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Short Communication

Synergistic activity of colistin with azidothymidine against colistin-resistant *Klebsiella pneumoniae* clinical isolates collected from inpatients in Greek hospitals

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ABSTRACT

Background: New antibiotics are urgently needed to treat multi-drug resistant infections; however, production of novel antibiotics is diminishing. Synergistic combination drug therapy to enhance the activity of available antibiotics may improve management of patients with resistant infections.
Methods: Colistin-resistant *Klebsiella pneumoniae* isolates were collected from inpatients in 10 Greek hospitals and used to study combination activity of colistin plus azidothymidine. Combination activity was evaluated with the sum of fractional inhibitory concentrations (ΣFIC) using the mini checkerboard broth microdilution method.
Results: A hundred individual strains were tested. Synergistic activity was noted in 79% (79/100) of isolates and additive activity in the remaining 21% (21/100). ΣFIC_{50} and ΣFIC_{90} were 0.28 and 0.56, respectively.
Conclusion: Colistin with azidothymidine exhibited promising synergistic activity against colistin-resistant *Klebsiella pneumoniae* isolates warranting further investigation of the combination.

1. Introduction

Multidrug-resistant Gram-negative bacteria are a significant cause of morbidity and mortality in hospitalized patients, mainly because of limited available treatment options and comorbidity [1]. Among them, carbapenem- and colistin-resistant *Klebsiella pneumoniae* isolates are prevalent. Colistin resistance may preclude a positive therapeutic result. Combination antibiotic regimens are potential treatment options, based mainly on their in vitro synergistic activities [2]. New or alternative treatment options with synergistic activity against colistin-resistant *K. pneumoniae* isolates are needed.

Azidothymidine (3′-azido-3′-deoxythymidine, AZT, Zidovudine) is a thymidine analogue taken orally or intravenously for treatment of human immunodeficiency virus (HIV) infection [3]. In early reports, azidothymidine-treated patients had a lower probability of *Salmonella* spp. infections [4], supporting the first studies that reported the antimicrobial properties of azidothymidine [5,6]. In these in vitro studies, azidothymidine was shown to have antibacterial activity against Enterobacteriaceae, including *K. pneumoniae,* because of its incorporation into bacterial genome and subsequent DNA chain termination [5-7].

Besides the antimicrobial properties of azidothymidine, various in vitro studies have shown at least additive activity of azidothymidine in combination with several antibiotics, such as tigecycline, ciprofloxacin and trimethoprim, against several Gram-negative

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Table 1
MIC range, 50th, 75th and 90th percentiles of MIC for colistin and azidothymidine against colistin-resistant *K. pneumoniae* isolates before and after combination of the two drugs.

	MIC (mg/L)				MIC (mg/L) in combination*			
	MIC range	MIC_{50}	MIC_{75}	MIC_{90}	MIC range	MIC_{50}	MIC_{75}	MIC_{90}
Colistin	4-128	16	32	32	0.25-16	1	2	4
Azidothymidine	0.125-4	1	2	2	0.0625-1	0.25	0.25	0.25

MIC – minimum inhibitory concentration
* Colistin in combination with azidothymidine.

bacteria [6,8,9]. Moreover, azidothymidine has shown synergistic antimicrobial activity even in combination with small antimetabolite molecules not currently used as antibiotics, such as hydroxyurea and floxuridine, against trimethoprim-resistant *Escherichia coli* and *K. pneumoniae* clinical isolates [9]. Recently, Hu et al. demonstrated in vitro synergistic activity of colistin in combination with azidothymidine against several Enterobacteriaceae clinical isolates, including ESBL- and NDM-1-producing *K. pneumoniae* strains [10]. The aim of the current study was to broaden knowledge of the synergistic activity of colistin/azidothymidine combination, examining this combination against a collection of colistin-resistant *K. pneumoniae* strains, including highly colistin-resistant strains (MIC_{75} = 32 mg/L), isolated from clinical specimens of inpatients in Greek hospitals. The names of the participating hospitals are mentioned in the **Members of the Colistin – Azidothymidine Hellenic Study Group** section.

2. Methods

Non-duplicate, colistin-resistant *K. pneumoniae* strains were collected from 10 Greek hospitals from 2015 to 2017. All isolates were stored at -70 °C prior to testing. Isolates were transferred and tested in the Laboratory of Department of Microbiology, National School of Public Health, Athens, Greece.

Experiments were performed using the checkerboard method in customized 96-well plates; minimum inhibitory concentration (MIC) values in the combination wells were determined using the microdilution method as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines. All isolates were tested twice. The drugs used were colistin sulphate (Sigma-Aldrich, US) and azidothymidine (Sigma-Aldrich, US). In the checkerboards, colistin concentrations (in mg/L) started from the tested MIC value up to four subsequent two-fold dilutions, and a control (drug-free) well; the range of concentrations for azidothymidine was 0.0625-4 mg/L, and a control (drug-free) well. Microdilution plates of azidothymidine and colistin were prepared and provided by Helperby Therapeutics, London, UK and subsequently transported in dry ice to the testing laboratory. The plates were stored at -70°C.

Plates were thawed for an hour before use. Cation-adjusted Mueller–Hinton broth (CAMHB) was used for further dilutions to inoculate a final bacterial cell suspension of 5×10^5 colony forming units (cfu)/mL. The plates were sealed and incubated for 20-22 h at 35 °C in a non-CO_2 incubator. *E. coli* specimen 4320 (MIC colistin 4 mg/L, distribution 4261, UK National External Quality Assessment Scheme) was the quality control strain used for laboratory testing.

MIC was determined visually as the lowest concentration of drug that inhibited bacterial growth. MIC_{50} and MIC_{90} are defined as concentrations of antimicrobial agent that inhibit 50% and 90% of tested isolates, respectively. The potential synergy of the two compounds was determined by the ΣFIC (sum of the fractional inhibitory concentration) method. ΣFIC is defined as the sum of the FIC (fractional inhibitory concentration) of the two combined antimicrobial agents, where FIC is the ratio of MIC of drug in combination (using the well with the maximal activity) over the MIC of drug alone. The estimated ΣFIC value indicates whether the combined effect is synergistic (ΣFIC <0.5), additive (0.5≤ ΣFIC ≤1), indifferent (1< ΣFIC ≤4) or antagonistic (ΣFIC >4) [11].

Table 2
Sum of fractional inhibitory concentrations (ΣFIC) of colistin with azidothymidine against 100 colistin-resistant *K. pneumoniae* isolates.

Sum of fractional inhibitory concentrations (ΣFIC)	n/N* (%)
ΣFIC <0.5	79/100 (79)
0.5≤ΣFIC ≤1	21/100 (21)
Median	0.31
Minimum	0.06
ΣFIC_{50}	0.28
ΣFIC_{75}	0.38
ΣFIC_{90}	0.56
Maximum	0.75

* n= number of isolates in each case
N= number of isolates tested

3. Results

One hundred colistin-resistant (MIC ≥ 4 mg/L) *K. pneumonia* isolates were studied. As shown in Table 1, colistin MIC_{50}, MIC_{75} and MIC_{90} decreased considerably after combination with azidothymidine (MIC_{50} from 4 mg/L to 1 mg/L, MIC_{75} from 32 mg/L to 2 mg/L and MIC_{90} from 32 mg/L to 4 mg/L). The azidothymidine MIC_{50} and MIC_{90} also decreased considerably after combination with colistin (MIC_{50} from 1 mg/L to 0.25 mg/L and MIC_{90} from 2 mg/L to 0.25 mg/L). Azidothymidine was noted to have antibacterial activity MIC_{50}= 1 mg/L and MIC_{90}= 2 mg/L.

ΣFIC_{50}, ΣFIC_{75} and ΣFIC_{90} were 0.28, 0.38 and 0.58, respectively, for the isolates tested (Table 2). Synergistic activity was seen in 79% of the isolates (79/100) and additive activity was noted in the remaining 21% (21/100). No antagonistic or indifferent effect was observed in any examined combination between the two antimicrobial agents.

4. Discussion

In this study, azidothymidine exhibited low MIC values (0.125-4 mg/L) against tested *K. pneumoniae* isolates. This confirms earlier observations that in addition to antiviral properties, azidothymidine has antibacterial activity against several members of Enterobacteriaceae [7,9]. Our study results for *K. pneumoniae* agree with two previous studies that showed azidothymidine MIC range between 0.1-3.1 mg/L and 0.1-1.67 mg/L [7,12].

Furthermore, the results of this study support the synergistic activity of colistin with azidothymidine, with low ΣFIC values, in line with a previous study examining a collection of *K. pneumoniae* clinical isolates [10]. Specifically, a synergistic antibacterial effect was noted against a substantial proportion (79%) of the tested colistin-resistant *K. pneumoniae* isolates and an additive effect was noted in the remaining isolates. No antagonism was observed between the two antimicrobial agents for any of the isolates tested. A few studies investigated the potential synergistic activity of azidothymidine in combination with other antimicrobials

against several Gram-negative bacteria (with similar azidothymidine MIC values to those of our isolates) [13]. However, higher ΣFIC values were reported in these studies, indicating lower synergistic activity between the examined compounds. A similar range of ΣFIC values to those in our study was reported for the combination of azidothymidine with gentamicin; however, this was based on a study of a small number of Enterobacteriaceae isolates [13].

Azidothymidine enters the HIV-infected cell and interferes with viral DNA production: it inhibits HIV reverse transcriptase enzyme after phosphorylation of azidothymidine to azidothymidine triphosphate by three deoxyribonucleoside kinases [14]. Similarly, in Enterobacteriaceae, phosphorylation of azidothymidine to azidothymidine-triphosphate by a thymidine kinase (when present) seems to be the crucial step to activate its bactericidal properties, inhibiting bacterial genome synthesis by acting as a DNA chain terminator [7].

In contrast, colistin acts mainly by disrupting cohesion of the outer membrane of susceptible Gram-negative bacteria, leading to cell envelope disruption [15,16]. The outer membrane protects the bacterial cells by hindering the penetration of hydrophobic or large antibiotic compounds [17]. Colistin promotes permeability of the outer membrane and this may explain the synergy of this drug in combination with other antimicrobials against several multidrug-resistant Gram-negative bacteria [17]. Azidothymidine is relatively lipophilic and permeates poorly into bacterial cells; modifications to the outer bacterial membrane by colistin may enhance permeation of azidothymidine into the cell, leading to synergistic antibacterial effects, even in colistin-resistant Gram-negative bacteria [18].

Azidothymidine pharmacokinetics have been evaluated mainly in HIV-infected adult patients, following either intravenous or oral administration [19,20]. Pharmacokinetic data from the azidothymidine phase I study showed that reaching steady state, after intravenous 1-h infusions of 2.5 mg/kg every 4 h, the mean peak (C_{max}) and trough (C_{min}) plasma concentrations of azidothymidine were 1.1 mg/L and 0.1 mg/L, respectively [19]. Also, the mean terminal half-life was about 1 h, which explains the small dosage intervals of 4 h [19]. Azidothymidine is eliminated primarily by hepatic glucuronidation to an inactive metabolite, supporting possible pharmacokinetic alterations in patients with hepatic disease [19,20].

Interestingly, in our study, azidothymidine exhibited synergistic bactericidal activity in combination with colistin at relatively low concentrations (0.0625-1 mg/L) that are similar to the clinically achievable plasma concentrations following approved intravenous therapeutic dosages [19,21]. Furthermore, the colistin MIC_{75} decreased considerably after combination with azidothymidine to a clinically desirable level (MIC_{75} from 32 mg/L to 2 mg/L). These data indicate that concomitant exposure to synergistic concentrations of colistin and azidothymidine against colistin-resistant *K. pneumoniae* is feasible in clinical practice and co-administration of these drugs deserves further investigation.

There are limited approved new antibiotics against multidrug-resistant Gram-negative bacteria [22]. Colistin, as monotherapy or in combination, is used frequently to treat patients with Gram-negative infections because it has considerable activity against Gram-negative bacilli with multiple concurrent resistance patterns [23,24]. However, there are increasing rates of colistin-resistant bacterial infections, mainly due to *K. pneumoniae* [25,26]. There is also an alarming increasing rate of Gram-negative infections with intrinsic resistance to colistin. In addition, there are concerns related to colistin toxicity, mainly nephrotoxicity and neurotoxicity [27]. These adverse events aggravate the compromised patient's health, and may result in antimicrobial therapy failure and a mandatory switch to other limited antibiotic options [28]. The potential clinical synergistic effect between colistin and azidothymidine may be efficacious against colistin-resistant *K. pneumoniae*, and enables lower doses of colistin, particularly in patients with renal dysfunction.

In conclusion, our study showed encouraging activity of a colistin-azidothymidine combination against colistin-resistant *K. pneumoniae* strains. Further clinical research is recommended to assess the potential application of this drug combination in clinical practice.

Members of the Colistin – Azidothymidine Hellenic Study Group

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Contributions

KT, PG and AV performed the microbiological experiments. MEF, GLV and MK performed the data analysis. MEF and GLV wrote the first draft of the article. All authors made substantial revisions and gave approval of the final version of the paper. YH and AC are the co-inventors of the antibiotic resistance breaker technology, particularly the combination of AZT and colistin/CMS (patents pending). They were the first to test this combination against highly-resistant Enterobacteriaceae (ECCMID 2018, Abstract 462). They originated the concept and performed the background work upon which this work is based.

Declarations

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The study was sponsored by Helperby Therapeutics Ltd.

Competing Interests

MEF participated in advisory boards of AstraZeneca, Infectopharm, Tetraphase, Shionogi, and Xellia; received lecture honoraria from Cipla, Merck, and Pfizer; and received research support from Shionogi, Tetraphase and Helperby. AC is founder, director and shareholder of Helperby Therapeutics Ltd. YH is also director of Research and shareholder of Helperby Therapeutics Ltd.

Ethical Approval

Not required.

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