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Effectiveness of purified methylene blue in an experimental model of *Mycobacterium ulcerans* infection

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ABSTRACT

Mycobacterium ulcerans is responsible for Buruli ulcer, characterised by extensive, disabling ulcers. Standard treatment combining rifampicin and streptomycin exposes patients to toxicity and daily painful injections. In this study, the in vitro susceptibilities of 3 *M. ulcerans* strains, 1 *Mycobacterium marinum* strain and 18 strains representative of eleven other *Mycobacterium* species and subspecies to methylene blue were determined. Whilst growth of *M. ulcerans* was inhibited by 0.0125 g/L methylene blue, growth of all other tested strains was not inhibited by 1 g/L methylene blue. The effectiveness of methylene blue in a murine model of *M. ulcerans* infection was then tested. Topical treatment by brushing a methylene blue solution on the skin lesion, systemic treatment by intraperitoneal injection of methylene blue, and a combined treatment (topical and systemic) were tested. The three treatment groups exhibited a significantly lower clinical score compared with the non-treated control group ($P < 0.05$). Moreover, subcutaneous nodules were significantly smaller in the systemic treatment group (excluding males) (3 ± 0.7 mm) compared with the other groups ($P < 0.05$). The *M. ulcerans* insertion sequence IS*2404* and the KR-B gene were detected in all challenged mice, but not in negative controls. The density of *M. ulcerans* (mycobacteria/cell) was significantly lower in the combined treatment group compared with the other groups. These data provide evidence for the effectiveness of purified methylene blue against the initial stage of Buruli ulcer.

1. Introduction

Buruli ulcer is an often-overlooked tropical disease characterised by gradual and extensive cutaneous and subcutaneous ulcers that evolve towards scars or amputations in the absence of treatment [1]. These lesions mainly result from the necrotising activity of the plasmid-encoded mycolactone toxin secreted by *Mycobacterium ulcerans*, the causative agent of Buruli ulcer [2], which evolved from a common ancestor of its closest parent *Mycobacterium marinum* by genomic reduction and acquisition of the pMUM001 plasmid [3,4]. The ultimate source of infection remains unknown, but several studies have significantly correlated the prevalence of Buruli ulcer with exposure to aquatic environments and aquatic insects that may act as a vector of the pathogen [5,6]. In Côte d'Ivoire, for example, multivariate analysis incorporating several geographic variables showed that irrigated rice fields were significantly associated with Buruli ulcer [7]. Accordingly, the only environmental isolate was obtained in Benin from an aquatic Hemiptera [8]. This notifiable disease has been reported in 33 countries, although the majority of cases occur in West Africa [9,10], with cases being also reported in South America [11,12], Japan [13] and in southern Australia [14] where Buruli ulcer was initially described and where *M. ulcerans* was initially isolated from four patients [15].

The prognosis of Buruli ulcer dramatically changed with the introduction of an effective 8-week course of rifampicin and streptomycin therapy [16,17]. However, the need for parenteral administration of potentially ototoxic and nephrotoxic streptomycin may limit administration of this efficient combination. Therefore, it would be interesting to further assess the potential efficacy of alternative drugs [17]. Methylene blue has been used for over a century as a topical treatment for infections [18] and has gained renewed interest following the availability of a pure form of the molecule [19,20]. No data have been reported regarding the effectiveness of methylene blue against *M. ulcerans* and Buruli ulcer, and only old data from unpurified forms of methylene blue have been reported for mycobacteria as a whole [18,21]. The aim of this study was to assess the in vitro activity of purified methylene blue against mycobacteria. Reproducible in vitro activity against *M. ulcerans* correlating with reproducible in vivo activity against experimental *M. ulcerans* infection in mice was observed.

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2. Materials and methods

2.1. Mycobacterium strains

A total of 22 isolates representing 13 *Mycobacterium* species and subspecies were studied in vitro, including: three *M. ulcerans* isolates (CU001, ATCC 33728 and ATCC 25900); *M. marinum* (one clinical isolate); two species from the tuberculosis complex [*Mycobacterium tuberculosis* (reference strain H37Rv and four clinical isolates including two Beijing strains) and '*Mycobacterium canettii*' (reference strain CIP 1400159)]; *Mycobacterium avium* (two isolates); *M. avium* subsp. *hominissuis* (two isolates); *Mycobacterium abscessus* subsp. *abscessus* (two isolates); *M. abscessus* subsp. *bolletii* (one isolate); *Mycobacterium chelonae* (one isolate); *Mycobacterium massiliense* (one isolate); *Mycobacterium fortuitum* (one isolate); *Mycobacterium xenopi* (one isolate); and *Mycobacterium kansasii* (one isolate). *Mycobacterium tuberculosis*, '*M. canettii*' and *M. ulcerans* were handled in a biosafety class 3 laboratory (NSB3), whilst the other species were handled in biosafety class 2 (NSB2) laboratories. All mycobacteria were subcultured on MOD9 medium (a serum-enriched derivative of Middlebrook 7H10 medium) [22], were suspended in sterile phosphate-buffered saline (PBS) and were then calibrated by measuring the optical density to reach a concentration of 10^5 CFU/mL. Regarding clumping bacteria, including *M. tuberculosis*, *M. abscessus* and *M. ulcerans*, homogenisation was achieved by vigorous vortexing using 3-mm glass beads (Sigma-Aldrich, Saint-Quentin-Fallavier, France) followed by several bypasses through a 25 G needle to disperse the remaining clumped bacteria.

2.2. Minimum inhibitory concentrations (MICs) of methylene blue against mycobacteria

Purified methylene blue (Provepharm, Marseille, France) was incorporated at 0 (positive control), 0.0125, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 g/L in Middlebrook 7H10 growth medium (Becton Dickinson, Pont-de-Claix, France) and then 30 mL of this medium was poured into sterile 90-mm Petri dishes (Gosselin, Hazebrouck, France). Plates were inoculated with 100 µL of *M. ulcerans* suspension (equivalent to 10^4 mycobacteria per plate) and were incubated at 37 °C in an atmosphere containing 5% CO_2 at 32 °C in a microaerophile atmosphere (Thermo Fisher Scientific, Villebon-sur-Yvette, France). Three plates were inoculated for each methylene blue concentration in parallel with a positive control plate without methylene blue. Plates were read by naked-eye observation after 15 days of inoculation, except for *M. ulcerans*-inoculated plates that were read at day 30. The MIC was defined by the method of proportions as the lowest concentration resulting in $<1.5 \times 10^{-3}$ colonies than the control plate (without methylene blue). Colonies were confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) [23].

2.3. Animal model

In this work, mice (*Mus musculus*) were used as an animal model for *M. ulcerans* infection according to recent publications [17,24]. The protocol was reviewed and approved by the French National Ethics Committee. A total of 40 BALB/c mice (20 male, mean weight at reception of 20.4 ± 1.9 g; 20 females, mean weight at reception of 15.2 ± 1.3 g) (Charles River Laboratories, l'Arbresle, France) were housed in the laboratory for 1 week before the beginning of the experiment. Animals were divided into four groups, each comprising five males and five females. Mice were anaesthetised for 1 h by intraperitoneal (i.p.) injection of 120 mg/kg ketamine and 8 mg/kg xylazine in order to shave the back of the mouse.

Subsequently, 40 µL of sterile PBS was inoculated intradermally into the upper part of the back (control) and 40 µL of 5×10^4 CFU/mL of *M. ulcerans* CU001 suspension in sterile PBS was inoculated intradermally into the lower part (Fig. 1A). Males and females were then accommodated separately in boxes placed in a ventilated cabinet, were fed and were observed daily until the first clinical signs of granuloma or ulcer appeared. Methylene blue treatment was initiated 2 weeks post-inoculation when clinical lesions developed in challenged mice. In group I, ten mice served as a control and received no treatment. In group II, the backs of ten *M. ulcerans*-inoculated mice were brushed with a methylene blue solution (1.25 mg/infected zone). In group III, ten mice received an i.p. injection of methylene blue (25 mg/kg). In group IV, ten mice received an i.p. injection of methylene blue (25 mg/kg) in addition to back brushing with a methylene blue solution (1.25 mg/infected zone).

Treatments were performed at 2-day intervals, with a total of five treatments over 10 days. Two weeks after the final treatment, mice were sacrificed by euthanasia using an i.p. injection of 100 mg/kg pentobarbital. Wounds were scored from zero to ten as follows: 0, non-inflammatory/swelling; 1–5, inflammatory/swelling; 6–8, inflammatory/swelling/hollow; and 9–10, ulcer. Excision biopsies from the PBS-inoculated zone and the infected zone were collected and were divided into equal parts. Mice were dissected and nodules were photographed in order to measure the nodule size using ImageJ software (http://imagej.nih.gov/ij/). Biopsies were divided into two, with one part being used for quantitative PCR and the other used for histological testing.

2.4. Quantitative real-time PCR (qRT-PCR)

To quantify the *M. ulcerans* load in skin biopsies, *M. ulcerans* was specifically detected by combining two RT-PCR assays targeting the insertion sequence IS*2404* and the KR-B gene as previously described [25,26]. Total DNA was extracted using a commercial kit (Tissue Kit; Macherey-Nagel, Hoerdt, France). PCR inhibition was assessed in each sample by adding 10 µL of internal control into 190 µL of sample as previously described [27]. In each extraction batch of 25 samples, two negative controls consisting of distilled water were included. Regarding RT-time PCR amplifications, a calibration curve was made for each of the two *M. ulcerans* DNA targets by using RT-PCR as described below, in a series of 10-fold *M. ulcerans* Agy99 suspensions from 10^6 CFU/mL to 1 CFU/mL; suspensions were extracted as described above in triplicate. Then, for each specimen, IS*2404* and the KR-B gene were tentatively amplified using qRT-PCR performed incorporating qRT-PCR reagents (Takyon™; Eurogentec, Liege, Belgium) and primers and probes as previously described [25] in a CFX96™ thermocycler and detection system (Bio-Rad, Marnes-la-Coquette, France). PCR was conducted in a 20 µL volume containing 5 µL of DNA, 0.5 µL of each primer, 0.5 µL of probe, 3.5 µL of sterile water and 10 µL of qRT-PCR MasterMix (Eurogentec). The qRT-PCR program comprised 1 cycle at 50 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 95 °C for 30 s and 60 °C for 1 min. Two negative controls for 25 samples were incorporated into each PCR run. All samples were tested in duplicate. A specimen was considered as positive for the detection of *M. ulcerans* when both the insertion sequence IS*2404* and the KR-B gene detection was positive (Ct ≤ 40 cycles) in replicates and the number of mycobacteria was derived from calibration curves made with *M. ulcerans* CU001. Simultaneously, mouse cells were quantified in each skin biopsy using RT-PCR targeting the glyceraldehyde-3-phosphate dehydrogenase gene as previously described [28]. The density of mycobacteria (expressed as mycobacteria/cell) was also tabulated by dividing the number of mycobacteria by the number of mouse cells (Supplementary Table S1).