# Azidothymidine enhances activities of carbapenems against New Delhi metallo-beta-lactamase 1 Enterobacteriaceae

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**Abstract**

**Objectives:** To investigate the efficacy of azidothymidine in combination with carbapenems against New Delhi metallo-beta-lactamase 1 (NDM-1) producing Enterobacteriaceae.

**Methods:** MIC was determined using the broth microdilution method. The combinatory effects of azidothymidine and carbapenems were examined using the checkerboard method and time-kill analysis.

**Results:** We found that the NDM-1 producing strains were resistant to all carbapenems tested. Fractional inhibitory concentration index from checkerboard assay demonstrated that azidothymidine synergized with carbapenems against all the NDM-1 strains. Time-kill analysis demonstrated significant synergistic activities when a low level of azidothymidine was combined with meropenem.

**Conclusions:** Azidothymidine in combination with carbapenems produced synergistic activities against NDM-1 Enterobacteriaceae strains in vitro.

**Running title:** Azidothymidine synergised with carbapenems against NDM-1 strains

**Introduction**

Carbapenemase producing Enterobacteriaceae (CPE) is associated with a reported mortality rate of up to 40-50% in those patients who are infected. 1 The rapid emergence of CPE, which are often resistant to many other antibiotics, has left the world with colistin as the last resort treatment option, although colistin is associated with both nephrotoxic and neurotoxic side effects. 2 Therefore, it is crucial to boost the effectiveness of carbapenems against CPE.

Previously, we showed that azidothymidine [3’-azido-3’-deoxythymidine] boosted the activity of colistin both *in vitro* and *in vivo* against multiple strains of resistant Enterobacteriaceae which produced ESBL, NDM-1 or carried the mobilized colistin resistance (mcr) gene. 3 Azidothymidine is an antiretroviral drug which is used in combination with other antivirals to prevent and treat HIV/AIDS. It inhibits viral reverse transcriptase and was the first effective treatment for HIV/AIDS. 4 Synergy between azidothymidine and other non-polymixin antibiotics has not been published previously.

In this study, we tested, for the first time, the *in vitro* activities of azidothymidine in combination with carbapenems against NDM-1 producing Enterobacteriaceae.

**Materials and Methods**

***Bacterial strains and growth conditions***

The bacterial strains harboring the blaNDM plasmid were ATCC BAA-2469 (*E. coli*), ATCC BAA-2470 (*K. pneumoniae*), ATCC BAA-2471 (*E. coli*), BAA-2472 (*K. pneumoniae*), ATCC BAA-2473 (*K. pneumoniae*) and NCTC 13443 (*K. pneumoniae*). The bacterial isolates were grown in nutrient broth (Oxoid, UK), on tryptone soya agar (Fluka, UK) or Chrome agar Orientation plates (BD, UK). Azidothymidine and carbapenems were obtained from Sigma-Aldrich, UK.

***Susceptibility tests of carbapenems and azidothymidine***

MIC of antibiotics and azidothymidine were determined using the broth microdilution method, in accordance with the Clinical and Laboratory Standards Institute guidelines. 5 MIC was performed using a 96-well micro-titre plate (Fisher Scientific, UK) as described previously. 3

***Checkerboard assays to determine combination effects of azidothymidine with carbapenems***

Combination of azidothymidine and carbapenems was prepared using 96 well micro-titre plates with drug concentrations starting two-fold higher than their MIC values, and were then serially diluted in a two-fold manner as described previously. 3 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 $\leq $4 and antagonism if the FICI was $>$4. 6For all of the wells of the micro-titre plates that corresponded to an MIC (no visible growth which were adjacent to wells with growth, isoeffective combinations), the sum of the FICs were calculated for each well using the above equation. The minimum FIC (ΣFICmin) and the maximum FIC (ΣFICmax) for all the isoeffective combinations were recorded in order to capture the activities of drug-drug interactions and to represent the pharmacodynamic interactions of the combinations. 7

***Time-Kill analysis of antibiotics alone and in combination with azidothymidine against log-phase bacteria***

A range of different concentrations of antibiotics and azidothymidine was prepared in a two-fold serial dilution and was added alone or in combination to a log phase bacterial culture containing 1- 5 x 107 cfu/mL, and incubated at 37°C. Viability expressed as log cfu/mL was determined at 0, 2, 4, 8 and 24 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$\geq $2-log10 decrease in cfu counts at 24 hours of the combination compared to the antibiotic alone, in addition to a $\geq $2-log10 decrease compared to the zero hour count. 8

**Results and Discussion**

The MICs for meropenem, imipenem, doripenem, ertapenem, biapenem and azidothymidine were determined against the 6 NDM-1 strains. As seen in Table S1, compared with the antibiotic breakpoints 9 resistance was found in all strains for all the carbapenems although the breakpoints for biapenem are not available. Azidothymidine MIC was 0.5 or 1 mg/L. Carbapenems are the most effective beta-lactams against Gram-negative bacteria containing most beta-lactamases including penicillinase and ESBL. Carbapenems are considered to be the most reliable last-resort treatment for bacterial infections. Here we showed that the NDM-1 strains rendered the last-resort of antibiotics ineffective. Therefore, it is important to rejuvenate the activities of carbapenems to bring the most effective drug back to patient bedside.

The effects of combining azidothymidine with meropenem, imipenem, doripenem, ertapenem and biapenem were determined using checkerboard assays against the 6 NDM-1 strains. As shown in Table 1, the ΣFICmin and ΣFICmax were significantly low showing the presence of both synergistic and no interaction in the combinations of azidothymidine with the five carbapenems at different drug concentrations (Table S2) against all six strains tested. No antagonism was observed. We also showed significant reduction (at least 4-fold) in MICs of the carbapenems after combination with azidothymidine (Table S2).

The bactericidal activities of the synergistic combination of azidothymidine and meropenem was performed using time kill assays against all the NDM-1 *E. coli* and *K. pneumoniae* strains. We used the concentrations of azidothymidine at 1 or 2 mg/L to combine with 4 different concentrations of meropenem starting at 2-fold or at the MIC level. As shown in Figure 1, meropenem at 32 (MIC) and 16 mg/L gave rise to 3 log reduction in cfu counts at 4 hours, regrowth was seen at 8 hours and 4 hours, respectively and meropenem at 8 mg/L reduced about 2 log cfu counts at 8 hours, meropenem at 4 mg/L reduced about 1.5 log cfu counts at 2 hours followed by regrowth. Azidothymidine at 2 mg/L reduced 2 logs of cfu counts at 8 hours followed by bacterial regrowth and at 1 mg/L inhibited bacterial growth for 8 hours followed by regrowth. However, when meropenem at 32 and 16 mg/L was combined with azidothymidine at 2 or 1 mg/L, complete elimination of cfu counts was observed at 4 hours (Figure 1a and 1b) and at 8 hours (Figure 1c and 1d), respectively. The combinations of meropenem at 8 and 4 mg/L with azidothymidine at 2 or 1 mg/L completely eliminated the cfu counts at 24 hours (Figure 1e, 1f, 1g, and 1h). Similar synergistic activities were observed for the other NDM-1 strains (Data not shown)

We demonstrated that azidothymidine synergized with 5 different carbapenems against NDM-1 strains, which is the most difficult-to-treat resistant type of Enterobacteriaceae (2 *E. coli* and 4 *K. pneumoniae*). We showed that in combination with azidothymidine, the MIC of each carbapenem was significantly reduced (Table S2).

It has been reported that after 600 mg oral dosing of azidothymidine, the Cmax reached 3.5 mg/L in humans. 10 When azidothymidine was given in a single intravenous dose of 120 mg on the first day, followed by a single oral dose of 200 mg on the second day, the maximum concentration of azidothymidine in serum was about 1.751 mg/L. 11 It is not known if the dose size used clinically are sufficient to boost the activities of carbapenems to treat bacterial infections in humans.

We used the published blood concentration of azidothymidine to combine with meropenem, and we found that azidothymidine at 1 or 2 mg/L significantly boosted the activities of the antibiotic against the NDM-1 strains. After observation of the meropenem time kill curve profile, we noticed that meropenem was bactericidal at the MIC level or below initially against the NDM-1 strains, then regrowth was seen. The early effect of meropenem might be due to lack of carbapenemase activity which was induced after treatment with the drug. 12 Combination of azidothymidine with meropenem significantly increased the activity of meropenem and showed sustained bacterial clearance over the 24-hour drug exposure. It is unknown how azidothymidine enhances the activities of carbapenems. Further studies are needed to uncover the mode of action of this combination.

Following on our initial proof of principal data, testing the novel synergistic effects between azidothymidine and carbapenems is under way using a panel of CPE isolated from patients with clinically relevant infections. In particular, the therapeutic activity of azidothymidine-carbapenem combinations against highly lethal CPE, including NDM-1 is clinically important. The combination therapies will be tested in animal models. This study paves the way for validation of azidothymidine-carbapenem combinations in future clinical trials with the aim for bench-to-bedside translation to benefit patients.

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**Transparency declarations**

AC is director, chief scientific officer and shareholder of Helperby Therapeutics Ltd. YH is the director of research and shareholder of Helperby Therapeutics Ltd.

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 **Figure legends**

**Figure 1**. Time Kill analysis showing the effects of azidothymidine (AZT) in combination with meropenem against NDM-1 *K. pneumoniae* BAA2472. AZT and meropenem 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 meropenem are AZT 2 mg/L + meropenem 32 mg/L (a), AZT 1 mg/L + meropenem 32 mg/L (b), AZT 2 mg/L + meropenem 16 mg/L (c), AZT 1 mg/L + meropenem 16 mg/L (d), AZT 2 mg/L + meropenem 8 mg/L (e), AZT 1 mg/L + meropenem 8 mg/L (f), AZT 2 mg/L + meropenem 4 mg/L (g), AZT 1 mg/L + meropenem 4 mg/L (h).

Table 1. Combination of azidothymidine with different carbapenems against NDM-1 producing *E. coli* and *K. pneumoniae*

|  |  |
| --- | --- |
|  |  FIC index AZT\* + |
| Bacterial strains  |  | Meropenem |  Imipenem |  Doripenem | Ertapenem | Biapenem |
| BAA2469 | ΣFICmin | 0.27 | 0.19 | 0.38 | 0.25 | 0.16 |
|  | ΣFICmax | 0.63 | 0.53 | 0.56 | 0.56 | 0.53 |
| BAA2470 | ΣFICmin | 0.19 | 0.31 | 0.19 | 0.13 | 0.19 |
|  | ΣFICmax | 0.56 | 0.56 | 0.56 | 0.53 | 0.53 |
| BAA2471 | ΣFICmin | 0.13 | 0.38 | 0.31 | 0.25 | 0.25 |
|  | ΣFICmax | 0.56 | 0.56 | 0.56 | 0.56 | 0.56 |
| BAA2472 | ΣFICmin | 0.50 | 0.31 | 0.38 | 0.50 | 0.25 |
|  | ΣFICmax | 0.63 | 0.56 | 0.56 | 0.56 | 0.56 |
| BAA2473 | ΣFICmin | 0.25 | 0.50 | 0.38 | 0.25 | 0.25 |
|  | ΣFICmax | 0.63 | 0.63 | 0.63 | 0.56 | 0.53 |
| NCTC13443 | ΣFICmin | 0.38 | 0.31 | 0.38 | 0.50 | 0.16 |
|  | ΣFICmax | 0.63 | 0.56 | 0.56 | 0.63 | 0.53 |

 \*AZT, azidothymidine

Figure 1

