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Screening a repurposing library for potentiators of antibiotics against *Staphylococcus aureus* biofilms

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ABSTRACT

Staphylococcus aureus biofilms are involved in a wide range of infections that are extremely difficult to treat with conventional antibiotic therapy. We aimed to identify potentiators of antibiotics against mature biofilms of *S. aureus* Mu50, a methicillin-resistant and vancomycin-intermediate-resistant strain. Over 700 off-patent drugs from a repurposing library were screened in combination with vancomycin in a microtitre plate (MTP)-based biofilm model system. This led to the identification of 25 hit compounds, including four phenothiazines among which thioridazine was the most potent. Their activity was evaluated in combination with other antibiotics both against planktonic and biofilm-grown *S. aureus* cells. The most promising combinations were subsequently tested in an in vitro chronic wound biofilm infection model. Although no synergistic activity was observed against planktonic cells, thioridazine potentiated the activity of tobramycin, linezolid and flucloxacillin against *S. aureus* biofilm cells. However, this effect was only observed in a general biofilm model and not in a chronic wound model of biofilm infection. Several drug compounds were identified that potentiated the activity of vancomycin against biofilms formed in a MTP-based biofilm model. A selected hit compound lost its potentiating activity in a model that mimics specific aspects of wound biofilms. This study provides a platform for discovering and evaluating potentiators against bacterial biofilms and highlights the necessity of using relevant in vitro biofilm model systems.

1. Introduction

Staphylococcus aureus is a notorious opportunistic pathogen that is involved in a wide range of infections, including chronic wounds, endocarditis and infections associated with indwelling medical devices [1]. Treatment of these infections is challenging due to the frequent occurrence of antimicrobial-resistant strains such as methicillin-resistant *S. aureus* (MRSA) [2]. In addition, *S. aureus* often reside within biofilms at the infection site [1]. Biofilms are cell communities embedded in a self-produced extracellular matrix in which the bacterial cells are protected from the host's immune system and the activity of antimicrobial agents [1]. Biofilm-related infections often fail to respond to antibiotic therapy, leading to chronic infections for which more effective treatments are urgently needed [1].

An approach to fill the antibiotic pipeline is screening repurposing libraries [3]. In drug repurposing or repositioning, drugs are used to treat diseases for which they were not initially developed [4]. This approach has several advantages over de novo drug development, e.g. long-term toxicity and metabolic properties are already known [4]. This reduces the risk, time and costs to bring the repurposed drug to the market [5]. Although several non-antibiotic drugs have antimicrobial activity [6], none of these are currently used in antibacterial therapy since their bactericidal activity is often only observed at very high concentrations, making them clinically less useful [7]. However, combination of antibiotics with non-antibiotics as potentiators ('helper' compounds) could result in a greater antimicrobial effect and less toxicity when lower concentrations of both can be used. For this reason, the search for antibacterial potentiators has recently gained interest [8].

Many whole-cell screenings are conducted using general growth medium and measure inhibition of bacterial growth as the endpoint [9]. However, growth conditions and endpoints used to measure antibacterial activity are important parameters, and different hits can be identified depending on whether compounds are screened for activity against planktonic or biofilm cells [9,10]. Investigating alternative endpoints, such as the ability to inhibit biofilm formation or quorum sensing, can lead to interesting results [11,12]. However, few screenings are performed against biofilms [13,14] and few studies have evaluated off-patent drugs for their possible potentiator activity towards antibiotics that could be used for topical treatment of biofilm-related infections, including chronic wound

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infections [15]. In addition, few studies have investigated the activity of hit compounds under conditions simulating the host environment. In the present study, a repurposing screen was performed in the presence of vancomycin against *S. aureus* biofilms, followed by validation of a selected hit in an in vitro chronic wound biofilm model.

2. Materials and methods

2.1. Strains and culture conditions

Staphylococcus aureus Mu50, a methicillin-resistant and vancomycin-intermediate-resistant strain, was used in this study. Pure cultures were maintained on tryptic soy agar (Lab M, Lancashire, UK) plates. Overnight broth cultures were obtained by inoculating Mueller–Hinton broth (Lab M) with several colonies and incubating for 18 h with shaking. All cultures were kept aerobically at 37 °C.

2.2. Drugs and antibiotics used

The National Institutes of Health Clinical Collection 1 and 2 (NIHCC 1&2) (Evotec, San Francisco, USA) are repurposing libraries containing, respectively, 446 and 281 components at a concentration of 10 mM, dissolved in 100% dimethyl sulphoxide (DMSO) and stored at –20 °C in 96-well microtitre plates (MTPs).

Ciprofloxacin (CIP) was obtained from Fluka (Buchs, Switzerland), tobramycin (TOB) was from TCI Europe (Antwerp, Belgium), and rifampicin (RIF), gentamicin (GEN), vancomycin hydrochloride hydrate (VAN), linezolid (LZD), tetracycline hydrochloride (TET), fusidic acid sodium salt (FA), flucloxacillin sodium (FLU), fluphenazine dihydrochloride (FLUPH), perphenazine (PERPH), trifluoperazine dihydrochloride (TRI) and thioridazine hydrochloride (TZ) were purchased from Sigma-Aldrich (Bornem, Belgium). Solutions were made in physiological saline (PS) (0.9% w/v NaCl) and ointments were made in 70% white Vaseline (Fagron, Waregem, Belgium) and 30% liquid paraffin (Fagron). PERPH was first dissolved in DMSO (Sigma-Aldrich) and was subsequently diluted in PS. For the minimum inhibitory concentration (MIC) and chequerboard tests, drugs were dissolved in ultrapure Milli-Q water (Millipore, Billerica, MA). All solutions were made fresh on the day of the experiment and were filter sterilised (0.22 μm; Whatman GmbH, Dassel, Germany).

2.3. Screening of the NIHCC 1&2 against mature biofilms of Staphylococcus aureus Mu50 grown in 96-well microtitre plates

Biofilms were formed as previously described [16]. In brief, round-bottomed 96-well MTPs (TPP, Trasadingen, Switzerland) were filled with 100 μL of a diluted overnight bacterial suspension [optical density (OD) at 595 nm of 0.05; ca. 5×10^7 CFU/mL] and were incubated for 4 h at 37 °C. Subsequently, wells were rinsed with PS to remove all planktonic cells and then 100 μL of fresh Mueller–Hinton broth was added. Plates were incubated for an additional 20 h at 37 °C. The 24-h-old biofilms were rinsed with 100 μL of PS. Subsequently, 49 μL of PS, 1 μL of compound (final concentration 100 μM) and 50 μL of a 80 μg/mL VAN solution were added (final concentration 40 μg/mL) in case of combination treatment. In the case of experiments carried out in the absence of VAN, 1 μL of compound was added to 99 μL of PS. All experiments were carried out at least in triplicate. A blank, a growth control and a control for VAN treatment alone were included on every plate. Following 24 h of incubation at 37 °C, the supernatant was removed and biofilms were rinsed with 100 μL of PS. The number of metabolically active cells was determined by resazurin staining (CellTiter-Blue® Cell Viability Assay; Promega, Leiden, The Netherlands) using a multilabel MTP reader at λ_{ex} 535 nm/λ_{em} 590 nm (EnVision; PerkinElmer LAS, Waltham, MA) as previously described [17]. The blank-corrected fluorescence signals generated by *S. aureus* biofilms treated with the combination of component and VAN were compared with the fluorescence signal generated after treatment with VAN alone. Successful hits were defined as combinations yielding a decrease in fluorescence signal of ≥90% compared with fluorescence generated by biofilms treated with VAN alone.

2.4. Determination of the minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI)

MICs of TZ, FLUPH, PERPH and TRI against *S. aureus* Mu50 were determined in triplicate using flat-bottom 96-well MTPs (TPP) as described previously [18]. The inoculum was standardised to ca. 5×10^5 CFU/mL. Plates were incubated at 37 °C for 24 h and the OD at 590 nm was measured using a multilabel MTP reader (EnVision). The MIC is the lowest concentration that generates a signal that is not significantly different from that of uninoculated growth medium. Interaction between TZ (0.78–50 μM), FLUPH (1.56–100 μM), PERPH (1.56–100 μM) or TRI (0.78–50 μM) and TOB (4–1024 μg/mL), GEN (2–512 μg/mL), VAN (0.125–32 μg/mL), CIP (1–256 μg/mL), LZD (0.125–32 μg/mL), TET (2–256 μg/mL), FA (0.004–1 μg/mL) or FLU (4–1024 μg/mL) was determined by chequerboard assay, and the FICI was calculated to determine whether the interaction was synergistic (FICI ≤ 0.5) or indifferent (FICI > 0.5).

2.5. Biofilm eradication tests in 96-well microtitre plates

The activity of the four phenothiazines against *S. aureus* biofilms was evaluated in a 96-well MTP biofilm model. Mature biofilms of *S. aureus* were grown as described in Section 2.3 and were subsequently treated with TZ, FLUPH, PERPH or TRI (10, 50 and 100 μM). The potentiating activity of TZ on antibiotics was investigated in the same model. Biofilms were treated with TZ (100 μM) in combination with TOB (1024 μg/mL), GEN (1024 μg/mL), FLU (1024 μg/mL), VAN (40 μg/mL), LZD (20 μg/mL), TET (128 μg/mL), CIP (250 μg/mL), FA (1000 μg/mL) and RIF (512 μg/mL). After 24 h, the treatment solutions were removed and biofilms were rinsed with 100 μL of PS. Cells were collected from the wells by two cycles of shaking for 5 min at 700 rpm (Titramax 1000; Heidolph Instruments GmbH, Schwabach, Germany) and sonicating for 5 min (Branson 3570E-MT; Branson Ultrasonic Corp., Danbury, CT). The contents of five wells were pooled in tubes containing 9 mL of PS and the number of CFU per biofilm (CFU/BF) was determined by plating serial 10-fold dilutions on tryptic soy agar plates. Experiments were performed in triplicate on at least 3 different days ($n \geq 3 \times 3$).

2.6. Validating the activity of tobramycin, linezolid or flucloxacillin and thioridazine in an in vitro chronic wound biofilm model

Preparation of the artificial dermis and biofilm formation was performed as described by Brackman et al. [19]. Following 24 h of biofilm formation at 37 °C, the infected artificial dermis were rinsed and subsequently solutions (100 μL) containing TOB (2048 μg/mL), LZD (400 μg/mL) or FLU (20.5 mg/mL) alone and with 100 μL of TZ (100 μM) were added. Alternatively, the artificial dermis were covered with 250 mg of ointment containing TOB (15 mg/g), alone or combined with 0.25 mg/g TZ for 24 h. The artificial dermis were kept at 37 °C during treatment. After treatment, the artificial dermis were placed into tubes containing 10 mL of PS, the sessile cells were removed from the artificial dermis by three cycles of vortexing (30 s) and sonication (30 s), and the number of CFU/dermis was determined by plating the resulting suspensions. Every condition was tested in triplicate.