Cell-wall-inhibiting antibiotic combinations with activity against multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli*

R. A. Hickman[1], D. Hughes[2], T. Cars[1], C. Malmberg[1] and O. Cars[1]

1) *Department of Medical Sciences, Section of Infectious Diseases and* 2) *Department of Medical Biochemistry and Microbiology, Biomedical Center, Uppsala University, Uppsala, Sweden*

Abstract

The increasing prevalence of hospital and community-acquired infections caused by multidrug-resistant (MDR) bacterial pathogens is rapidly limiting the options for effective antibiotic therapy. Systematic studies on combinations of already available antibiotics that could provide an effective treatment against MDR bacteria are needed. We tested combinations of antibiotics that target one important physiological function (peptidoglycan synthesis) at several steps, and studied Enterobacteriaceae (*Klebsiella pneumoniae* and *Escherichia coli*) for which multidrug resistance associated with ESBL-producing plasmids has become a major problem. To measure the effectiveness of antibiotics alone and in combination, we used checkerboard assays, static antibiotic concentration time-kill assays, and an improved *in-vitro* kinetic model that simulates human pharmacokinetics of multiple simultaneously administered antibiotics. The target strains included an MDR *K. pneumoniae* isolate responsible for a recent major hospital outbreak. A double combination (fosfomycin and aztreonam) and a triple combination (fosfomycin, aztreonam and mecillinam) were both highly effective in reducing bacterial populations in all assays, including the *in vitro* kinetic model. These combinations were effective even though each of the MDR strains was resistant to aztreonam alone. Our results provide an initial validation of the potential usefulness of a combination of antibiotics targeting peptidoglycan synthesis in the treatment of MDR Gram-negative bacteria. We suggest that a combination of fosfomycin with aztreonam could become a useful treatment option for such infections and should be further studied.

Keywords: Combination treatment, Enterobacteriaceae, *in vitro* kinetic model, pharmacodynamics, pharmacokinetics, synergy, time kill assay

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Corresponding author: O. Cars, Department of Medical Sciences, Section of Infectious Diseases, Uppsala University, Box 256, 751 05 Uppsala, Sweden

E-mail: otto.cars@medsci.uu.se

Introduction

Access to effective antibiotic chemotherapy is critical to the success of many life-saving procedures in modern medicine, including invasive surgery, cancer chemotherapy, transplantations and the treatment of preterm babies [1]. The global evolution of multidrug-resistant pathogens, driven by the overuse and misuse of antibiotics, and coupled with the lack of new antibiotics in the pipeline [2], could push modern medicine into a post-antibiotic era.

Major resistance problems are being caused by multidrug-resistant (MDR) Gram-negative pathogens [3]. Today, nosocomial infections caused by Enterobacteriaceae in patients with underlying diseases are difficult to treat with available antibiotics and are associated with significantly increased mortality [4]. In part the difficulty in treatment is caused by the prevalence of extended spectrum β-lactamases (ESBL) in Enterobacteriaceae, compromising the effectiveness of the β-lactam antibiotics. The most common class of ESBL in Enterobacteriaceae in Europe is CTX-M-15 [5], usually encoded on plasmids and spread by horizontal gene transfer.

Beginning in 2005 an outbreak of a *Klebsiella pneumoniae* strain producing the CTX-M-15 ESBL occurred within Uppsala University Hospital [6], causing major infection problems for patients [7]. The genetic sequence of the outbreak strain plasmid showed that it was composed of a pKPN3 *K. pneumoniae* plasmid backbone combined with *bla* $_{\text{CTX-M-15}}$ encoded on a multidrug-resistant cassette, probably acquired from the outbreak *Escherichia coli* ST131 strain [8]. The clinical relevance of this isolate, the paucity of treatment options, and the availability of detailed molecular data on resistance mechanisms, motivated us to study whether effective therapy could be achieved using combinations of available antibiotics. As comparator strains we used a fully susceptible *E. coli* and an *E. coli* into which the ESBL plasmid had been conjugated. The ESBL-producing *E. coli* was used in this study as a control of interest as some patients hospitalized during the outbreak were double infected with an ESBL-producing *K. pneumoniae* clone and the acquired *E. coli* strain.

The use of antibiotics in combination is already a common hospital procedure in empirical treatment of severe infections [9], but often guidelines on suitable combinations to use are sparse. We decided to focus exclusively on antibiotics that inhibited one major target (peptidoglycan synthesis), the hypothesis being that targeting one cellular system at multiple points perturbs it beyond recovery and prevents resistance formation from single step compensatory mutations ('The multi-targeting hypothesis') [10]. We also wished to include antibiotics that are not currently widely used, so-called underused or 'forgotten antibiotics' [11], to increase the potential of finding novel synergies. Thus, the antibiotics we concentrated on were fosfomycin, aztreonam and mecillinam. Both fosfomycin and mecillinam (in the prodrug form of pivmecillinam) have been used to treat uncomplicated UTIs, whilst aztreonam has been used to treat complicated Gram-negative infections in hospitalized patients.

Fosfomycin was our main antibiotic of interest, due to its potential as a driving force in antibiotic combination therapy. Fosfomycin displays several interesting pharmacokinetic/pharmacodynamic aspects, i.e. lack of cross-resistance by its unique inhibition of UDP-N- acetyleglucosamin-1-carboxyvinyltransferase [12] and potential to permeabilize bacterial cells to increase uptake of other antimicrobial agents by acting as a chelating agent for magnesium in the outer membrane [13]. Furthermore, fosfomycin has been shown to have good activity against ESBL-producing *K. pneumoniae* isolates, with 81.3% reported positive in a recent study [14]. Aztreonam was selected as a combination partner due to its PBP 3 (and slight PBP1) inhibiting action and previously reported *in-vitro* synergy with fosfomycin against *Pseudomonas aeruginosa* [13]. Mecillinam was selected due to its low levels of resistance, according to a recent study [15]where 89% of CTX-M15-producing *E. coli* strains were reported susceptible, but also because its mechanism of action is inhibition of PBP2 in Gram-negative cells. Thus, the mode of action of mecillinam complements that of aztreonam, which should minimize cross-resistance due to single mutations affecting PBP affinity or expression levels.

Many potentially valuable antibiotics are currently not a therapeutic option in many countries, often because they are not licensed for human use. We believe studies that investigate their therapeutic potential, individually or in combination, can provide support for the political and economic interests required to motivate the changes that can make these drugs more widely available to meet increasingly urgent clinical needs. In the course of this study, we used checkerboard assays, static antibiotic concentration time-kill assays, and an improved *in-vitro* kinetic model that simulates human pharmacokinetics of multiple simultaneously administered antibiotics.

Materials and Methods

Bacterial strains and plasmids

Three strains were used in this study (Table 1): (i) DA15000, a clinical isolate of *K. pneumoniae* from Uppsala University Hospital carrying the multi-resistance plasmids pUUH239.1 (5243 bp) and pUUH239.2 (220 824 bp) [8]; (ii) MG1655, an *E. coli* K-12 wild-type [16]; and (iii) DA14833, *E. coli* MG1655 F-, λ-, *ilvG*, *rfb-50*, *rph-1*, *nalR*, *strR*/pUUH239.2 (transconjugant). The plasmid pUUH239.2 encodes resistance to β-lactams (bla$_{\text{CTX-M-15}}$, bla$_{\text{TEM-1}}$ and bla$_{\text{OXA-1}}$), aminoglycosides [aac-(6′)-1b-cr and aadA2], tetracyclines [tet(A) and tetR], trimethoprim (dhfrXII), sulphonamides (sul1), quaternary ammonium compounds (qacEΔ1), macrolides [mph (A)-mxr-mphR(A)] and the heavy metal ions silver, copper and arsenic [8]. Bacteria were cultivated in Mueller–Hinton II broth (MHII), a cation-adjusted broth (Becton, Dickinson and Company, Sparks, MD, USA) and plated on MHII agar (Difco Laboratories, Detroit, MI, USA).

TABLE 1. Susceptibility testing and FICI for antibiotic combinations

	MIC[a] (*n*=3)			FICI (*n*=3)		
	FOF	ATM	MEC	FOF +ATM	FOF +MEC	ATM +MEC
MG1655	8 (S)	0.625 (S)	0.25 (S)	0.33	0.27	0.2
DA14833	16 (S)	64 (R)	1 (S)	0.23	0.22	0.38
DA15000	32 (S)	64 (R)	4 (S)	0.58	0.32	0.50

[a]S, sensitive; R, resistant; according to EUCAST SIR data.
FICI and MIC data are based on at least three independent assays in each case.