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Pharmacological synergism of bee venom and melittin with antibiotics and plant secondary metabolites against multi-drug resistant microbial pathogens

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ABSTRACT

The goal of this study was to investigate the antimicrobial activity of bee venom and its main component, melittin, alone or in two-drug and three-drug combinations with antibiotics (vancomycin, oxacillin, and amikacin) or antimicrobial plant secondary metabolites (carvacrol, benzyl isothiocyanate, the alkaloids sanguinarine and berberine) against drug-sensitive and antibiotic-resistant microbial pathogens. The secondary metabolites were selected corresponding to the molecular targets to which they are directed, being different from those of melittin and the antibiotics.

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were evaluated by the standard broth microdilution method, while synergistic or additive interactions were assessed by checkerboard dilution and time-kill curve assays. Bee venom and melittin exhibited a broad spectrum of antibacterial activity against 51 strains of both Gram-positive and Gram-negative bacteria with strong anti-MRSA and anti-VRE activity (MIC values between 6 and 800 μg/ml). Moreover, bee venom and melittin showed significant antifungal activity (MIC values between 30 and 100 μg/ml). Carvacrol displayed bactericidal activity, while BITC exhibited bacteriostatic activity against all MRSA and VRE strains tested (reference strains and clinical isolates), both compounds showed a remarkable fungicidal activity with minimum fungicidal concentration (MFC) values between 30 and 200 μg/ml. The DNA intercalating alkaloid sanguinarine showed bactericidal activity against MRSA NCTC 10442 (MBC 20 μg/ml), while berberine exhibited bacteriostatic activity against MRSA NCTC 10442 (MIC 40 μg/ml).

Checkerboard dilution tests mostly revealed synergism of two-drug combinations against all the tested microorganisms with FIC indexes between 0.24 and 0.50, except for rapidly growing mycobacteria in which combinations exerted an additive effect (FICI = 0.75–1). In time-kill assays all three-drug combinations exhibited a powerful bactericidal synergistic effect against MRSA NCTC 10442, VRE ATCC 51299, and *E. coli* ATCC 25922 with a reduction of more than $3\log_{10}$ in the colony count after 24 h. Our findings suggest that bee venom and melittin synergistically enhanced the bactericidal effect of several antimicrobial agents when applied in combination especially when the drugs affect several and differing molecular targets. These results could lead to the development of novel or complementary antibacterial drugs against MDR pathogens.

Introduction

Honeybee venom is a complex mixture of pharmacologically active chemicals (Gajski and Garaj-Vrhovac 2013). Bee venom has been widely used in traditional medicine to treat various inflammatory disorders such as rheumatism, arthritis, tendonitis, fibrosis, lupus, multiple sclerosis and to dissolve scar tissue (Ali 2012). In addition, several studies have reported its cytotoxic properties against various types of tumor cells such as lung, liver, kidney, prostate, bladder, breast, and leukemic cancer cells (Jo et al. 2012; Tu et al. 2008). However, bee venom has some drawbacks such as strong neurotoxicity, cardiotoxicity, nephrotoxicity, hemolytic and immunogenic effects which limits its application (Cojol and Join 2000).

Besides 88% water, bee venom consists of at least 18 pharmacologically active components including a variety of peptides [melittin, apamin, adolapin, and mast cell deregulating (MCD) peptide], enzymes (phospholipase A2 and hyaluronidase), bioactive amines (histamine and epinephrine), and other non-peptide components

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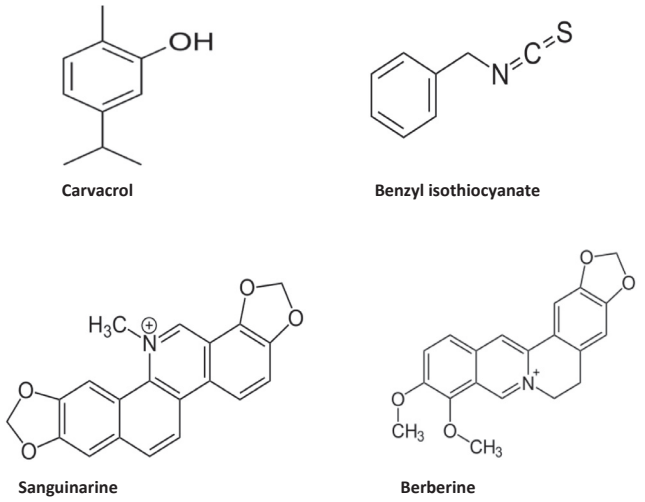

Fig. 1. Chemical structures of plant secondary metabolites used in this study.

(lipids, carbohydrates and free amino acids), minerals and volatiles (Tu et al. 2008; Zhou et al. 2010). The principal toxin and the major active peptide is melittin (52% of bee venom dry weight), a polypeptide consisting of 26 amino acid residues with a molecular weight of 2849 Da. Melittin exerts multiple effects towards various cell types, including anticancer, anti-inflammatory, antibacterial, and antiviral effects (Falco et al. 2013). Recently, it was demonstrated that melittin which had been loaded onto nanoparticles was able to target and inactivate human immunodeficiency virus (HIV) without harming healthy human cells (Hood et al. 2013).

The emergence and rapid spread of MDR bacterial infections, especially methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), extended-spectrum-beta-lactamase producing *E. coli* and multidrug-resistant *Mycobacterium tuberculosis* have increased the rate of patient mortality and morbidity and has become a major challenge in clinical treatment of patients with conventional antibiotics (Phougat et al. 2014).

The purpose of this study was to investigate the antimicrobial effect of bee venom and its major component melittin alone or in two- and three-drug combinations with antibiotics (vancomycin, oxacillin, and amikacin) or known antimicrobial plant secondary metabolites (carvacrol, benzyl isothiocyanate, sanguinarine, and berberine; Fig. 1) (Van Wyk and Wink 2004) against drug-sensitive and multidrug resistant pathogens including Gram-positive bacteria, Gram-negative bacteria, yeasts, and rapidly growing mycobacteria (RGM) which have a similar cell wall structure as the pathogen *M. tuberculosis*. The idea was to study antimicrobial compounds which affect molecular targets (biomembranes, proteins, DNA) that are different from those of the antibiotics. In this investigation we employed the broth microdilution method to determine MIC and MBC values of single antimicrobial compounds, while checkerboard dilution and time kill assays were used to evaluate the potential synergistic properties of multidrug combinations.

Materials and methods

Mass spectrometry of bee venom and melittin

Molecular weight determinations of peptides were performed using a ESI QTOF hybrid mass spectrometer (QStar, Applied Biosystems) as described previously (Tu et al. 2008). Briefly, samples were trapped on a porous R1 reversed phase column, washed with 0.1% formic acid and eluted with 80% acetonitrile, 0.1% formic acid and ionized by electrospray. Acquired spectra were deconvoluted using the software Bayesian Protein Reconstruct, supplied with the instrument.

Chemicals

Lyophilized powders of honeybee venom (*Apis mellifera*), melittin (≥85% purity), and phospholipase A2 (PLA_2) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Oxacillin, amikacin, benzyl isothiocyanate (98% purity), carvacrol (98% purity), sanguinarine, and berberine were obtained from Sigma–Aldrich (Steinheim, Germany), streptomycin, vancomycin from Applichem (Darmstadt, Germany), and nystatin from Cellpharm (Hannover, Germany).

Microorganisms

A total of thirty reference strains (Gram-positive, Gram-negative, fungi, and RGM) from American Type Culture Collection (ATCC) were used in this study and are listed in Tables 1–3. In addition, three strains of MRSA, three strains of VRE, six strains of Gram-negative bacteria and four strains of fungi isolated from clinical sources were included in this study. The latter strains were supplied by Medical Microbiology Lab., Hygiene Institute, Heidelberg University, Germany.

Culture media and inoculum preparation

All bacterial strains were cultivated on Columbia Agar supplemented with 5% sheep blood (Becton Dickinson, Germany) and cation-adjusted Muller-Hinton broth (CAMHB) (Fluka, Buchs, Switzerland) except VRE and streptococci, for which Brain Heart Infusion (BHI) (Merck, Darmstadt, Germany) was used. CHROMagar *Candida* medium (Becton Dickinson, Heidelberg, Germany) and Sabouraud Dextrose broth (SDB) (Merck, Darmstadt, Germany) were employed for the cultivation of fungi. Inocula were prepared from pure cultures grown on agar plates; one or two colonies of microorganisms were suspended in sterile saline until a turbidity equivalent of 0.5 McFarland standard was achieved (1×10^8 colony forming unit per ml (cfu/ml) for bacteria and 1×10^6 cfu/ml for fungi). These suspensions were diluted in broth to get final organism density of approximately 1×10^6 cfu/ml and 1×10^5 cfu/ml for bacteria and fungi, respectively.

Determination of minimum inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC)

MIC and MBC analyses were performed according to CLSI standards (Clinical and Laboratories Standards Institute 2006). Bacteria or fungi were cultured on appropriate agar plates to prepare inoculum suspensions with a final concentration of approximately 1×10^6 bacteria and 1×10^5 yeast cells which were added to each well of a 96-well microtiter plate containing two-fold serial dilution of the samples. The plates were incubated at 37 °C for 24 h, 48 h, and 72 h for bacteria, yeast, and RGM respectively. The MIC values were scored as the lowest concentration of compounds that inhibited growth of the microorganisms after a given incubation period. MBC values were determined by subculturing 10 μl of each well greater than or equal to the MIC in appropriate agar medium and incubated under the appropriate condition as mentioned above. The MBC was defined as the lowest concentration of the sample showing no visible growth on agar plates after the incubation period. Vancomycin, streptomycin, nystatin, and amikacin were also included as positive controls or reference drugs.

Checkerboard dilution

Serial 2-fold dilutions of bee venom or antimicrobial agents were mixed in each well of a 96-well microtiter plate. Fifty-microliter aliquots of the first antimicrobial agent and second antimicrobial agent were added in vertical and horizontal orientation, respectively. A 100 μl of fresh bacterial suspension (1×10^6 cfu/ml) was added to each well and incubated for the specified time and temperature as