistin but retain maximal therapeutic
276 efficacy hence minimize its toxic profile. These data suggest that further clinical
277 development of a colistin plus AZT combination may be able to achieve an effective lower
278 dose colistin therapy against colistin-sensitive and colistin-resistant infections.

279 Bacterial infections caused by carbapenem resistant strains are life threatening and
280 effective treatment is difficult to achieve. The last resort treatment option is to use colistin
281 (9, 49). Previous studies have shown that in bacteria AZT needs to be converted to the
282 nucleotide to inhibit bacterial DNA replication (50) and that bacterial thymidine kinase is
283 responsible for the initiation of the activation process - phosphorylation of AZT (23, 50).
284 Other antibiotics such as ciprofloxacin also inhibit DNA replication by blocking GyrA. But
285 comparison of the resistance profiles of ciprofloxacin and AZT are very different (see Table
286 3). This suggests that AZT has a different mechanism of action to other antibacterial agents
287 which are in the market. Rather, AZT is likely to act on a new target in bacteria. Further
288 studies on how AZT acts against Gram-negative bacteria are underway in our laboratories
289 by analysis of AZT mutants with next-generation sequencing and investigation of the AZT
290 effect on bacteria by performing Bacterial Cytological Profiling (BCP).

291 The therapeutic effectiveness of AZT and colistin combinations was also examined using a
292 mouse peritoneal infection model. As a potential therapeutic agent, AZT has been used to
293 treat HIV. Its bactericidal activity has been reported *in vivo* (19). Here we demonstrate that
294 AZT at 5 mg/kg inhibited the NDM-1 *K. pneumoniae* BAA2472 and the *mcr-1 E. coli* strain
295 Af40 growth in the mouse peritoneal infection. However, the combination of AZT with
296 colistin improved the therapeutic activities of each single agent with significant kill of the
297 bacteria at 2 or 6 hours in mouse peritoneal cavity. Most importantly, when colistin
298 methanesulfonate was completely ineffective up to 6 hours of treatment, the addition of
299 AZT was able to significantly reduce bacterial counts and attenuate the clinical signs in the

300 animals. Here we used colistin methanesulfonate instead of colistin sulfate. The reason
301 was that colistin methanesulfonate is used clinically and is less toxic than colistin sulfate in
302 mice (51). Colistin methanesulfonate is a prodrug which needs to convert to the active form
303 of colistin (52). The conversion normally delays the activity of the drug (52). Here we
304 demonstrated that with the addition of AZT, the effect of colistin methanesulfonate and AZT
305 was significantly increased. Collectively, the data show that the application of AZT and
306 CMS combination therapy *in vivo* offers the potential to increase both colistin and AZT
307 activities against antibiotic-resistant Enterobacteriaceae.

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

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325

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496 497 498 499 **Figure legends**

500 Figure 1. Time Kill analysis showing the effects of AZT in combination with colistin against
501 NDM-1 *K. pneumoniae* BAA2472. AZT and colistin alone or in combination were added to
502 the log phase cultures and CFU counts were carried out at different time points.
503 Combination concentrations of AZT and colistin are colistin 2 mg/L + AZT 4 mg/L (A),
504 colistin 2 mg/L + AZT 2 mg/L (B), colistin 2 mg/L + AZT 1 mg/L (C), colistin 1 mg/L + AZT
505 4 mg/L (D), colistin 1 mg/L + AZT 2 mg/L (E) and colistin 1 mg/L + AZT 1 mg/L (F). The
506 dash line is the limit of detection in the assay (30 CFU/ml).

507 Figure 2. Time Kill analysis showing the effects of AZT in combination with colistin against
508 *mcr-1* colistin resistant *E. coli* Af40. AZT and colistin alone or in combination were added to
509 the log phase cultures and CFU counts were carried out at different time points.

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

514 Figure 3. Effects of AZT in combination with colistin against the NDM-1 *K. pneumoniae*
515 BAA2472 and the *mrc-1 E. coli* strain Af40 in a mouse peritoneal infection model. A. Mice
516 were infected with strain BAA2472. Treatment was initiated 30 minutes after infection with
517 AZT (5 mg/kg), CMS (10 mg/kg) and AZT plus CMS. B. Mice were infected with strain
518 Af40. Treatment was initiated 30 minutes after infection with AZT (5 mg/kg), CMS (20
519 mg/kg) and AZT plus CMS. Bacterial counts in the peritoneal cavity were determined from
520 4 mice for each group at 0 hour before and 2 and 6 hours post-treatment. The data has
521 been repeated once. ** indicates $p \leq 0.01$. *** indicates $p \leq 0.001$.

522

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

Antibiotics	MIC (mg/L)						
	NCTC13443 <i>K. pneumoniae</i>	BAA – 2468 <i>E. cloacae</i>	BAA – 2469 <i>E. coli</i>	BAA – 2470 <i>K. pneumoniae</i>	BAA – 2471 <i>E. coli</i>	BAA – 2472 <i>K. pneumoniae</i>	BAA – 2473 <i>K. pneumoniae</i>
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
Nitrofurantoin	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*

	<i>E. coli</i> (23)			<i>K. pneumoniae</i> (31)			<i>mcr-1 E. coli</i> (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

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





