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Natural isothiocyanates express antimicrobial activity against developing and mature biofilms of *Pseudomonas aeruginosa*

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ABSTRACT

Background: The antimicrobial properties of natural isothiocyanates (ITCs) found in plants such as nasturtium (*Tropaeolum majus*) and horseradish (*Armoracia rusticana*), and the need of new chemotherapeutic options for treatment of infections caused by multidrug-resistant and biofilm-forming Gram-negative bacteria such as *Pseudomonas aeruginosa* (Pa), led us to evaluate the effects of three major ITCs, allylisothiocyanate (AITC), benzylisothiocyanate (BITC), and phenylethyl-isothiocyanate (PEITC), and a mixture (ITCM) adapted to the ITC composition after release of active components out of natural sources.

Material/methods: Out of 105 Pa isolates 27 isolates with increased biofilm formation were selected for testing. The effects of ITCs on Pa were evaluated regarding (1) planktonic bacterial proliferation, (2) biofilm formation, (3) metabolic activity in mature biofilms, and (4) synergism of ITCs and antibiotics.

Results: (1) Each ITC had anti-Pa activity. Mean minimum inhibitory concentrations (MICs) were (μg/ml, mean ± standard deviation): AITC 103 ± 6.9; BITC, 2145 ± 249; PEITC 29,423 ± 1652; and ITCM, 140 ± 5. (2) Treating bacteria with PEITC and ITCM in concentrations below the MIC significantly inhibited biofilm formation. Particularly, ITCM reduced biofilm mass and bacterial proliferation. (3) ITCs significantly inhibited metabolic activity in mature biofilms. (4) Combining ITCs with meropenem synergistically increased antimicrobial efficacy on Pa biofilms.

Conclusions: ITCs represent a promising group of natural anti-infective compounds with activity against Pa biofilms.

1. Introduction

Pseudomonas aeruginosa (Pa) is one of the major bacterial pathogens causing nosocomial infections, and in recent years, multidrug-resistant (MDR) and extensively drug-resistant (XDR) lineages of Pa have emerged in the hospital setting. Owing to many intrinsic resistance mechanisms, only few therapeutics remain to effectively treat Pa infections. The increasing prevalence rates of nosocomial MDR and XDR Pa lineages contribute to this problem [1–3].

Bacterial microorganisms can adhere to surfaces and build up a bacterial biofilm consisting of bacterial cells and a surrounding extracellular polymer substance (EPS). Biofilm-forming microorganisms as well as biofilms are a recognized cause of hospital-acquired infections, such as urinary tract and catheter-related bloodstream infections or infections based on implanted medical devices including indwelling catheters, artificial heart valves, orthopedic prostheses, or osteosynthesis materials [4–8]. Additionally the ability of Pa strains to form biofilms is known as independent predictive factors for mortality in patients with Pa bacteremia as well as for the establishment of recurrent urinary tract infections [9,10]. At the same time, bacteria in biofilms are more resistant to common antibacterial compounds and substances than bacteria in the planktonic state [5,11–13]. The fact that Pa is known as an outstanding biofilm former that is additionally equipped with a broad palette of resistance mechanisms results in very limited therapeutic options. There is a need to identify new antimicrobial substances with less potential to trigger development of resistance in the target organism as well as anti-biofilm efficacy.

Antimicrobial compounds and substances isolated from natural sources with known antibacterial properties are therefore of special interest. Examples of natural compounds with antimicrobial activity are isothiocyanates (ITC), being a promising alternative for conventional antibiotics due to their expected antimicrobial activity [14–16]. ITCs

Abbreviations: AITC, allylisothiocyanate; BITC, benzylisothiocyanate; CIP, ciprofloxacin; EPS, extracellular polymer substance; ITC, isothiocyanate; ITCM, isothiocyanate mixture; MDR, multidrug-resistant; MEM, meropenem; MIC, minimum inhibitory concentration; Pa, *Pseudomonas aeruginosa*; PEITC, phenylethylisothiocyanate; XDR, extensively drug-resistant

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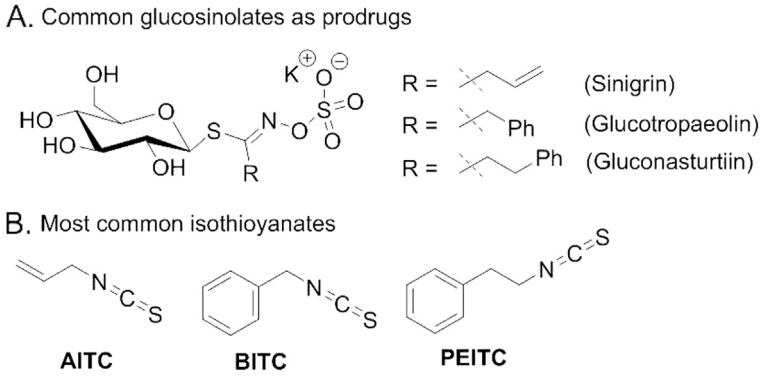

Fig. 1. ITCs occur in nature usually in form of glucosinates which are glycoside-derived prodrugs that bear the biologically interesting ITC group attached to the anomeric center (A). AITC, BITC or PEITC are released mostly by enzyme-mediated hydrolysis of the plant-derived glucosinolates furnishing ITCs with the substitution pattern of amino acids in their side chain (B).

are found in plants such as nasturtium (*Tropaeolum majus*) and horseradish (*Armoracia rusticana*) with distinct compositions of the major components allylisothiocyanate (AITC), benzylisothiocyanate (BITC), and phenylethylisothiocyanate (PEITC) respectively their corresponding glucosinolates Sinigrin, Glucotropaeolin and Gluconasturtiin [14,17]. ITCs occur in nature usually in form of glucosinates which are glycoside-derived prodrugs that bear the biologically interesting ITC group attached to the anomeric center (Fig. 1A) According to their biosynthesis from amino acids, several types of the glucosinate prodrugs are known, including aromatic and aliphatic side chains. The antimicrobial target compounds (e.g. AITC, BITC or PEITC) are released mostly by enzyme-mediated hydrolysis of the plant-derived glucosinolates furnishing ITCs with the substitution pattern of amino acids in their side chain [18] (Fig. 1B).

The objective of our study was to assess the potential of ITCs for the treatment of biofilm-related infections caused by the outstanding biofilm former Pa. We therefore assessed the antimicrobial efficacy of three ITCs, AITC, BITC, and PEITC, as well as a mixture of all three compounds (ITCM) reflecting the proportions of active agents in natural sources and derived phytomedical preparations such as Angocin® (Repha Gmbh, Langenhagen, Germany). The impact of ITCs was analyzed on mature and developing biofilms of clinical Pa lineages isolated either from clinical patients with signs and symptoms of infections or from the hospital environment. Furthermore, we assessed possible additive effects of ITC and the antibiotic meropenem (MEM).

2. Materials/methods

2.1. Stock solutions

Stock solutions of the ITCs, AITC, BITC, and PEITC (Sigma–Aldrich, Taufkirchen, Germany) were prepared in dimethylsulfoxide (Sigma–Aldrich) at 50 mg/ml. Synthesized nature-identical ITC compounds were used to ensure highest possible purity and to exclude unspecific effects i.e. interactions with other plant compounds. The tested ITCs conformed to a purity of 97% for AITC, 98% for BITC and 99% for PEITC according to the manufacturers' specifications. For each assay, the stock solution was diluted to the appropriate working concentration in culture medium. The ITCM mixture consisted of 38% (v/v) AITC, 50% BITC, and 12% PEITC according to the proportion of nasturtium (*Tropaeolum majus*) and horseradish (*Armoracia rusticana*) in the phytomedical preparation Angocin® and the composition of glucosinolates in both plants [19,20]. Stock solutions of MEM (Dr. Friedrich Eberth Arzneimittel, Ursensollen, Germany) were prepared in sterile deionized water.

2.2. Bacterial isolates

We tested the capacity for biofilm development of 105 Pa isolates. Twenty-seven isolates with increased biofilm-forming capacity were selected and used for further experiments. The selected Pa isolates showed a 2- to 6-fold increased capacity to produce EPS compared with standard isolates PAO1 (ATCC 15692) and PA14 (DSM 19882). The selected isolates consisted of five environmental strains, five blood culture isolates, and 17 MDR and XDR Pa strains from invasive and noninvasive clinical samples and were classified as MDR, XDR or susceptible according to Magiorakos et al. [21]. For comparative purposes, the well-characterized strains PAO1 and PA14 were included in this study.

2.3. Determination of minimum inhibitory concentrations of ITCs

Serial doubling dilutions of the proprietary ITCs were prepared in M63 minimal medium consisting of 0.015 M ammonium hydrogen sulfate (Carl Roth, Karlsruhe, Germany), 0.1 M potassium dihydrogen sulfate (Sigma–Aldrich, Steinheim, Germany), 1.8 µM iron sulfate heptahydrate (Carl Roth), 1 mM magnesium sulfate heptahydrate, 2 ml/l glycerol (VWR Chemicals, Darmstadt, Germany), and 1 g/l casein hydrolysate standard (Carl Roth). Subsequently, 50 µl of each dilution was transferred to 96-well microtiter plates (Sarstedt, Nümbrecht, Germany). Wells containing only M63 medium were used as growth controls, and 50-µl aliquots of standardized bacterial preparations were added to the microtiter plates. Plates were incubated for 24 h at 37 °C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of ITC at which no bacterial growth was observed. Resazurin (Sigma–Aldrich, Hamburg, Germany) was used as an indicator of bacterial growth, and a resazurin conversion to resorufin of at least 5% of controls was defined as positive for bacterial growth (MIC_{95}).

2.4. Preparation of bacterial biofilms

Pa isolates were cultured on Columbia blood agar plates at 37 °C for 24 h. Colonies were harvested and transferred into M63 minimal medium and were adjusted to approximately 1×10^9 colony-forming units (CFU)/ml. Biofilms were cultured for 18 h in 96-well plates (Sarstedt) under static conditions at 37 °C at saturated humidity. After incubation, the biofilms were washed twice with 0.9% (w/v) NaCl solution, and biofilms were stained using a 0.1% (w/v) solution of crystal violet (Sigma–Aldrich) in 0.9% (w/v) NaCl. Biofilms were then rinsed twice with distilled water and dried at 37 °C for 1 h. The biofilm-bound crystal violet was solubilized in 80% ethanol. Quantification was performed by determining crystal violet absorbance at 590 nm using a Sunrise RC photometer (Tecan, Männedorf, Switzerland).