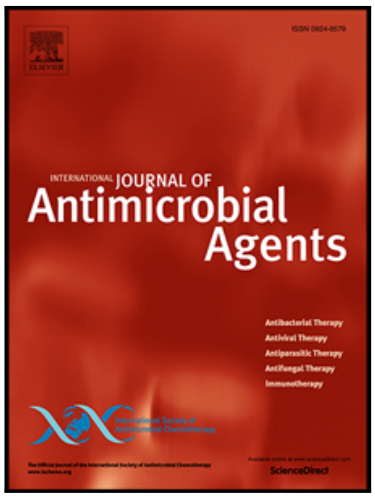

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Synergistic activity of colistin with azidothymidine against colistin-resistant *Klebsiella pneumoniae* clinical isolates collected from inpatients in Greek hospitals

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ABSTRACT

Background: New antibiotics are urgently needed for the treatment of multidrug resistant infections. However, the production of novel antibiotics is diminishing. The enhancement of activity of available antibiotics through synergistic combination drug therapy may help in the management of patients with resistant infections.

Methods: Colistin-resistant *Klebsiella pneumoniae* isolates were collected from inpatients in 10 Greek hospitals and used in the study of combination activity of colistin plus azidothymidine. The combination activity was evaluated with the sum of fractional inhibitory concentrations (ΣFIC), using the mini checkerboard broth microdilution method.

Results: 100 individual strains were tested. Synergistic activity was noted in 79% of the isolates (79/100) and additive activity in the rest 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 carbapenem resistant *Klebsiella pneumoniae* isolates warranting further investigations of the combination.

Key words: colistin, azidothymidine, synergism, repurposing, Enterobacteriaceae, antiviral, Gram-negative, carbapenem

INTRODUCTION

Multidrug resistant (MDR) Gram-negative bacteria constitute a significant cause of morbidity and mortality among hospitalized patients mainly due to limited available treatment options and comorbidity [1]. Among them, carbapenem- and colistin-resistant *Klebsiella pneumoniae* isolates are prevalent. As colistin-resistance may hamper a positive therapeutic result, combination antibiotic regimens have emerged as potential treatment options, based mainly on their *in vitro* synergistic activities [2]. Therefore, 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 used for the treatment of patients with human immunodeficiency virus infection (HIV) by oral or intravenous administration [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, 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 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]. In this study we tried to broad the knowledge so far about the synergistic activity of colistin/azidothymidine combination examining them against a collection of colistin-resistant and carbapenem-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.

METHODS

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

The experiments were performed using the checkerboard method in customized 96-well plates to determine the MIC values in the combination wells using the microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines. All isolates were tested twice. The used drugs were colistin sulphate (Sigma-Aldrich, US) and azidothymidine (Sigma-Aldrich, US). In the checkerboards, the used concentrations (in mg/L) for colistin started from the tested MIC value up to four subsequent two-fold dilutions, and a control (drug free) well; the range of the 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 for subsequent usage.

Plates thawed for an hour before use. Cation adjusted Mueller–Hinton broth (CAMHB) was used for further dilutions in order to inoculate a final bacterial cell suspension of 5×10^5 cfu/ml. The plates were sealed and incubated for 20-22 h at 35 ºC in a non-CO_2 incubator. *Escherichia 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.

The MIC concentration was determined visually as the lowest concentration of drug which inhibited the bacterial growth. The MIC_{50} and MIC_{90} are defined as the 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].

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 the 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 the 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}= 1mg/L and MIC_{90}= 2 mg/L.

$\Sigma FIC_{50,}$ 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) while additive activity was noted in the rest 21% (21/100). No antagonistic or indifferent effect was observed in any examined combination between the two antimicrobial agents.

DISCUSSION

In this study, azidothymidine exhibited low MIC values (0.125-4 mg/L) against *K. pneumoniae* isolates tested. This confirms earlier observations that in addition to antiviral properties, azidothymidine has antibacterial activity against several members of Enterobacteriaceae [7, 9]. Specifically, for *K. pneumoniae* our study results are in agreement with two previous relative 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, and of interest, with low ΣFIC values keeping in line with a previous

study examining a collection of *K. pneumoniae* clinical isolates [10](30373798). Specifically, synergistic antibacterial effect was noted against a substantial proportion (79%) of the tested colistin-resistant *K. pneumoniae* isolates while additive effect was noted in the rest of the isolates. No antagonism was observed between the two antimicrobial agents for any of the tested isolates. A few previous 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. Similar range of ΣFIC values, with those observed 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 exhibits its antiviral properties by interfering with viral DNA production as it inhibits HIV reverse transcriptase enzyme activity after the phosphorylation of azidothymidine to azidothymidine triphosphate by three deoxyribonucleoside kinases [14]. Similarly, in Enterobacteriaceae, the phosphorylation of azidothymidine to azidothymidine-triphosphate by a thymidine kinase (when present) seems to be the crucial step in order to activate its bactericidal properties, inhibiting bacterial genome synthesis by acting as a DNA chain terminator [7].

On the other hand, colistin acts mainly by disrupting the cohesion of the outer membrane of susceptible Gram-negative bacteria and leading to cell envelope disruption [15, 16] . Outer membrane protects the bacterial cells by hindering the penetration of hydrophobic or large antibiotic compounds [17]. The ability of colistin to promote the permeability of the outer membrane has been previously discussed in trying to interpret the potential mechanism of synergy of colistin in combination with other antimicrobials against several multidrug-resistant Gram-negative bacteria [17]. Hypothetically, as azidothymidine is relatively lipophilic [18], outer bacterial membrane modifications due to colistin activity, may have enhanced azidothymidine's limited cell permeating capability, lead to synergistic antibacterial effects, even partially in colistin-resistant Gram-negative bacteria and lead to synergistic antibacterial effects.

Azidothymidine pharmacokinetics has been evaluated mainly in HIV-infected adult patients, following either IV or oral administration [19, 20]. Pharmacokinetic data from the azidothymidine phase I study showed that reaching the steady state, after intravenous one-hour infusions of 2.5 mg/kg every 4 hours, the mean peak (C_{max}) and through (C_{min}) plasma concentrations of azidothymidine were 1.1 mg/L and 0.1 mg/L, respectively [19]. Also, the mean terminal half-life is about 1 hour, explaining the small dosage intervals of 4 hours [19]. Azidothymidine is primarily eliminated 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 the combination with azidothymidine to a clinical desirable level (MIC_{75} from 32 mg/L to 2 mg/L). These data suggest that the concomitant exposure to synergistic concentrations of colistin and azidothymidine against colistin-resistant *K. pneumoniae*, is feasible in clinical practice and a potential co-administration of these drugs may deserve further investigation.

There are limited approved new antibiotics against multi-drug resistant Gram-negative bacteria [22]. Subsequently, colistin, as monotherapy or in combination, is used frequently for the treatment of patients with Gram-negative infections due to the 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]. Also, there is an alarming trend of increasing rates of Gram-negative infections with intrinsic resistance to colistin . In addition, there are concerns related to the toxicity of [27]colistin, mainly nephrotoxicity and neurotoxicity. The development of such adverse events may result to aggravation of the already compromised patient's health, antimicrobial therapy failure and mandatory switch to other limited antibiotic options [28]. Therefore, the potential clinical synergistic effect between colistin and azidothymidine may lead to a desirable therapeutic effect against colistin-resistant *K. pneumoniae* with lower

colistin exposure by administering a lower dose of colistin, especially in patients with renal dysfunction.

In conclusion, our study showed encouraging activity of the combination of colistin with azidothymidine against colistin-resistant *K. pneumoniae* strains. These observations suggest that this combination deserves further studies in humans towards the potential application 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, and in particular of 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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Ethical Approval: Not applicable

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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 the 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

*Colistin in combination with azidothymidine.

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