Diagnostic Microbiology and Infectious Disease 79 (2014) 125–134

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Evaluation of the best method to assess antibiotic potentiation by phytochemicals against *Staphylococcus aureus*

Ana Cristina Abreu [a], Sofia C. Serra [b,c], Anabela Borges [a,d], Maria José Saavedra [d], António J. Salgado [b,c], Manuel Simões [a,*]

[a] LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr Roberto Frias, s/n, 4200–465 Porto, Portugal
[b] Life and Health Sciences Research Institute (ICVS), School of Health Sciences,University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal
[c] ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal
[d] CECAV, Centro de Ciência Animal e Veterinária, Departamento de Ciências Veterinárias, Universidade de Trás-os-Montes e Alto Douro, Quinta de Prados, 5000-801 Vila Real, Portugal

ARTICLE INFO

Article history:
Received 10 October 2013
Received in revised form 18 February 2014
Accepted 2 March 2014
Available online 12 March 2014

Keywords:
Alkaloids
Co-therapy
Efflux pump
Potentiation
Resistance-modifying agents

ABSTRACT

The increasing occurrence of bacterial resistance to antibiotics has now reached a critical level. Finding antibiotic coadjuvants capable to inhibit the bacterial resistance mechanisms would be a valuable mid-term solution, until new classes of antibiotics are discovered. Selected plant alkaloids were combined with 5 antibiotics against 10 *Staphylococcus aureus* strains, including strains expressing distinct efflux pumps and methicillin-resistant *S. aureus* strains. The efficacy of each combination was assessed using the microdilution checkerboard, time-kill, Etest, and disc diffusion methods. The cytotoxicity of the alkaloids was evaluated in a mouse fibroblast cell line. Potentiation was obtained in 6% of all 190 combinations, especially with the combination of: ciprofloxacin with reserpine (RES), pyrrolidine (PYR), and quinine (QUIN); tetracycline with RES; and erythromycin with PYR. The highest cytotoxicity values were found for QUIN (half maximal inhibitory concentration [IC_{50}] = 25 ± 2.2 mg/L) and theophylline (IC_{50} = 100 ± 4.7 mg/L).

1. Introduction

The evolution of bacterial resistance increased over the last years, and given the difficulty of new drug discovery by the traditional methods, new strategies are urgently needed. The use of paired and even triple combinations of antimicrobial drugs with positive *in vitro* interactions has become increasingly important in clinical applications. The combinatorial therapy has also been a common practice in order to prevent the emergence and widespread of multi-drug–resistant (MDR) infections (Sopirala et al., 2010). Indeed, the biological system is less able to compensate for the action of 2 or more drugs simultaneously (Zimmermann et al., 2007). Also, the cost of developing a new antimicrobial versus the cost of finding a combination between known ones is an easy justification for research into drug potentiation (Lambert et al., 2003).

While several bioactive compounds have a significant antimicrobial activity, other compounds have been found to be synergistic enhancers of antibiotics, despite they may not have any antimicrobial properties alone (Abreu et al., 2012b). The modes of action underlying the synergistic activity of these antibiotic adjuvants can be diverse. Two important mechanisms include the serial or orthogonal inhibition of vital physiological pathways or the dispersion of a biofilm to planktonic cells, resulting in an increased susceptibility to antibiotics (Kalan and Wright, 2011). Also, several antibiotic adjuvants have been evaluated for their action as resistance-modifying agents (RMAs) (Hemaiswarya et al., 2008), i.e., compounds that can modify or inhibit the bacterial mechanisms of resistance, so that antibiotics can efficiently kill the resistant bacteria. Several RMAs were already described (Chan et al., 2011; Gibbons et al., 2003; Khan et al., 2006; Roccaro et al., 2004; Schmitz et al., 1998; Yam et al., 1998). Efflux pump inhibitors (EPIs) are important examples of RMAs since they can prevent the extrusion of an antibiotic to the exterior of the cell and thus allow the antibiotic to act efficiently against bacteria. If such compounds were clinically approved, many inefficient and old antibiotics (for which resistance mechanisms are already disseminated) could be recycled. Therefore, many of the current problems on the lack of new antibiotics and new classes of antimicrobials would be possible to solve.

It is unquestionable that natural compounds have been a major source of new bioactive agents. In fact, natural compounds were already "prescreened" over millions of years ago by natural selection, which puts them ahead in the race for the discovery of new antimicrobials. Plants have been traditionally used for centuries to treat human diseases and inhibit microbial growth. They are important sources of a wide variety of secondary metabolites such as alkaloids, isothiocyanates, peptides, phenolics, polyacetilenes, and terpenoids, which have been well-established to possess

* Corresponding author. Tel.: +351-225081654; fax: +351-225081449.
E-mail address: mvs@fe.up.pt (M. Simões).

http://dx.doi.org/10.1016/j.diagmicrobio.2014.03.002
0732-8893/

antimicrobial properties (Phatthalung et al., 2012). Alkaloids are heterocyclic nitrogen compounds (Cowan, 1999). There is an excellent rationale that plant alkaloids should possess antibacterial activity, particularly given the number of cytotoxic drugs and templates from this source (Gibbons, 2004).

The objective of this work was to determine whether several alkaloids (caffeine [CAF], reserpine [RES], pyrrolidine [PYR], theophylline [THEO], and quinine [QUIN], Fig. 1) were able to improve the activity of common antibiotics belonging to several classes (ampicillin [AMP] and oxacillin [OXA] – β-lactams; ciprofloxacin [CIP] – fluoroquinolone; erythromycin [ERY] – macrolide; and tetracycline [TET]). Several resistant strains of *Staphylococcus aureus* were used. *S. aureus* and, specifically, MRSA strains are important pathogens in clinical settings, responsible for a high level of hospital-acquired infections (Oluwatuyi et al., 2004), due to their great capacity of acquiring resistance genes. The methods for detecting potentiation used in this work were the broth microdilution checkerboard, time-kill assay, Etest, and the disc diffusion method (DDM). A comparison of the results of potentiation given by these 4 methods was evaluated.

2. Materials and methods

2.1. Bacterial strains

Three clinical MRSA (MJMC001, MJMC002, MJMC004) and 3 clinical MSSA (MJMC003, MJMC009, MJMC010) were isolated from patients with diabetic feet at the Centro Hospitalar de Trás-os-Montes e Alto Douro, EPE (Portugal). *S. aureus* SA1199B, which overexpresses the NorA MDR efflux pump, *S. aureus* RN4220, which contains plasmid pU5054 (that carries the gene encoding the MsrA macrolide efflux protein), and *S. aureus* XU212, which possesses the TetK efflux pump and is also an MRSA strain, were kindly provided by S. Gibbons (University College London, UCL) (Gibbons and Udo, 2000; Gibbons et al., 2003; Oluwatuyi et al., 2004; Smith et al., 2007). The collection strain *S. aureus* CECT 976, already used as model microorganism for antimicrobial tests with phytochemical compounds(Abreu et al., 2012a; Saavedra et al., 2010; Simões et al., 2008), was included as a quality control strain. Prior to use, each strain at −80 °C was transferred onto Mueller–Hinton (MH; Merck, Darmstadt, Germany) agar plate, grown overnight, and inoculated into MH broth at 37 °C and under agitation (150 rpm).

2.2. Antibiotics and alkaloids

AMP, CIP, ERY, OXA, and TET were obtained from Sigma (Portugal) and prepared according to the manufacturer recommendations. CAF, RES, PYR, THEO, and QUIN were purchased from Sigma and the stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma, Sintra, Portugal). Etest strips of the antibiotics (AB Biodisk, Solna, Sweden) were obtained from Izasa (Portugal, Barcelona, Spain).

2.3. Antibacterial susceptibility testing

The MIC of each agent was determined by microdilution techniques according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (NCCLS/CLSI, 2003). For *S. aureus* SA1199B, RN4220, and XU212, only CIP, ERY, and TET were tested, respectively. Bacteria (~10^6 CFU/mL) were inoculated into MH broth and dispensed at 200 μL/well in 96-well microtiter plates, along with 2-fold dilutions of the compounds to test. Several intermediate concentrations were also prepared in order to minimize the errors in MIC determination. MIC was defined as the lowest concentration of the antimicrobial compound that inhibited bacterial growth after 24 h of incubation at 37 °C. The bacterial growth was determined at 600 nm using a microplate reader (Spectramax M2e; Molecular Devices, Inc., Sunnyvale, USA). Three independent experiments were performed for each compound. The highest concentration of DMSO remaining after dilution (10% (v/v)) caused no inhibition of bacterial growth (data not shown).

2.4. Checkerboard microdilution assay

Checkerboard testing was performed in 96-well microtiter plates in triplicate according to Chan et al. (2011) with some modifications. After an overnight incubation into MH broth at 37 °C, bacterial cultures were adjusted in fresh broth to approximately 10^6 CFU/mL.

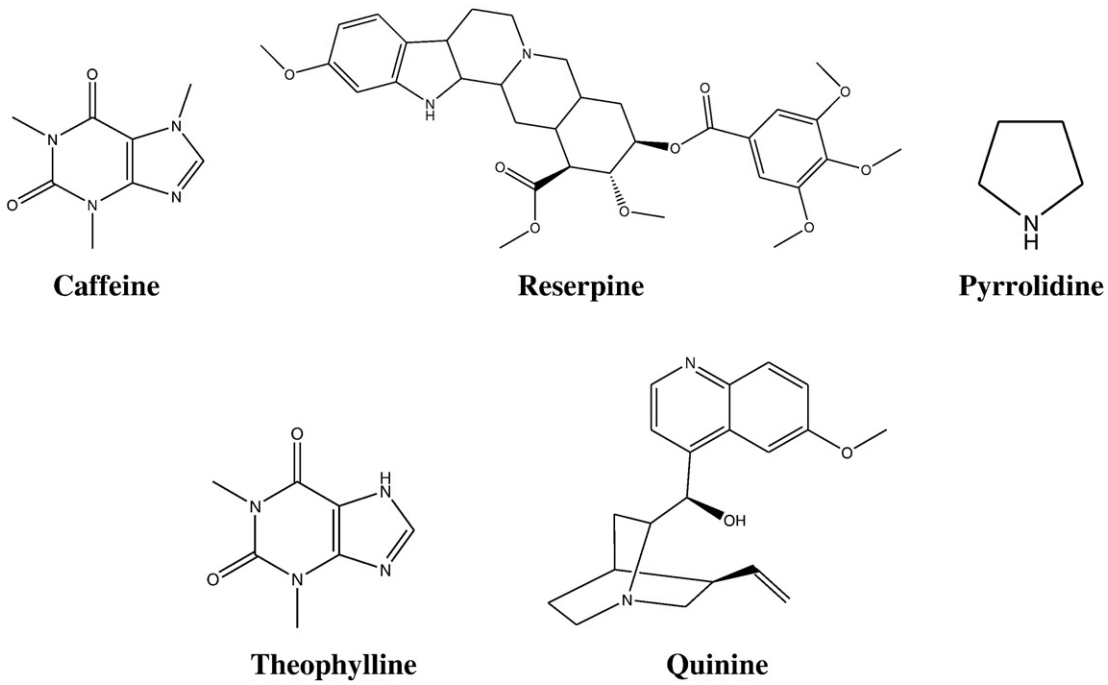

Fig. 1. Chemical structures of the alkaloids used in this study.