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Effects of clofazimine on planktonic and biofilm growth of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*

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ABSTRACT

Mycobacteria form lipid-rich biofilms that restrict the efficacy of antimicrobial chemotherapy, possibly necessitating the use of lipophilic antibiotics. In the current study, the activity of one such agent, clofazimine, against *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* planktonic cells and biofilms was investigated. Minimum inhibitory concentrations (MICs) of clofazimine were determined for planktonic cultures, whilst minimum bactericidal concentrations (MBCs) were determined for planktonic, biofilm-producing and biofilm-encased organisms using standard bacteriological procedures. The effects of clofazimine on biofilm formation and the stability of pre-formed biofilm were measured using a crystal violet-based spectrophotometric procedure. In the case of *M. smegmatis*, clofazimine was found to be active against planktonic phase (MICs and MBCs of 2.5 mg/L and >20 mg/L, respectively) and biofilm-producing organisms (MBC of 2.5 mg/L); clofazimine demonstrated greater activity against *M. tuberculosis*, corresponding values of 0.06, 5 and 0.3 mg/L. Although clofazimine inhibited biofilm production both by *M. tuberculosis* and *M. smegmatis* ($P < 0.05$ at ≥0.07 mg/L and ≥0.3 mg/L, respectively) and appeared to reduce the pre-formed *M. tuberculosis* biofilm, addition of antimicrobial agent to pre-existing biofilm matrices failed to kill biofilm-encased organisms. In conclusion, clofazimine is more effective against *M. tuberculosis* than against *M. smegmatis*, exhibiting bactericidal activity both for actively growing and slowly replicating bacilli but not for non-replicating organisms of both species.

1. Introduction

Unlike the biofilms of most bacteria, which consist of an assemblage of exopolysaccharides, lipids, proteins and DNA, those of mycobacteria have a particularly high lipid content, including glycolipids and mycolic acids [1–3]. Mycobacterial subpopulations resident in biofilm matrices consist of planktonic (aerated, exponentially or actively growing) and sessile (slowly/non-replicating, dormant, persistent) bacteria that vary with respect to antibiotic susceptibility, the former being generally sensitive and the latter resistant [2–4]. Notwithstanding the slowly/non-replicating state, resistance of the sessile population to antimicrobial agents is also attributable to poor penetration of the biofilm by ostensibly effective antibiotics [5]. In the setting of pulmonary tuberculosis (TB), the sessile organisms are insulated in sac-like granuloma lesions, somewhat similar to encasement in biofilm observed in in vitro cultures [6–8]. During TB chemotherapy with rifampicin and isoniazid, planktonic bacteria are killed rapidly (within 2 weeks), whilst eradication of sessile organisms necessitates extended exposure to higher concentrations of these agents [1,4,9]. Clearly, antibiotics that penetrate the biofilm and/or inhibit its formation [10,11] have the potential to improve the efficacy of TB chemotherapy.

The antimycobacterial riminophenazine agent clofazimine is categorised by the World Health Organization (WHO) as a last-resort option (group 5 drug) in the treatment of multidrug-resistant TB (MDR-TB), largely because of its poor pharmacokinetic properties [12,13]. However, this antibiotic possesses several properties that may enable it to target both biofilm-forming as well as biofilm-insulated organisms, including those that are MDR [12–14]. Foremost amongst these are its lipophilicity and low-level

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resistance profile, as well as recently described inhibitory activity against mycobacterial persisters [15–18]. To address this issue, this study was undertaken with the primary objectives of determining: (i) the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of clofazimine against actively growing planktonic and slowly replicating biofilm-producing mycobacteria as well as non-replicating biofilm-encased organisms; and (ii) the inhibitory effects of clofazimine both on biofilm formation and the structural resilience of pre-formed biofilm.

2. Materials and methods

2.1. Strains and growth media

Mycobacterium tuberculosis H37Rv ATCC 25618 *and Mycobacterium smegmatis* Mc^2155 laboratory strains were used in this study. BD Difco™ 7H10 agar (BD Diagnostics, Sparks, MD) containing 0.5% glycerol/10% oleic acid–dextrose–catalase (OADC) (BD Diagnostics) as well as BD Difco™ 7H9 broth (BD Diagnostics) containing 0.2% glycerol/10% OADC/0.05% Tween 80 were prepared following the manufacturers' instructions, whilst Sauton broth was prepared as described previously [1].

2.2. Chemicals, reagents and antibiotics

Unless otherwise stated, all other chemicals and reagents, including clofazimine, were purchased from Sigma Chemical Co. (St. Louis, MO). Clofazimine was dissolved in dimethyl sulphoxide (DMSO) and was used at concentrations ranging from 0.01 mg/L to 20 mg/L. The final concentration of DMSO in the drug-containing and control systems was 1%, and solvent controls were included in all experiments.

2.3. Inoculum preparation

M. tuberculosis and *M. smegmatis* were inoculated into 7H9 broth and were incubated at 37 °C for 7 days or 24 h under stirring conditions, respectively. Thereafter, the cultures were centrifuged at 3500 × *g* for 10 min at room temperature and the supernatants were discarded. The pellets were washed twice, re-suspended in Sauton broth and the optical density at 540 nm was adjusted to 0.6, yielding ca. 10^7–10^8 CFU/mL.

2.4. Minimum inhibitory concentration determination

The MICs of clofazimine for *M. tuberculosis* and *M. smegmatis* were determined using broth and plate dilution procedures in a BD BACTEC™ TB system (BD Diagnostics) and on 7H10 plates, respectively, based on the proportion method as described previously [19,20]. The clofazimine concentrations used ranged from 0.01 mg/L to 5 mg/L. For *M. tuberculosis*, the procedure used was as described previously [19]. In the case of *M. smegmatis*, the various concentrations of clofazimine were incorporated into 7H10 agar plates. One set of drug-free and all drug-containing plates were inoculated with 10^4 CFU/mL, whilst another set of drug-free plates, serving as controls, was inoculated with 100× dilution of the inoculum (10^2 CFU/mL). The plates were incubated at 37 °C for 72 h to allow for the appearance of colonies. The lowest concentration of the drug that yielded fewer colonies than those that grew on the 100× diluted controls was regarded as the MIC.

2.5. Planktonic and biofilm culture preparation

Approximately 10^5 CFU/mL were inoculated into tissue culture plates containing 7H9 or Sauton broth with no detergent for planktonic or biofilm cultures, respectively. For *M. tuberculosis*, 1.5 mL volumes of bacterial cultures were added to the centre wells of 24-well tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany), whilst peripheral wells were filled with the same volume of sterile distilled water to prevent desiccation. In the case of *M. smegmatis*, 5 mL volumes of bacterial cultures were added to the wells of 6-well tissue culture plates (Greiner Bio-One GmbH). All cultures were thoroughly mixed and incubated at 37 °C in the dark.

Planktonic culture plates were incubated for 10 days or 24 h with frequent shaking, whilst the corresponding biofilm culture plates were wrapped with Parafilm® and were incubated without shaking for 10 weeks or 5 days for biofilm formation and for 5 weeks or 5 days post biofilm development for pre-formed biofilm cultures for *M. tuberculosis* and *M. smegmatis*, respectively. Biofilm formation resulted in the formation of a white layer on the surface of the culture medium [1,21,22].

The rates of growth of the bacteria were determined by sampling, diluting and plating the cultures every 24 h and 6 h for *M. tuberculosis* and *M. smegmatis* planktonic organisms, respectively, and bi-/weekly or daily for biofilm bacilli for each mycobacterial species, and monitoring thereafter for the appearance of colonies, which were compared with the number of viable bacteria in the initial inoculum.

2.6. Minimum bactericidal concentration determination and rate of killing

Clofazimine MBCs were determined for planktonic and biofilm cultures of both mycobacterial species. Varying concentrations of clofazimine were added to one set of wells, whilst drug-free control systems received DMSO. Planktonic organisms were treated with clofazimine (0.3–20 mg/L) for 10 days or 24 h, whilst the biofilm-forming bacilli were treated (0.01–5 mg/L) for 10 weeks or 5 days for *M. tuberculosis* and *M. smegmatis*, respectively. In the case of biofilm-encased bacilli, the corresponding treatment (0.15–20 mg/L) times were 5 weeks and 5 days post biofilm development. The number of planktonic bacilli was determined by sampling each well and dilutions plated on 7H10 agar plates, followed by incubation at 37 °C in the dark for 3 weeks or 72 h for the appearance of colonies for *M. tuberculosis* or *M. smegmatis*, respectively. The number of clofazimine-treated biofilm-producing bacilli was determined by adding 100 μL of 0.2% Tween 80 to culture wells followed by incubation on a shaker at 37 °C for 24 h or 1 h for *M. tuberculosis* and *M. smegmatis*, respectively, to solubilise biofilm. The cultures were sampled and plated as described previously for planktonic organisms. The number of clofazimine-treated biofilm-encased bacilli was determined for 5 weeks or 5 days following addition of the antimicrobial agents for *M. tuberculosis* or *M. smegmatis*, respectively.

The rate of clofazimine-mediated killing of the various bacterial populations was determined by evaluating the MBCs daily or every 6 h for planktonic organisms, and bi-/weekly or daily for biofilm-producing and biofilm-encased *M. tuberculosis* or *M. smegmatis*, respectively. The MBC was regarded as the lowest concentration of clofazimine showing ≥2 log reduction in CFU/mL of the initial inoculum (Day 0) [20].

2.7. Biofilm quantification

Biofilms were quantitated using a crystal violet-based staining procedure [23]. Supernatants were removed and the residual biofilm biomasses were washed twice with distilled water and were air-dried. Crystal violet (1%) was added to each well and was incubated for 30 min at room temperature. Unbound dye was removed by washing with distilled water and the wells were

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