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Research article

Intereactions between doripenem and clavulanate — Application of minimal inhibitory concentration analysis and cytometry flow for bactericidal studies

Katarzyna Ciemniak [a], Judyta Cielecka-Piontek [b], Daria Szymanowska [a,*], Gabriela Wiergowska [b]

[a] Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Wojska Polskiego 48, 60-627 Poznań, Poland
[b] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Poznan University of Medical Sciences, Grunwaldzka 6, 60-780 Poznań, Poland

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ABSTRACT

Background: In view of the current low efficacy of bacterial infection treatment the common trend towards searching for antibiotic systems exhibiting synergistic action is well justified. Among carbapenem analogues a particularly interesting option is provided by combinations of clavulanic acid with meropenem, which have proven to be especially effective.
Results: Determination of the minimal inhibitory concentration (MIC) along with the method based on flow cytometry constitutes an important tool in the identification of bacterial sensitivity to active substances. Within this study the inhibitory effect of doripenem, clavulanic acid and the doripenem-clavulanate acid system was analyzed in relation to such bacteria as *Salmonella enteritidis*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Clostridium butyricum* and *Clostridium pasteurianum*, *Acinetobacter baumannii*, *Enterobacter aerogenes*. The lowest MIC, amounting to 0.03 μg/mL, was observed for the doripenem-clavulanate acid system in the case of *E. coli* ATCC 25922. In turn, the lowest MIC for doripenem applied alone was recorded for *K. pneumoniae* ATCC 31488, for which it was 0.1 μg/mL. The strain which proved to be most resistant both to doripenem and the doripenem-clavulanate acid system, was *A. baumannii*, with MIC of 32 μg/mL (clinical isolate) and 16 μg/mL (reference strain). Cytometric analysis for *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 showed changes in cells following exposure to limiting concentrations of the active substance.
Conclusions: Analysis of MIC supplies important information concerning microbial sensitivity to active substances, mainly in terms of limiting concentrations causing mortality or vitality of the tested species, which is essential when selecting appropriate antibiotic therapy.

1. Introduction

Doripenem is a carbapenem (a β-lactam antibiotic) and contains a carboxyl group in the C-2 position and a trans-β-hydroxyethyl group in the C-6 position. Similar to the other carbapenems excluding ertapenem, it is used in the acid form and exhibits pharmacokinetic parameters comparable to those of meropenem and imipenem [1]. The mode of action of doripenem, similarly as in the case of other carbapenems, consists in the inhibition of bacterial cell wall biosynthesis by binding with penicillin binding proteins (PBPs) [2]. Affinity of doripenem to various PBP types depends on microorganisms. In the case of *Escherichia coli* doripenem shows the greatest affinity to PBP2, in *Pseudomonas aeruginosa* it is to PBP3, while in *Staphylococcus aureus* it is to PBP1, PBP2 and PBP4 [2,3]. Among carbapenems the spectrum of microbiological activity of doripenem is closest to that of meropenem [4]. Doripenem was authorized for use by the U.S. Food and Drug Administration (FDA) in 2007 and by the European Medicines Agency (EMA) in 2008 [5,6]. In Europe it was registered as a drug for treatment of adult patients suffering from such infections as hospital acquired pneumonia, including ventilator-associated pneumonia (VAP), complicated infections within the abdominal cavity and complicated infections of the urinary tract, including pyelonephritis.

Resistance of bacteria to β-lactam antibiotics is determined by diverse mechanisms e.g. the production of β-lactamases, enzymes hydrolyzing β-lactam molecules. This mechanism may be eliminated by supplementing the active substance with a respective inhibitor. Clavulanic acid (CA) may be an example of such a compound. CA is the first clinically useful β-lactam inhibitor with a high affinity to class

* Corresponding author.
E-mail address: daria.szymanowska@up.poznan.pl (D. Szymanowska).
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A β-lactamase [7,8,9,10] and, in effect, an irreversible inhibitor of intracellular and extracellular β-lactamases. Consequently, combinations of clavulanic acid with penam analogs (amoxicillin) are widely used to treat bacterial infections of various etiology. CA exhibits activity against a broad spectrum of Gram-positive and Gram-negative bacteria. However, in comparison to other antibiotics this activity is relatively low. As a result, CA may not be used as an independent bactericide. The application of CA is based on the formation of its complexes with other broad-spectrum antibiotics susceptible to the action of β-lactamases [11].

In view of the current post-antibiotic era with the observed low efficacy of bacterial infection treatment the common trend towards searching for antibiotic systems exhibiting synergistic action is well justified. Among carbapenem analogues a particularly interesting option is provided by combinations of CA with meropenem, which have proven to be especially effective in treatment of multidrug-resistant *Mycobacterium tuberculosis* [12]. Literature on the subject also presents reports in the combination of meropenem with systems of other chemotherapeutics, e.g. amoxicillin-clavulanate and linezoid-calvulanate combinations [13,14] in order to increase bactericidal response to *M. tuberculosis*. Moreover, we need to mention here a stronger bactericidal response for the meropenem-clavulanate system in relation to bacterial strains posing a clinical problem in therapies targeting *P. aeruginosa, S. aureus* and *L. monocytogenes* [15].

Nowadays, one of the biggest fields of interest is to develop methods which enable fast, accurate assessment of presence of microorganisms and also its viability and functionality [16]. Traditional analytical methods provide information about whole population of microorganisms and there is no possibility to distinguish intermediate states of evaluated objects. In comparison, methods using fluorescent markers allow single-cell analysis and enable distinguish various subpopulations [17,18]. Flow cytometry is an advanced method used for quantitative, as well as qualitative assessment of individual microbial cells. Cytometric measurement can be based both on light dispersion and fluorescence emitted by tested cells. Direct analytical tools of this type don't require growth of microorganisms on artificial media, therefore it gives an opportunity to assess the existence of viable but non-culturable microbial cells [18,19,20].

This method provides an information about cell complexity and physiology, there are many fluorescent probes to evaluate such parameters as membrane potential and integrity, enzyme activity and intracellular pH [16,17]. It is possible to assess few parameters of tested cells at once, using simultaneous staining with more than one fluorescent dye. Multiparametric analysis provides quick and precise information about dynamics and physiological differentiation within the population [20,21]. At first, flow cytometry was developed for different clinical applications, like oncology or hematology. Now use of this technique expands on microbiology field with great results and enables obtaining information about very complex, heterogenous samples in short period of time [16,17].

The aim of this study was to determine the minimal inhibitory concentration for selected indicator microorganisms along with the cytometric analysis illustrating changes in limiting concentrations inhibiting microbial growth for the binary system of doripenem and clavulanic acid, while bactericidal activity of doripenem and clavulanic acid was considered as the reference value.

2. Material and methods

2.1. Microorganisms

The bacterial strains analyzed using MIC analysis and flow cytometric assay included ATCC reference strains and clinical isolates (*) from the Institute of Laboratory Medicine at the Clinic Poznan, Poland. A total of 22 microorganisms were included in this study. The reference strains were obtained from the American Type Culture Collection (ATCC) and included: *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 31488, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 8427, *Clostridium butyricum* ATCC 860, *Clostridium pasterianum* ATCC 6013, *Acinetobacter baumannii* ATCC 19606, *Enterobacter aerogenes* ATCC 13048.

2.2. Microbiological analysis

Minimal Inhibitory Concentration (MIC) was determined for each reference strain from the American Type Culture Collection and clinical isolates. MICs for doripenem, clavulanate acid and their system were assayed using serial dilutions on the Mueller–Hinton liquid medium (Merck, Germany). Microbial culture with a standardized optical density was used in that experiment. The applied method follows the standards of the National Committee for Clinical Laboratory Standards (NCCLS) [22]

2.3. Flow cytometry

In the flow cytometric susceptibility assay two bacterial reference strains were investigated, i.e. *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. Samples were analyzed using a Guava easyCyte™ 8 (Merck Millipore) flow cytometer, equipped with 2 lasers (488 nm and 640 nm), 6 fluorescence detectors, forward scatter (FSC) and side scatter (SSC) detectors. The instrument setup (optical alignment) was performed using the guava® easyCheck Kit (Merck Millipore). The particles were characterized by two non-fluorescent parameters: forward scatter (FSC) and side scatter (SSC), and one fluorescent parameters - red fluorescence from propidium iodide (PI). A 640 nm red laser was employed in excitation of the fluorescent reagent. Forward scatter (FSC) and side scatter (SSC) measurements were applied in the analyses. FSC and SSC parameters are designated to cell size and complexity. Flow cytometry analyses were performed using logarithmic gains and specific detector settings (5000 events were recorded per analysis). The threshold was set at the FSC signals. Data were acquired in a four-decade logarithmic scale as height signals and analyzed with the guavaSoft 2.6 software (Merck Millipore). Samples were analyzed in triplicates.

3. Results

The primary aspect of the study comprised analyses of the effect of doripenem and clavulanic acid as well as the system of doripenem with clavulanic acid on eleven selected species of indicator microorganisms. Analyses were performed on reference strains and clinical isolates (Table 1). *C. pasteurianum, K. pneumoniae* and *E. aerogenes* were the microbial species exhibiting the greatest sensitivity to doripenem. The minimal inhibitory concentrations were 0.25, 0.1 and 0.25 μg/mL for reference strains and 0.25, 2 and 1 μg/mL, respectively, for clinical isolates. The highest MIC values were recorded for strains of *A. baumannii* and *S. aureus* both in the case of reference strains and clinical isolates. For *A. baumannii* ATCC 19606 MIC it was 16 μg/mL in relation to doripenem, while for the clinical isolate of that species it was 32 μg/mL. In the case of *S. aureus* MIC was 8 μg/mL both for the reference strain and the clinical isolate. Moreover, the effect of clavulanic acid was investigated for selected microbial species exhibiting a pathogenic potential. No effect of the β-lactamase inhibitor on bacteria from the genus *Salmonella,* or such species as *K. pneumoniae, C. butyricum* and *A. baumannii.* For the other species MIC values were much higher than for doripenem. In the case of *E. aerogenes* MIC for CA was 250 μg/mL, for *P. aeruginosa* MIC amounted to 125 μg/mL, while for *P. vulgaris* it was 32 μg/mL both for reference strains and clinical isolates. A combination of doripenem and clavulanic acid in many cases made it possible to