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Clofazimine protects against *Mycobacterium tuberculosis* dissemination in the central nervous system following aerosol challenge in a murine model

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ABSTRACT

Tuberculosis (TB) has been the scourge of the human race for many decades, claiming countless number of lives. This is further complicated by the ability of *Mycobacterium tuberculosis* to infect extrapulmonary sites, specifically the brain. These extrapulmonary forms of TB are difficult to treat owing to problems associated with drug delivery across the blood–brain barrier. Linezolid (LIN) and clofazimine (CFZ) are two of the more promising anti-TB drugs in recent times. In this study, BALB/c mice were aerosol-infected with *M. tuberculosis* H37Rv and were treated for 4 weeks with LIN [100 mg/kg body weight (BW)] or CFZ (100 mg/kg BW). Concurrently, it was investigated whether an aerosol TB infection would lead to dissemination of TB bacilli into the brain. Post-treatment brain and lung CFUs were determined together with serum, lung and brain drug concentrations. CFZ displayed a strong bactericidal effect in the lung, whilst LIN had a bacteriostatic effect. *Mycobacterium tuberculosis* appeared at 2 weeks post-infection in the untreated group (2.38 ± 0.43 $\log_{10}$ CFU) and more surprisingly at 3 weeks post-infection in the LIN-treated group (1.14 ± 0.99 $\log_{10}$ CFU). TB bacilli could not be detected in the brains of the CFZ-treated group. To the best of our knowledge, this is the first study showing the appearance of *M. tuberculosis* in the brain following a murine aerosol TB infection. This study may advocate the use of CFZ as prophylactic treatment to prevent the development of extrapulmonary TB of the central nervous system using a two-pronged approach.

1. Introduction

For many years, tuberculosis (TB) has been a manageable disease thanks to the advent of short-course chemotherapy [1]. However, in recent times *Mycobacterium tuberculosis* has evolved resistance, resulting in the emergence of multidrug-resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tuberculosis* [2]. New drugs and regimens are improving outcomes for patients with pulmonary TB caused by MDR and XDR strains, but these have not been studied in patients with extrapulmonary forms of TB that are associated with high mortality and morbidity rates [2].

The most severe forms of extrapulmonary TB are caused by the presence of bacilli in the brain. *Mycobacterium tuberculosis* first colonises the lung and then enters the systemic circulation resulting in dissemination to the brain [3]. Intracranial TB can cause various pathologies, including intracranial tuberculomas, tubercular encephalitis, brain abscesses and TB meningitis [3]. Evidence-based guidelines exist for the treatment of susceptible intracranial TB, but less is known about the appropriate treatment of drug-resistant forms that have a very high mortality rate. This is largely due to uncertainty about the penetration and distribution of second-line TB drugs into the brain.

The limited availability of new agents has led to the repurposing of drugs used principally for diseases other than TB. Linezolid (LIN) and clofazimine (CFZ) have emerged as two of the most promising repurposed drugs to enter clinical trials, with minimum inhibitory concentrations (MICs) of 0.5 μg/mL and 0.25 μg/mL, respectively for *M. tuberculosis*. CFZ is an established antimycobacterial agent that has been used extensively to treat leprosy [4] but has

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been resurrected to treat drug-resistant TB and has recently been recommended by the World Health Organization (WHO) for use in a new short-course treatment for MDR-TB [5,6]. Studies investigating its effects in a murine model have demonstrated the ability of CFZ to reduce the treatment time for susceptible TB. It is a lipophilic riminophenazine dye and is particularly attractive for the treatment of intracranial TB because it accumulates extensively in tissue, as a result of which it exhibits bactericidal activity even after the cessation of treatment [7–9]. LIN is an oxazolidinone antibiotic that inhibits the translation step of protein synthesis [10]. Owing to its excellent bioavailability and pharmacokinetics, it is used to treat a number of resistant bacterial infections, including those of the central nervous system (CNS) [11,12]. LIN has good activity in murine models of TB, alone and in combination, and is now being used to treat XDR-TB.

The successful adoption of LIN and CFZ to treat pulmonary forms of TB led us to study their potential for the treatment of CNS TB. In previous studies using mass spectrometry imaging (MSI), we have shown the brain distribution of LIN in a healthy rodent model [13]. In addition to this, using liquid chromatography tandem mass spectrometry (LC-MS/MS) we were able to provide evidence for the presence of CFZ in a healthy murine model [14]. The aims of the current study were to extend our work using LC-MS/MS to determine how tissue pharmacokinetics in the brain and lung are affected by the presence of TB bacilli and also to determine whether the drugs influence the dissemination of TB to the murine brain.

2. Materials and methods

2.1. Animal experimentation

All animal experimentation was carried out with the approval of the Animal Research Ethics Committee of the University of KwaZulu-Natal (Durban, South Africa) and all procedures were in accordance with the standard approved protocols for animal research. All experiments involving *M. tuberculosis* cultures were carried out in biosafety level 3 facilities of the African Health Research Institute (Durban, South Africa). Animals were housed under BSL-3 conditions in individually ventilated cages under standards conditions of temperature and humidity, a 12-h light/dark cycle, and with ad libitum access to standard mouse feed and water. Animal welfare was monitored daily using institutional humane end-points.

A total of 65 female BALB/c mice (20–30 g) were aerosol-infected with *M. tuberculosis* H37Rv grown in Middlebrook 7H9 broth (Sigma-Aldrich, St Louis, MO) using a Glas-Col full-body inhalation chamber (Glas-Col, LLC, Terre Haute, IN). Mice were infected with a high dose of *M. tuberculosis* (8.08 $\log_{10}$ CFU) and treatment began 3 days later (Day 0).

2.2. Drug treatment

LIN was purchased as a Zyvox® intravenous infusion (Pfizer Pharmaceuticals, Pearl River, NY). The drug was extracted using dichloromethane (yield 98%) and was confirmed by LC-MS and high-performance liquid chromatography–ultraviolet (HPLC-UV). Activity of the drug was confirmed by conducting resistance testing of the drug. CFZ was purchased from Sigma-Aldrich Inc. (Darmstadt, Germany). Animals were grouped randomly and were treated via oral gavage for 4 weeks (5 days a week from Monday–Friday) with a daily dose of 100 mg/kg body weight (BW) of each drug prepared in a 0.05% (w/v) agarose suspension. Both of these doses have previously shown excellent brain and tissue penetration in a healthy rodent model [13,15]. Despite their proven efficacy when used in combination with other established drugs, in this study CFZ and LIN were given as a monotherapy in order to determine the extent of their individual protective effect in the CNS of an infected model. Treatment was initiated 3 days after infection. Prior to drug administration, five animals were sacrificed to determine pre-treatment lung and brain CFUs. The study also included an untreated (UT) control group that was administered the vehicle only [0.05% (w/v) agarose] for the duration of the experiment.

2.3. Assessment of antimicrobial activity

CFU counts of lung and brain homogenates were measured to determine the antimicrobial activity of LIN and CFZ. Three sets of brain tissue and the left lung from each animal were collected at each time point for homogenisation and subsequent plating. Quantitative CFUs were determined by plating serial dilutions of lung ($n = 5$) and brain ($n = 3$) homogenates on Middlebrook 7H11 selective medium (Sigma-Aldrich) as previously described [8]. The dilution that yielded CFU counts closest to 50 was used to determine total organ counts.

2.4. Tissue harvest

Five animals from each group were sacrificed weekly post-infection. The methods used to monitor drug concentrations in serum via LC-MS/MS have been described previously, and drug distribution in brain and lung was assessed using MSI [13,14,16]. Prior to removal of brain tissue, ca. 1 mL of blood was removed via cardiac puncture, which accounts for >90% of the circulatory volume of the mouse. To prevent further contamination, tissue was rinsed in saline before being placed in storage vessels.

2.5. Tissue preparation

Lung and brain tissue was homogenised in Milli-Q® water (Merck Millipore, Burlington, MA) (1 mL/g of tissue). Serum, lung and brain homogenates where then extracted using a modified solid-phase extraction process as described previously [13–15].

MSI uses the m/z ratio of an analyte to plot its exact spatial distribution in tissue sections. For this series of experiments, 12-μm brain sections were prepared using an isolated cryomicrotome (Zeiss, Oberkochen, Germany) in a BSL-3 environment. Sections were thaw-mounted into indium–titanium oxide-coated slides. Matrix-assisted laser desorption/ionisation (MALDI) MSI was used to analyse lung and brain sections, which uses a matrix (α-cyano-hydroxycinnamic acid) to aid ionisation of the analyte. Matrix was then applied onto the conductive slides in a uniform manner using a Bruker ImagePrep (Bruker Daltonics, Bremen, Germany). Image acquisition was conducted using a rapifleX™ system (Bruker Daltonics), with a 50 μm pixel size and 800 laser shots per pixel.

2.6. Data and statistical analysis

Data analysis was performed using GraphPad Prism 7.0b (GraphPad Software Inc., San Diego, CA). For the in vivo early bactericidal effect experiment, CFU count data were analysed using two-way analysis of variance (ANOVA) corrected with Tukey's test for multiple comparisons. Serum CFZ concentrations were analysed using linear trend one-way ANOVA (dose–response) and using one-way ANOVA corrected with Tukey's test for multiple comparisons (interdose comparisons).

3. Results and discussion

A total of 65 BALB/c mice were infected by aerosol with a high inoculum of *M. tuberculosis* H37Rv. At 3 days post-infection (Day 0), five animals were sacrificed and their lung tissue was plated to establish the pre-treatment bacillary load. Assessment of CFU counts showed a mean count of 4.23 ± 0.37 $\log_{10}$ CFU. Treatment was