

Inhaled Microparticles Containing Clofazimine Are Efficacious in Treatment of Experimental Tuberculosis in Mice

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Inhalable clofazimine-containing dry powder microparticles (CFM-DPI) and native clofazimine (CFM) were evaluated for activity against *Mycobacterium tuberculosis* in human monocyte-derived macrophage cultures and in mice infected with a low-dose aerosol. Both formulations resulted in 99% killing at 2.5 µg/ml *in vitro*. In mice, 480 µg and 720 µg CFM-DPI inhaled twice per week over 4 weeks reduced numbers of CFU in the lung by as much as log₁₀ 2.6; 500 µg oral CFM achieved a log₁₀ 0.7 reduction.

Clofazimine (CFM) is a riminophenazine possessing activity against various species of both drug-sensitive and -resistant mycobacteria, including *Mycobacterium leprae*, *M. tuberculosis*, and *M. avium-M. intracellulare* (1). Its MIC against *M. tuberculosis* H37Rv is 0.15 to 2.5 µg/ml (2). CFM is very hydrophobic, and its gastrointestinal absorption is low. At therapeutic oral doses, it may cause discoloration of the skin. High lipophilicity, long half-life (~70 days), and high retention in tissues may cause toxicity (3, 4). These properties of CFM contribute to its low ranking (category 5) on the WHO list of second-line therapy choices for multidrug- and extensively drug-resistant tuberculosis (TB) (5).

We hypothesize that CFM as a dry powder microparticle formulation for inhalation (CFM-DPI) might hold advantages for generating drug levels at the primary site of infection that would not otherwise be achievable (6). We present preliminary findings showing that (i) CFM-DPI retains the antimicrobial properties of native CFM *in vitro* and (ii) CFM-DPI is more efficacious in treating experimental tuberculosis than if given orally.

CFM-DPI was prepared by spray drying, which was carried out independently at two locations: in South Africa using a Buchi mini-spray dryer (model B-290) and in India using a Labultima LU 20 spray dryer. Commercially purchased CFM (Sigma) was dissolved in dimethyl sulfoxide (DMSO) or ethanol and L-leucine in distilled water. Spray drying conditions (7) were similar at both locations. The resulting particle size (1 to 5 µm) of CFM-DPI was suitable for deep lung delivery (Fig. 1A and B).

In order to confirm the intracellular killing kinetics of CFM-DPI, *M. tuberculosis* bacilli residing in human monocyte-derived macrophages were comparatively exposed to CFM in both native and dry-powder form. Monocytes were isolated from heparinized human blood collected with informed consent (Pretoria University Ethics Committee approved). Standard procedures of density gradient/plastic adherence were employed and monocytes cultured in RPMI 1640, supplemented with 5% autologous serum, interleukin-3, and granulocyte-macrophage colony-stimulating factor for 7 days at 37°C in 5% CO₂. Approximately 10⁵ macrophages were infected with H37Rv at a multiplicity of infection of 10 over 18 h,

and unbound bacilli were removed with phosphate-buffered saline (pH 7.4). CFM and CFM-DPI (concentrations ranging from 0.15 to 2.5 µg/ml) were added to the infected macrophages and incubated for 2 days. Intracellular killing of bacilli was determined by measuring the ratio of the number of surviving bacteria at each drug concentration to that of drug-free controls at the beginning and end of the 2-day period. Both CFM-DPI and native CFM formulations exhibited 99% killing at 2.5 µg/ml (Table 1).

Animal experiments were approved and supervised by the Animal Ethics Committee of the National JALMA Institute, Agra, India. Forty-eight Swiss mice were infected with ~100 log-phase *M. tuberculosis* H37Rv bacilli through the aerosol route (Glascol inhalation exposure system) and randomly divided into groups of eight (Table 2). An untreated control group and a group treated with a combination of isoniazid and rifabutin (INH-RFB) for comparison formed part of the experiment. Treatment started 21 days postinfection, with doses in all treatment groups administered twice per week for 4 weeks (total of 8 doses). CFM was administered orally by gavage as a suspension in soybean oil at dose levels of 5, 10, and 20 mg/kg of body weight (about 125, 250, and 500 µg/animal). From preliminary observations (8), the nature of the oil used in the formulation is not expected to affect terminal biodistribution of the drug, but this needs further validation. It was noted that CFM is available for clinical use in the form of hard gelatin capsules containing micronized drug in a base composed of oil and wax and displays between 45% and 62% oral bioavailabil-

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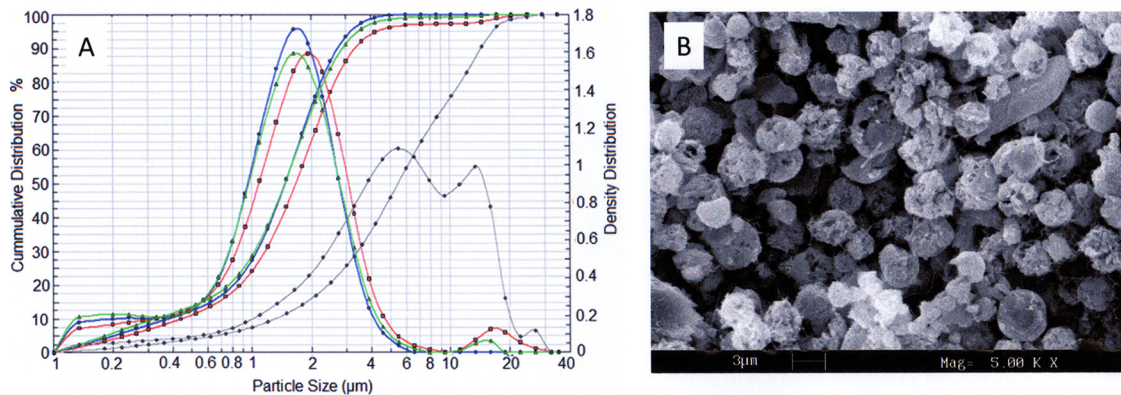


FIG 1 (A) Particle size distribution of spray-dried formulations prepared in South Africa containing 100% (green), 90% (blue), and 80% (red), relative to leucine fraction, compared to native (gray) clofazimine; (B) scanning electron micrograph showing particles containing a 80:20 ratio of CFM to leucine (prepared by spray drying in India).

ity in this form (9). CFM-DPI in an amount equivalent to the emitted drug content of about 240, 480, and 720 μg was administered to infected mice using an in-house, biosafe inhalation apparatus standardized and calibrated for reproducibility by dose delivery as reported elsewhere (10). The dosing schedule allowed for two dose levels from each of the oral and inhalation groups to be closely comparable (~ 250 μg and ~ 500 μg , respectively) (Table 2, groups 2, 3, 6, and 7). The lower oral dose (125 μg) and higher inhaled dose (720 μg) in the series allowed for observations of CFM activity over a wide dosing range. INH-RFB was dosed at 10 mg/kg each (250 $\mu\text{g}/\text{animal}$).

Lungs and spleen were harvested on day 48 postinfection and homogenized (Polytron) separately in sterile Middlebrook 7H10 medium. Aliquots of 100 μl were inoculated neat at 1:100 and 1:1,000 dilutions onto Middlebrook 7H10 agar plates supplemented with oleic acid-albumin-dextrose-catalase, 25 $\mu\text{g}/\text{ml}$ cycloheximide, 10 $\mu\text{g}/\text{ml}$ amphotericin B, and 5 $\mu\text{g}/\text{ml}$ vancomycin (Himedia, India). Plates were incubated at 37°C in a biochemical oxygen demand incubator and CFU scored after 4 weeks.

Significant, dose-dependent reductions in bacterial loads in the lungs and spleen were observed in treated mice compared with untreated controls. However, CFM-DPI seemed more effective than oral CFM in eliminating bacteria from the lungs, but neither formulation in this dosing regimen was effective in

reducing bacterial load in the spleen. In Table 2, reductions in numbers of lung CFU by 1.33, 2.14, and 2.59 \log_{10} for CFM-DPI groups 2, 3, and 4 and by 0.38, 0.58, and 0.73 \log_{10} for oral CFM groups 5, 6, and 7, respectively, are shown. In the spleens, in contrast, the reductions in numbers of CFU following CFM-DPI were 0.59, 0.9, and 1.02 \log_{10} , and for oral CFM, they were 1.19, 1.36, and 1.4 \log_{10} , respectively. The reduction in lung (but not spleen) CFU counts by CFM-DPI was comparable to that achieved in the oral INH-RFB group. No signs of toxicity, behavioral or histological, were observed within the limited scope of these experiments.

In a recently reported study (11), oral CFM treatment of infected mice (20 mg/kg, 5/7 days) resulted in a >2 - \log_{10} reduction after 30 days and, at 200 mg/kg, a 3- \log_{10} reduction. These observations might additionally serve as reference points for further assessment of inhaled formulations of CFM.

It is concluded that spray-dried CFM-DPI is suitable for deep lung delivery, retains the *in vitro* kill characteristics of the native compound, and demonstrates efficacy in preliminary *in vivo* experiments. Definitive studies to ascertain the optimal dose and frequency of administration of the inhalable formulations are needed.

TABLE 1 Numbers of *M. tuberculosis* CFU surviving per ml of cell lysate of human monocyte-derived cultured macrophages exposed to native CFM-DPI for 2 days

CFM concn ($\mu\text{g}/\text{ml}$)	No. of bacilli surviving (CFU/ml) with ^a :			
	Native CFM		CFM-DPI	
	Mean	SEM	Mean	SEM
0 (control)	1.2×10^5	18,173.8	1.2×10^5	18,173.8
0.15	2.8×10^4	3,908.9	6.4×10^4	15,260.3
0.3	2.2×10^4	6,487.5	4.9×10^4	7,545.7
0.6	2.0×10^4	5,153.3	4.1×10^4	14,031.0
1.25	5.6×10^3	2,967.4	5.5×10^3	1,483.3
2.5	3.5×10^2	250.7	2.5×10^2	11.2

^a Results are from four experiments performed in triplicate.

TABLE 2 Numbers of *M. tuberculosis* CFU recovered from lungs and spleens of mice harvested after 28 days of a twice-weekly treatment starting 21 days postinfection

Treatment group	Log ₁₀ no. of CFU/g of tissue \pm SD ^a	
	Lungs	Spleen
Group 1 (untreated control)	6.11 \pm 0.02	5.79 \pm 0.08
Group 2 (inhalation of CFM-DPI at ~ 240 μg)	4.78 \pm 0.21	5.19 \pm 0.05
Group 3 (inhalation of CFM-DPI at ~ 480 μg)	3.97 \pm 0.24*	4.89 \pm 0.10
Group 4 (inhalation of CFM-DPI at ~ 720 μg)	3.5 \pm 0.65*	4.76 \pm 0.08
Group 5 (oral CFM at ~ 125 μg)	5.73 \pm 0.16	4.60 \pm 0.11
Group 6 (oral CFM at ~ 250 μg)	5.53 \pm 0.26	4.43 \pm 0.18
Group 7 (oral CFM at ~ 500 μg)	5.38 \pm 0.25	4.39 \pm 0.12
Group 8 (oral INH + RFB at ~ 250 μg each)	3.83 \pm 0.46*	2.82 \pm 0.92*

^a *, significant difference at the 0.05 level from infected, untreated controls and from the 500- μg -oral-dose group.

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