Synergy against extensively drug-resistant Acinetobacter baumannii in vitro by two old antibiotics: colistin and chloramphenicol

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\textbf{A B S T R A C T}

Combination antimicrobial therapy is an important option in the fight against Gram-negative ‘superbugs’. This study systematically investigated the synergistic effect of colistin (CST) and chloramphenicol (CHL) in combination against extensively drug-resistant \textit{Acinetobacter baumannii} (XDR-AB). The microtitre plate chequerboard assay was used to test synergy against 50 XDR-AB clinical strains. Then, three XDR-AB clinical isolates and the type strain \textit{A. baumannii} ATCC 19606 were chosen for further synergy studies using time–kill assay, mutant prevention concentration (MPC) assay and real-time population analysis profile (PAP) assay. In the chequerboard assays, synergistic or additive effects [defined as a fractional inhibitory concentration index (FICI) of ≤0.5 and 0.5 < FICI < 1, respectively] were observed in all 50 isolates. In further synergy testing, the results of time–kill assays indicated that CHL monotherapy produced rapid bacterial killing followed by rapid re-growth, with the emergence of CST resistance; CHL monotherapy was largely ineffective. The combination CST/CHL, however, showed a synergistic effect and enhanced bacterial killing in the four tested strains. It also significantly delayed re-growth and suppressed the emergence of CST resistance. In the MPC assay, a decrease in MPCs for CST was observed in the two CST-resistant strains. PAP assay showed that both CST-resistant strains were heteroresistant.

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\section{1. Introduction}

During the last decade, infections due to extensively-drug resistant \textit{Acinetobacter baumannii} (XDR-AB) have presented an enormous challenge to health care [1]. The only agents that remain consistently active in vitro versus XDR-AB are colistin (CST) and tigecycline. Although CST is active against \textit{A. baumannii}, recent studies have shown that CST resistance emerges rapidly in \textit{A. baumannii} isolates that are treated with CST alone. Very worryingly, heteroresistance to CST in \textit{A. baumannii} has been reported [2].

Recently, Liu et al published observations on the emergence of a plasmid-mediated CST resistance mechanism, MCR-1 [3]. Like the NDM-1 resistance mechanism, MCR-1 can also be transferred to strains with a high in vitro horizontal transfer rate ($10^{-1}$–$10^{-2}$). Falgenhauer et al reported similar findings. They detected the \textit{mcr-1} gene in four \textit{Escherichia coli} isolates, three originating from swine and one from a human wound infection [4]. These findings emphasise the urgent need for new strategies in the fight against pandrug-resistant Gram-negative bacteria.

Chloramphenicol (CHL) is a bacteriostatic antimicrobial originally derived from the bacterium \textit{Streptomyces venezuelae} and introduced into clinical practice in 1949 [5]. Similar to CST, CHL fell out of favour with clinicians owing to its potential adverse events, which include aplastic anaemia. However, it has a wide antimicrobial spectrum and excellent tissue penetration. Some research has indicated that CHL has acceptable activity against \textit{Enterococcus faecium} and methicillin-resistant \textit{Staphylococcus aureus} (MRSA) but shows very poor activity against the non-fermenting pathogens such as \textit{Pseudomonas aeruginosa} and \textit{A. baumannii} [6]. In an era of increasing resistance to many antibiotic classes, CHL may have a role in the treatment of intra-abdominal and respiratory tract infections. However, in vitro data regarding the combination of CHL with other agents against Gram-negative micro-organisms have some limitations. The currently available data are scant, old, mostly carried out with penicillin and do not reproduce the in vivo pharmacokinetic behaviour of the drugs [6].

Due to the current situation, combination therapy has been proposed to increase antimicrobial activity and reduce the emergence of resistance to CST, although comprehensive clinical data are lacking. A recent report demonstrated that the combination of CHL and CST exhibits enhanced in vitro activity against multidrug-resistant \textit{Klebsiella pneumoniae} [7]. We suspect that this combination may exhibit
similar bactericidal or synergistic effects against XDR-AB. Thus, the aim of this study was to investigate, using clinically relevant concentrations of each antibiotic, the antibacterial activity and emergence of CST resistance associated with CST monotherapy and with CST/CHL combination therapy against XDR-AB.

2. Materials and methods

2.1. Bacterial isolates and antibiotics

This study included 50 XDR-AB clinical isolates derived from specimens of 50 patients from 30 different tertiary care hospitals in Anhui Province, China, between September 2013 and September 2014. All the strains were XDR, which was defined as resistance to at least one antimicrobial agent from three or more antimicrobial categories. Each isolate was stored at −20 °C until use and was subcultured on Mueller–Hinton agar (MH) (Oxoid Ltd., Basingstoke, UK) for in vitro testing. Both colistin sulphate salt and CHL were obtained from Sigma-Aldrich China, Inc. (Shanghai, PR China).

2.2. Determination of minimum inhibitory concentrations (MICs)

MICs of CST and CHL against the 50 XDR-AB isolates were determined by a serial two-fold agar dilution method using MHA according to Clinical and Laboratory Standards Institute (CLSI) recommendations [9]. Isolates were considered susceptible or resistant at respective MICs of ≤8 mg/L or ≥32 mg/L for CHL and ≤2 mg/L or ≥4 mg/L for CST as per the CLSI guidelines for Enterobacteriaceae and A. baumannii. Acinetobacter baumannii ATCC 19606 and E. coli ATCC 25922 were used as quality control strains. The MIC50 and MIC90 values (MICs for 50% and 90% of the organisms, respectively) and the MIC range for the 50 XDR-AB isolates are shown in Table 1.

2.3. Colistin + chloramphenicol checkerboard assay

First, the synergistic effect of CST/CHL combination against the 50 XDR-AB clinical strains was tested. Then, three XDR-AB clinical isolates, including the CST-susceptible (CST+) strain GN2088 and two CST-resistant (CST−) strains (GN1719 and GN0651) selected according to the MICs presented in Table 1, as well as the type strain A. baumannii ATCC 19606 were chosen to study the synergistic effect of CST/CHL combination in depth. The checkerboard microdilution method was performed as described previously [9]. In brief, 96-well microtitre plates (Sigma-Aldrich China, Inc.) were set-up with increasing concentrations of CHL (0–256 mg/L) in the horizontal wells and CST (0–32 mg/L) in the vertical wells and were inoculated with 106 CFU/mL of A. baumannii prepared in Mueller–Hinton broth (MHB) (Oxoid Ltd.). Plates were incubated at 37 °C for 24 h and were visually inspected for turbidity to determine growth. Synergy was assessed by calculation of the fractional inhibitory concentration index (FICI) and the susceptible breakpoint index (SBPI) as previously described [10,11]. The FICI was interpreted as follows: FICI ≤0.5, synergy; FICI < 1, superior to additive; FICI = 1.0, additive; 1 < FICI < 2, subadditive; FICI = 2.0, indifference; and FICI > 2.0, antagonism. Synergy was also defined as a SBPI of >2. The cumulative inhibition ratio (CIR) was defined as the percentage of isolates that were inhibited at a certain antibiotic concentration [12].

2.4. Detection of resistance genes by PCR

Genes encoding resistance to carbapenems (blaOXA-23-like, blaOXA-24-like, blaOXA-51-like, blaOXA-58-like and metallo-β-lactamases) and CST (mcr-1) were identified by PCR and sequencing for all isolates as previously described [9].

2.5. Determination of the mutant prevention concentration (MPC)

The MPCs of the two drugs alone and in combination were determined by a modified method described by Zhao and Drlica [13] and Zhanel et al [14]. In brief, all CST+ isolates were grown overnight on MHA at 37 °C in ambient air. Overnight growth was then swabbed into MHB and was incubated for 24 h at 37 °C in ambient air in order to achieve an inoculum of ca. 106 CFU/mL. Samples (100 mL) were inoculated onto MHA plates containing 0.5 × 1, 2 ×, 4 ×, 8 ×, 16 ×, 32 ×, 64 ×, 128 × or 256 × their CST MIC alone and in combination with CHL (32 mg/L). Finally, the inoculated plates were incubated for 48 h at 37 °C and were screened visually for growth. The MPC was recorded as the lowest antimicrobial concentration at which no colonies grew on an agar plate after 48 h of incubation. The MIC and MPC data were used to calculate the size of the mutant prevention index (MPI = MPC/MIC) and the mutant selection window (MSW = MPC–MIC) for CST alone and in combination with CHL.

2.6. Population analysis profiles (PAPs)

PAPs were used to determine heteroresistance to CST in the A. baumannii strains examined [15]. Each CST+ A. baumannii strain was cultured in MHB to an inoculum of >106 CFU/mL before viable counting on MHA plates containing CST (0.5, 1, 2, 3, 4, 6, 8 and 10 mg/L). Plates were incubated for 24 h at 37 °C. The limit of detection was 20 CFU/mL (equivalent to one colony per plate). PAPs for CST were also constructed following 24 h of CST treatment in the time–kill studies (see below).

2.7. Time–kill assays

Time–kill assays were conducted for each strain using CST alone and in combination with CHL according to a previously described methodology [1]. The final concentrations used were 2 mg/L for CST in order to mimic the steady-state plasma concentration with pharmacokinetically optimised dosing of CST [16]. CHL monotherapy of 16 mg/L and 32 mg/L was examined against all of the strains. Thus, two combination regimens were examined across the four strains: CST 2 mg/L + CHL 16 mg/L; and CST 2 mg/L + CHL 32 mg/L. MHB was inoculated with 5 × 105 CFU/mL of the strain and was incubated at 37 °C. Colony counts were determined at 0.5, 1, 2, 4, 8 and 24 h to determine the viable CFU/mL. Synergism between two antimicrobials was defined as ≥2 log10 decrease in bacterial growth compared with the most active single agent and the number of surviving organisms in the presence of the combination must be ≥2 log10 CFU/mL.

Table 1

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Susceptibility (%)</th>
<th>MIC (mg/L)</th>
<th>FICI</th>
<th>SBPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>MIC50</td>
</tr>
<tr>
<td>CST</td>
<td>94</td>
<td>0</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>CHL</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>256</td>
</tr>
<tr>
<td>CST + CHL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.25–0.5/0.5–128</td>
</tr>
</tbody>
</table>

S, susceptible; I, intermediate; R, resistant; MIC, minimum inhibitory concentration; MIC50/90, MICs for 50% and 90% of the organisms, respectively; FICI, fractional inhibitory concentration index; SBPI, susceptible breakpoint index; S+, synergy; A, additive effect.
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