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Assessment of the efficacy of clofazimine alone and in combination with primary agents against *Mycobacterium tuberculosis* in vitro

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ABSTRACT

Objectives: The chemotherapeutic regimens of patients with drug-susceptible (DS)- tuberculosis (TB) comprise four primary anti-TB drugs: rifampicin (RMP), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA), administered for six-to-nine months. These drug regimens target the various microbial populations that include actively replicating (AR), slow-replicating (SR) and non-replicating (NR) organisms. Clofazimine (CFZ) has showed benefit in shortening DS-TB treatment in vivo from six to four months when used in combination with this regimen in murine models of experimental infection. However, its antimicrobial efficacy when used in combination with the primary drugs against the various microbial populations of *Mycobacterium tuberculosis* has not been demonstrated.

Methods: In the current in vitro study, the inhibitory and bactericidal activities of CFZ in combination with the primary anti-TB drugs, RMP, INH and EMB against the AR and SR organisms in planktonic and biofilm-forming cultures, respectively, were evaluated by fractional inhibitory concentration index (FICI) and fractional bactericidal concentration index (FBCI) determinations, using the Loewe Additivity Model.

Results: In planktonic cultures, CFZ demonstrated synergistic growth inhibitory activity in combination with RMP and INH individually and collectively. With respect to bactericidal activity, CFZ exhibited synergistic activity only in a two-drug combination with RMP. However, in biofilm-forming cultures, all CFZ-containing anti-TB drug combinations exhibited synergistic inhibitory and bactericidal effects, particularly in combination with RIF and INH.

Conclusion: Clofazimine exhibited synergistic effects in combination with primary anti-TB drugs against both planktonic and biofilm-forming cultures, showing potential benefit in augmenting treatment outcome when used during standard TB chemotherapy.

1. Introduction

Despite ongoing intense efforts to achieve its control, tuberculosis (TB), a disease caused by the *Mycobacterium tuberculosis* (*M. tuberculosis*) bacterium, remains a major threat to global public health, leading to high morbidity and mortality especially in low socioeconomic countries [1]. Treatment of TB patients encompasses those infected with drug-susceptible (DS)- and drug-resistant (DR)- TB isolates. The DS-TB-infected patients are treated for an overall period of six-to-nine months divided into an intensive phase covering the initial two months, when patients are given four primary anti-TB agents, rifampicin (RMP), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA), and a continuation phase in the remaining four- to seven-month period involving administration of RMP and INH only [1]. The two treatment phases are aimed at targeting the bacteria with different metabolic rates, with the intensive phase being effective in eliminating the actively replicating (AR) organisms, whereas the continuation phase, which targets the slow- (SR) and non-replicating (NR) populations has not, however, been successful in completely eliminating these bacterial subpopulations [2].

Despite this, the DS-TB treatment regimen is usually effective, resulting in a greater than 95% treatment success rate, with the remaining 5% representing treatment failure, even in those patients

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who completed treatment [3]. A major factor contributing to poor treatment outcome is the prolonged treatment schedule, which is associated with non-compliance in patients, leading to emergence of drug resistance in *M. tuberculosis* isolates [4]. These DR-mycobacterial strains have varying susceptibility profiles to anti-TB agents, including multidrug-resistant (MDR: *M. tuberculosis* strain resistant to both INH and RMP), MDR plus (MDR+: an MDR-TB strain resistant to at least one additional antibiotic other than a fluoroquinolone [ofloxacin and ciprofloxacin] and second-line injectable agent [amikacin, capreomycin, kanamycin]), extensively drug-resistant (XDR: MDR-TB strain resistant to at least one fluoroquinolone and any injectable antibiotic) and totally drug-resistant (TDR: *M. tuberculosis* strain resistant to all four of the aforementioned primary anti-TB agents and the second-line anti-TB antibiotics, which include fluoroquinolone and injectable antibiotics, as well as streptomycin, ethionamide, para-aminosalicylic acid and cycloserine) TB strains [5,6]. Drug resistance in *M. tuberculosis* has led to challenges in the chemotherapy of TB patients harbouring these resistant strains, with these patients receiving less effective therapeutic regimens over an extended period of 18–24 months, which disappointingly yields poor treatment success rates of 11%–44% [1,7]. However, an improvement in the cure rates of these patients has been achieved since 2018, when the World Health Organization (WHO) recommended the use of a standardised, nine-month, shorter, chemotherapeutic regimen that included clofazimine (CFZ), the prototype riminophenazine agent, throughout the treatment phase of MDR-TB patients [3,8–10], which resulted in high treatment success rates of 84%–89%. This shorter treatment regimen was associated with low relapse rates in patients, which has been attributed to many properties of CFZ including its high antimicrobial activity against the different metabolic subpopulations of the pathogen [7,10,11].

Despite these improvements in the treatment of DR-TB patients, CFZ is not, however, used in the treatment of DS-TB patients, and no clinical studies have seemingly been described to date. Nevertheless, the potential benefit of CFZ in the treatment of DS-TB has been demonstrated in vivo in murine models of experimental TB [12]. In these studies, DS-TB-infected mice were treated with a primary anti-TB-drug regimen containing CFZ, which resulted in the effective treatment period being shortened from six to four months [12,13]. This treatment benefit was also associated with low bacterial regrowth in animal tissues. Despite these in vivo activities, the antimicrobial effects of CFZ, when used in combination with primary agents against the DS-TB *M. tuberculosis*, have not been evaluated.

In this context, *M. tuberculosis* organisms with varying metabolic rates are isolated in different microenvironments during infection, including intracellularly in macrophages and extracellularly in granuloma lesions [14,15]. These metabolic variants of *M. tuberculosis* can be generated artificially by using different bacteriological culture procedures in vitro, with the AR, SR and NR bacteria being predominantly isolated in planktonic, biofilm-forming and matured, pre-formed biofilm cultures, respectively [2,16]. Using these procedures, the antimicrobial activities of CFZ alone, as well as the individual primary antimicrobial agents, against these metabolic variants of *M. tuberculosis* in vitro have been described. In this context, we have previously shown that CFZ has high inhibitory/low bactericidal activity against AR organisms, but high inhibitory/bactericidal activities against the SR bacteria, while it showed no effect against the NR organisms [7,16]. In contrast, other studies have demonstrated high bactericidal activity of CFZ against the NR variant of the pathogen in vitro, using an aerated, streptomycin-starved (SS18b) model of dormancy [7]. The primary agents, on the other hand, have shown high antimicrobial activity against the AR organisms [17,18], but lesser activity against the SR and NR variants [19]. These activities vary, however, according to the type of antibiotic, with RMP showing high bactericidal activity against AR and NR organisms; INH showing high bactericidal activity against AR, high inhibitory/low bactericidal activity against SR and poor activity against NR organisms; EMB showed low bacteriostatic/bactericidal activity against the AR and SR variants and a poor effect against NR organisms, while PZA showed low inhibitory/bactericidal activity against AR, but high bactericidal activity against the NR organisms [19].

Given the paucity of data on the interactive antimicrobial activity of CFZ with the primary anti-TB agents, we have investigated the anti-mycobacterial effects of this agent when used in combination with RMP, INH and/or EMB against metabolically distinct phenotypes of drug-sensitive *M. tuberculosis* in vitro. Our experimental approach involved a comparison of the antimicrobial activities of these agents individually and in combination against AR planktonic and SR biofilm-forming bacteria using the Loewe Additivity Model as described previously [20–22].

2. Materials and methods

2.1. Strain of Mycobacterium tuberculosis and growth media

The *M. tuberculosis* H37Rv laboratory reference strain, ATCC 25618, which is susceptible to all primary anti-TB antibiotics, was used. Middlebrook 7H9 broth (Difco, MI, USA) and 7H10 agar (Difco), supplemented with 10% oleic acid, dextrose, catalase (OADC) growth supplement (Becton Dickinson, NJ, USA) and 0.2/0.5% glycerol with/without Tween 80, were used for preparation of planktonic cultures and colony development, respectively. Sauton's broth medium (pH 7.2) was used for preparation of biofilm-forming cultures [16].

2.2. Antibiotics and chemicals

Unless otherwise stated, all antibiotics and chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The antibiotics used included CFZ and three primary anti-TB agents; INH, RMP and EMB. Clofazimine and RMP were each prepared in 100% dimethyl sulphoxide (DMSO) to stock concentrations of 2 mg/mL, while INH and EMB were each dissolved in sterile distilled water to stock concentrations of 10 mg/mL. The final working concentration ranges for each antibiotic were prepared by two-fold serial dilutions with appropriate solvents [16,19,23]. The concentration ranges were: CFZ, 0.002–20 μg/mL; RMP, 0.00003–1 μg/mL; INH, 0.00009–1 μg/mL and EMB, 0.003–40 μg/mL in planktonic cultures and at 0.009–1.25 μg/mL; 0.009–1 μg/mL; 0.0007–1 μg/mL and 0.06–16 μg/mL, respectively, for biofilm-forming cultures. The final DMSO concentration used in all the assays was 1%, and appropriate solvent controls were included in all assays.

2.3. Inoculum preparation

The *M. tuberculosis* inoculum was prepared as described previously [16,24]. Briefly, bacterial cells from frozen stock cultures were grown in 7H9 broth for seven days until they reached the mid-logarithmic phase corresponding to an optical density (OD) of 0.6 to 1 at 540 nm, measured spectrophotometrically using a Spectronic Helios UV-Vis spectrophotometer (Merck, New Jersey, USA). The cells were harvested by centrifugation (2851 × *g*, room temperature), washed twice and resuspended in phosphate-buffered saline (PBS: pH 7.4) to an OD of 0.6 at 540 nm [yielding approximately 10^7–10^8 colony-forming units (cfu)/mL]. The bacterial suspensions were stored at -20 °C and used to provide an inoculum size of approximately 10^5 cfu/mL in all assays.

2.4. Preparation of planktonic and biofilm-forming cultures

2.4.1. Bacterial growth

The cultures were prepared as described previously [24]. Planktonic cultures were prepared in 7H9 broth in 96-well micro-tissue culture plates to final volumes of 200 μL. An inoculum of 10^5 cfu/mL bacterial cells was added to the growth medium in each well, and the plates were incubated at 37 °C in the presence of 5% CO_2 for six days in the dark with frequent mixing to promote bacterial growth. Bacterial growth was determined using the microplate-based Alamar Blue Assay (MABA) method, which uses the colourimetric indicator Alamar (resazurin) that remains blue in the absence of growth and turns pink during growth. To determine growth, on day six (D6), Alamar Blue reagent (blue) (10% (v/v), final) was added to each well and the plates were incubated for a further 24-h period to allow for a change in colour from blue to pink in growing cultures [24].

The biofilm-forming cultures were prepared by inoculating the bacterial cells (10^5 cfu/mL) into Sauton broth medium in 24-well micro-tissue culture plates to a final volume of 2 mL per well, and the contents of the wells were thoroughly mixed. The plates were wrapped in three layers of parafilm, followed by incubation at 37 °C in the presence of 5% CO_2 in the dark for five weeks with no shaking. Biofilm formation was evaluated visually, by formation of a dense white irregular layer of rough appearance on the surface of Sauton's broth medium.

2.4.1.1. Biofilm quantification. The biofilm biomass in the biofilm-forming cultures was quantified using a crystal violet-based staining procedure as described previously [16,24]. The supernatants, containing planktonic cells in the biofilm-forming cultures, were removed, and the residual biomass in the wells was stained with 1% crystal violet (1 mL) and the biofilm-associated crystal violet was then extracted with 70% ethanol (1 mL), and 10-fold dilutions were made followed by measurement of the ODs at 570 nm using the Spectronic Helios UV-Vis spectrophotometer (Merck).The quantities of biofilm were determined at the end of week five (W5). The amount of biofilm $>$0.5 corresponded to visible biofilm formation on visual examination.

2.4.2. Bacterial viability

Bacterial viability was determined using a colony-counting procedure as described previously [16,24], and the cultures were prepared as for bacterial growth. For planktonic cultures, the contents of each well were thoroughly mixed and sampled followed by the preparation of serial 10-fold dilutions in PBS. The dilutions of planktonic cultures were plated on 7H10 agar medium and the plates incubated at 37 °C in 5% CO_2 for three weeks to allow for the development of colonies.

In the case of the biofilm-forming cultures, prior to plating, the biofilm-encased cells were released into the growth medium by dissolving the biofilm matrix in each well with Tween 80 (0.05% final) under shaking conditions at 37°C for 6 h. The contents of the wells were then plated as described for planktonic cultures. The cultures were plated on the initial and last days of each experiment, and these time points were recorded as day zero (D0) and day seven (D7) and as week zero (W0) and W5 for planktonic and biofilm-forming cultures, respectively. The number of colonies per plate were counted and the numbers of bacteria (cfu/mL) determined using the dilution theory as described [16,24].

2.5. Drug combination assays

Drug combinations of CFZ with the primary anti-TB agents were evaluated for their inhibitory and bactericidal effects using the Loewe Additivity Model as described previously [20,21]. This was achieved by determining the minimum inhibitory concentrations (MICs)/minimum bactericidal concentrations (MBCs) of the individual agents, which were used to determine the combination index (CI) as fractional inhibitory concentration index (FICI)/fractional bactericidal concentration index (FBCI) values, respectively [20,25].

2.5.1. Minimum inhibitory concentration (MIC) determination

The inhibitory activities of antibiotics in planktonic and biofilm-forming cultures were evaluated by determining MIC values by growth determination. For planktonic cultures, the cultures were prepared as described earlier (Section 2.4.1), and the various concentrations of each antibiotic, prepared in double-dilutions, were added to a set of drug-treatment wells, while appropriate solvent was added into a set of drug-free wells, as to the corresponding control systems. The cultures were incubated, and growth was determined using the MABA method as described earlier (Section 2.4.1). The MIC of each antibiotic for planktonic bacteria was taken as the lowest concentration of the antibiotic at which no growth was detected.

For biofilm-forming cultures, these were prepared and mixed with the different concentrations of each antibiotic, as described for the planktonic cultures, followed by incubation for biofilm development. The effects of the antibiotics on biofilm formation were determined by comparing the amount of biofilm, determined visually and by the crystal violet procedure, in the drug-treated systems with those of the drug-free controls. The MIC of each antibiotic for biofilm formation was regarded as the lowest concentration of the antibiotic that resulted in the absence of visible formation of biofilm, as well as a significant reduction in the amounts of biofilm formation to an OD of $<$0.5 using the crystal violet procedure.

2.5.2. Minimum bactericidal concentration (MBC) determination

The bactericidal activity of each antibiotic was evaluated by determining their MBC values according to viability determination. The cultures used for MIC determination were evaluated for MBC for both planktonic and biofilm cultures. For planktonic cultures, the contents of each well were plated onto 7H10 agar medium for determining the numbers of bacteria in cfu/mL, as described previously [16,24]. For biofilm-forming cultures, prior to plating, the biofilm matrix was dissolved with sterile Tween 80 (0.05% final concentration), followed by plating of the contents of the wells as described for the planktonic cultures [24].

The numbers of bacteria were determined at the initial (D0/W0) and final time points (D7/W5) for planktonic and biofilm-forming cultures, respectively. The lowest concentration of an antibiotic yielding at least a 2-log reduction in bacterial numbers at final time points in comparison to those of the initial time points for both cultures was taken as the MBC for that antibiotic.

2.5.3. Combination index (CI) determination: Fractional inhibitory concentration index (FICI) and fractional bactericidal concentration index (FBCI) values

The CI values were determined using the Loewe Additivity Model as described previously [20]. For FICI determinations, for both cultures, the antibiotic mixtures consisting of ratios of 1/2x, 1/3x and 1/4x the MIC values of each antibiotic in a combination for two-, three- and four-drug combinations, respectively, and their two-fold dilutions were added to the cultures followed by growth determination. The lowest dilution of each set of drug combinations that demonstrated bacterial inhibition was used for FICI determinations. The FICI was determined as the sum of the fractional inhibitory concentrations (FICs) of the individual antibiotics as described in the following formula:

$$\mathrm{FICI} = \sum \mathrm{FIC_N}$$

where: Σ = sum of FIC values of individual antibiotics.

FIC = CIC/MIC
N = number of antibiotics in a combination.
CIC = combined inhibitory concentration representing lowest concentration of an antibiotic that inhibits growth when it is in a combination.
MIC = lowest concentration of an antibiotic that inhibits growth when it acts alone.

For FBCI determinations, the cultures were prepared as described earlier for the FICI determination and the numbers of bacteria were determined as for MBC determinations. The lowest dilution of each antibiotic combination that yielded a 2-log reduction in the numbers of bacteria relative to those of the D0/W0 controls was used for determining the FBCI, as described earlier for the FICI determination [26,27].

The effect of antibiotics in a combination was regarded as synergistic, additive, indifferent or having antagonism when the CI values were ≤0.5, 0.5–1.0, 1.0–4.0 or >4.0, respectively [26,27].

2.6. Statistical analysis

Statistical analyses were performed on all data using the GraphPad Instat 3 Programme, and the results are expressed as the mean values ± standard deviations (SDs). The inhibitory and bactericidal activities of the antibiotics individually and in combination against planktonic and biofilm-forming cultures were determined using dose-response plots. Comparisons between drug-free controls and drug-treated systems were performed using the unpaired *t* test/Mann Whitney U-test. Each assay was repeated three times with triplicate determinations for each experimental system. In all assays in planktonic and biofilm-forming cultures, where the three different experiments showed different MIC/MBC and FICI/FBCI values, the experiments were repeated until three sets of experiments of antibiotics singly or in combinations achieved inhibitory/bactericidal effects at similar concentrations. Statistical significance for the effect of the antibiotics on bacterial cultures was taken as a $P < 0.05$. As the determinations of the MIC/FICI values in planktonic cultures were not based on quantitation of the colourimeric MABA readings but on similar concentrations showing minimum inhibitory effects, no statistical significance calculations were used in determining of these values.

3. Results

3.1. Drug combinations

Combining two, three and four of the test antibiotics generated six, four and one different combinations, respectively. These were tested for both inhibitory and lethal activities in planktonic and biofilm-forming cultures of *M. tuberculosis*.

3.2. Inhibitory activities

3.2.1. Minimum inhibitory concentration (MIC) determinations

In this study, in order to evaluate the antimicrobial activities of the individual antibiotics, a cut-off value of 1 µg/mL was used, with the inhibitory activity of an antibiotic regarded as being high or low when the MIC of the antibiotic was lower or higher than this cut-off value, respectively.

Using the MABA method, CFZ and the primary drugs, RMP and INH, showed high inhibitory activities against planktonic cultures of *M. tuberculosis*. However, the inhibitory activities of the two primary drugs were significantly greater than that of CFZ (Table 1 and Supplementary Fig. S1). Ethambutol demonstrated the least inhibitory activity of the test antibiotics and was also the only anti-TB agent with low inhibitory activity against planktonic bacteria, achieving an MIC value >1 µg/mL.

In the case of biofilm-forming organisms, similar to planktonic growth, the results showed that based on its low MIC value, CFZ also has high inhibitory activity against biofilm-forming bacteria. However, the MIC value was similar to that observed for planktonic bacteria (Table 1 and Supplementary Fig. S2). In the case of the primary drugs, RMP and INH also exhibited high inhibitory activity against biofilm-forming bacteria. However, these MIC values were higher than those achieved for planktonic bacteria, increasing by 100- and 20-fold, respectively. These increases in the antibiotic concentrations of these two agents resulted in their MIC values being comparable to those of CFZ against the biofilm-forming bacteria. For EMB, its activity in biofilm-forming cultures was again low, resulting in an MIC value higher than that of planktonic bacteria increasing by two-fold. Furthermore, it was the least active agent, being the only one with an MIC value of >1 µg/mL.

3.2.2. Inhibitory drug combination assays: Fractional inhibitory concentration index (FICI) determination

The inhibitory drug combination results are shown in Tables 2 and Supplementary Fig. S3 and Table 3 and Supplementary Fig. S4 (Table 2: planktonic cultures; Table 3: biofilm cultures) and are plotted in Fig. 1.

3.2.2.1. Two-drug combinations. Clofazimine exhibited synergistic inhibitory effects on the growth of the planktonic cultures when added in combination with either RMP or INH, with the CFZ/RMP combination being the most effective. An additive effect was observed for the combination of CFZ with EMB. However, for the primary anti-TB agents, synergistic inhibition of bacterial growth was observed when RMP was combined with either INH or EMB in the absence of CFZ. These combinations were more effective than those that included CFZ. Interestingly, the combination of the primary agents, EMB and INH, achieved an additive effect on planktonic bacteria.

For biofilm cultures, all three of the CFZ-containing combinations demonstrated synergistic inhibitory effects against the pathogen, which, similar to that of the planktonic cultures, were most effective in combination with RMP and INH. In the case of the primary antibiotics in the absence of CFZ, synergistic inhibitory activity was observed with combinations of RMP with either INH or EMB, which were similar to the effects observed on planktonic growth. Similar to planktonic bacteria, the combination of EMB and INH showed an additive inhibitory effect on biofilm-forming bacteria.

3.2.2.2. Three-drug combinations. All three-drug combinations demonstrated synergistic inhibitory activities against the planktonic growth of *M. tuberculosis*. For the CFZ-containing sets, the greatest synergistic activity was observed when this agent was used in combination with RMP and INH. The combination of the three primary drugs also exhibited high synergistic inhibition of mycobacterial growth, which was, however, comparable to that of CFZ with RMP and INH.

In the case of biofilm cultures, as with planktonic cultures, all three-drug combinations showed synergistic inhibitory effects. However, in contrast to that which was observed with the planktonic cultures, two of the three CFZ-containing combinations (CFZ+RMP+INH and CFZ+RMP+EMB) were the most effective (Table 2), while the combination of the three primary drugs was higher than that of the least effective CFZ-containing three-drug combination.

3.2.2.3. Four-drug combination. The combination of all four antibiotics also resulted in synergistic inhibition of planktonic growth of the mycobacteria that was comparable to the most effective two-drug combination of RMP+INH and the most effective

Table 1
The minimum inhibitory concentration values of clofazimine and primary anti-TB agents against planktonic and biofilm-forming *Mycobacterium tuberculosis*. The results are of three separate experiments performed in duplicate with one representative experiment performed for the MABA and crystal violet experiments in triplicate for planktonic and biofilm-forming cultures, respectively.

Antibiotics	MIC planktonic cultures (μg/mL)	MIC biofilm-forming cultures (μg/mL)	Crystal violet measurementsOD_{570} nm ± SD
Control	ND	ND	28 ± 0.5
CFZ	0.15	0.15	0.26 ± 0.009
INH	0.006	0.03	0.543 ± 0.071
RMP	0.002	0.125	0.28 ± 0.015
EMB	2.5	4	0.132 ± 0.133

ND, not done; MIC, minimum inhibitory concentration; OD, optical density.

Table 2
The fractional inhibitory concentration indices of clofazimine in combination with the primary anti-TB agents against planktonic *Mycobacterium tuberculosis*. The results are of three separate experiments performed in duplicate with one representative replicate performed for the MABA procedure in triplicate.

Antibiotic combinations	Ratios of individual antibiotics at FIC values	CIC of individual antibiotics (μg/mL)	FICI	Antibiotic interaction effects
CFZ + INH	1/4 + 1/4	0.03 + 0.001	0.5	Synergistic
CFZ + RMP	1/8 + 1/8	0.01 + 0.0002	0.25	Synergistic
CFZ + EMB	1/2 +1/2	0.07 + 1.25	1.0	Additive
EMB + INH	1/2 +1/2	1.25 + 0.003	1.0	Additive
RMP + EMB	1/16 + 1/16	0.0001 + 0.16	0.125	Synergistic
RMP + INH	1/32 + 1/32	0.00006 + 0.0002	0.0625	Synergistic
CFZ + INH + EMB	1/24 + 1/24 + 1/24	0.006 + 0.0003 + 0.1	0.125	Synergistic
CFZ + RMP + EMB	1/12 + 1/12 + 1/12	0.01 + 0.0001 + 0.2	0.25	Synergistic
CFZ + RMP + INH	1/48 + 1/48 + 1/48	0.003 + 0.00004 + 0.0001	0.0625	Synergistic
RMP + INH + EMB	1/48 + 1/48 + 1/48	0.00004 + 0.0001 + 0.05	0.0625	Synergistic
CFZ + RMP + INH + EMB	1/64 + 1/64 + 1/64 + 1/64	0.002 + 0.00003 + 0.00009 + 0.003	0.0625	Synergistic

CIC, combined inhibitory concentration; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index; ND, not done.

Table 3
The fractional inhibitory concentration indices of clofazimine in combination with the primary anti-TB agents against biofilm-forming *Mycobacterium tuberculosis*. The results are of three separate experiments performed in duplicate with one representative replicate performed for the crystal violet procedure in triplicate

Antibiotic combinations	Ratios of individual antibiotics at FIC values	CIC of individual antibiotics (μg/mL)	FICI	Antibiotic interaction effects	Crystal violet measurements OD_{570} nm ± SD
CONTROL	ND	ND	ND	ND	29.90 ± 0.02
CFZ + INH	1/16 + 1/16	0.009 + 0.002	0.125	Synergistic	0.532 ± 0.071
CFZ + RMP	1/16 + 1/16	0.009 + 0.008	0.125	Synergistic	0.531 ± 0.028
CFZ + EMB	1/8 + 1/8	0.02 + 0.5	0.25	Synergistic	0.497 ± 0.041
RMP + EMB	1/8 + 1/8	0.016 + 0.5	0.25	Synergistic	0.519 ± 0.047
INH + EMB	1/2 + 1/2	0.015 + 2	1	Additive	0.502 ± 0.152
RMP + INH	1/16 + 1/16	0.008 + 0.002	0.125	Synergistic	0.543 ± 0.212
CFZ + RMP + EMB	1/24 + 1/24 + 1/24	0.005 + 0.005 + 0.15	0.125	Synergistic	0.541 ± 0.125
CFZ + RMP + INH	1/24 + 1/24 + 1/24	0.006 + 0.005 + 0.001	0.125	Synergistic	0.186 ± 0.016
CFZ + INH + EMB	1/6 + 1/6 + 1/6	0.02 + 0.005 + 0.66	0.5	Synergistic	0.542 ± 0.073
RMP + INH + EMB	1/12 + 1/12 + 1/12	0.01 + 0.025 + 0.3	0.25	Synergistic	0.456 ± 0.213
CFZ + RMP + INH + EMB	1/64 + 1/64 + 1/64 + 1/64	0.002 + 0.001 + 0.0004 + 0.06	0.0625	Synergistic	0.365 ± 0.003

FIC, fractional inhibitory concentration; CIC, combined inhibitory concentration; FICI, fractional inhibitory concentration index; ND, not done; OD, optical density.

three-drug combinations of RMP+INH+EMB and RMP+INH+CFZ (FICI = 0.0625, all three sets). In the case of the biofilm cultures, addition of CFZ to the three primary antibiotics resulted in synergistic inhibition of the growth of biofilm-forming bacteria.

The four-drug combinations were found to be the most effective of all the inhibitory combinations tested against both cultures (FICI = 0.0625) (Table 4).

3.3. Bactericidal activities

3.3.1. Minimum bactericidal concentration (MBC) determinations

For MBC determinations, similar to the growth inhibitory activity as described earlier, a cut-off value of 1 μg/mL was used to evaluate the activity of the individual agents against the bacterial cultures (Table 5 and Supplementary Fig. S5). Clofazimine exhibited low bactericidal activity against planktonic bacteria, achieving an MBC value of 5 μg/mL, which is 30-fold higher than its corresponding MIC value, confirming findings previously reported by Mothiba et al. [16]. In the case of the primary drugs, RMP and INH exhibited the highest bactericidal activities, while the activity of EMB was low, showing an MBC similar to that of CFZ. The MBC values of these three primary anti-TB agents were two-fold higher than their MIC values against the planktonic bacteria.

Clofazimine, on the other hand, showed high bactericidal activity against biofilm-forming mycobacteria, with an MBC value that was similar to its MIC value against these organisms. In the case of the primary drugs, RMP and INH, as with planktonic bacteria, demonstrated the highest bactericidal activities with MBC values that were lower than those observed for CFZ. The MBC for RMP was similar to its MIC, while that of INH was increased by two-fold to that of its MIC against biofilm-forming bacteria (Tables 1 and 5 and Supplementary Fig. S6). However, despite the high bactericidal activities, the MBC values for these two primary anti-TB agents against biofilm-forming bacteria were higher than those shown against the planktonic bacteria increasing by 32- and 4.8-fold for RMP and INH, respectively. Similar to the results obtained

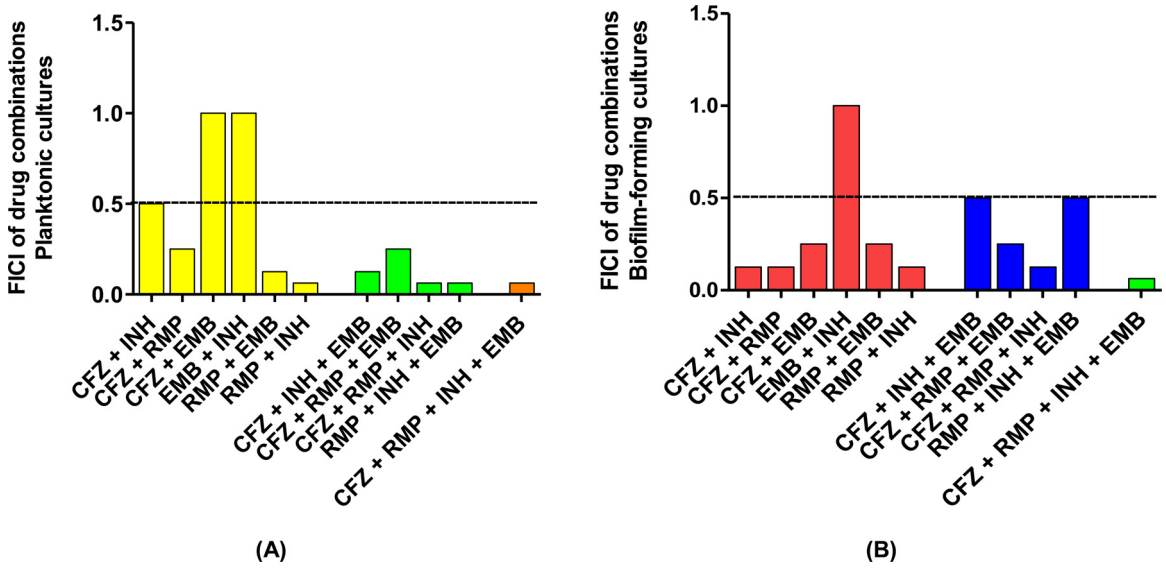

Fig. 1. Inhibitory interactions of clofazimine (CFZ) in combination with the primary anti-tuberculosis drugs on the growth of (A) planktonic and (B) biofilm-forming *Mycobacterium tuberculosis* using the fractional inhibitory concentration indices (FICIs). The graph was plotted using the final FICI values shown in Table 1. Synergistic inhibitory effect representing a FICI value ≤0.5 is shown by the horizontal dashed line (—).

Table 4
Summary of clofazimine-containing combinations, according to the degree of synergism, against planktonic and biofilm-forming *Mycobacterium tuberculosis*, with highly synergistic combinations corresponding to low CI values

	Planktonic bacteria		Biofilm-forming bacteria	
CI value achieved	Inhibitory effect	Bactericidal effect	Inhibitory effect	Bactericidal effect
0.0625	CFZ + RMP + INH CFZ + RMP + INH + EMB	NA	CFZ + RMP + INH + EMB	NA
0.125	CFZ + INH + EMB	NA	CFZ + RMP + INH	CFZ + RMP + INH + EMB
0.25	CFZ + RMP CFZ + RMP + EMB	NA	CFZ + RMP CFZ + RMP + EMB	CFZ +RMP CFZ + RMP + EMB CFZ + RMP + INH
0.5	CFZ + INH	CFZ + RMP	CFZ + EMB CFZ + INH CFZ + INH + EMB	CFZ + EMB CFZ + INH CFZ + INH + EMB

CI, combination index for FICI/ FBCI for inhibitory or bactericidal effect respectively; NA, not achieved.

Table 5
The minimum bactericidal concentration values of clofazimine and primary anti-TB agents against planktonic and biofilm-forming *Mycobacterium tuberculosis*. The results are of three separate experiments performed in duplicate with one representative replicate performed in triplicate

	MBC planktonic cultures			MBC biofilm-forming cultures		
Antibiotics	μg/mL	cfu/mL ± SD (D0)	cfu/mL ± SD (D7)	μg/mL	cfu/mL ± SD (W0)	cfu/mL ± SD (W5)
CONTROL	ND	4.8×10^4	$9.6 \times 10^6 \pm 1.3 \times 10^6$	ND	6.9×10^5	$4.6 \times 10^7 \pm 2.8 \times 10^6$
CFZ	5	±	$3.2 \times 10^2 \pm 19$	0.15	±	$4.6 \times 10^2 \pm 20$
INH	0.0125	1.6×10^3	$2.99 \times 10^2 \pm 58$	0.06	6.3×10^4	$3.1 \times 10^2 \pm 34$
RMP	0.004		$1.17 \times 10^2 \pm 10.1$	0.125		$1.1 \times 10^3 \pm 1.1 \times 10^2$
EMB	5		$3.8 \times 10^2 \pm 59$	8		$1.2 \times 10^3 \pm 5.1 \times 10^2$

MBC, minimum bactericidal concentration; D0/D7, day 0/day 7; ND, not done; SD, standard deviation; W0/W5, week 0/week 5.

for planktonic organisms, EMB was the least effective agent against biofilm-forming bacteria, with an MBC value two-fold higher than that for planktonic bacteria. The MBC value for EMB was the highest achievable concentration among the anti-TB agents against both planktonic and biofilm bacteria (8 μg/mL, Table 5).

3.3.2. Bactericidal drug combination assays: Fractional bactericidal concentration index (FBCI) determination

The bactericidal drug combination results are shown in Tables 6 and 7 and plotted in Fig. 2. In all antibiotic bactericidal combinations tested against both planktonic (Table 6 and Supplementary Fig. S7) and biofilm-forming (Table 7 and Supplementary Fig. S8) cultures, CFZ was used at a concentration of 0.15 μg/mL, which was the sub-MBC concentration found for the planktonic cultures (MBC: 5 μg/mL) but corresponding to its MBC against biofilm-forming organisms.

3.3.2.1. Two-drug combinations. Synergistic bactericidal activity against the planktonic cultures was observed in one of the three CFZ-containing two-drug combination sets, *viz*., CFZ+RMP, and in another combination consisting of the primary agents, RMP+INH. Both combinations exhibited comparable levels of synergy. Clofazimine showed poor bactericidal effects when used in combination with either INH or EMB. However, for combinations of the primary anti-TB agents alone, poor activity was shown by combinations of EMB with RMP or INH. Combinations of EMB with either CFZ or INH were the least effective two-drug combinations, with the FICI not being achievable. This would imply an antagonistic effect since

Table 6
The fractional bactericidal concentration indices of clofazimine in combination with the primary anti-TB agents against planktonic *Mycobacterium tuberculosis*. The results are of three separate experiments performed in duplicate with one representative replicate performed in triplicate

Antibiotic combinations	cfu/mL ± SD (D0)	cfu/mL ± SD (D7)	Ratios of individual antibiotics at FBC values	CBC of individual antibiotics (μg/mL)	FBCI	Antibiotic interaction effects
CONTROL	$5.6 \times 10^4 \pm 2.5 \times 10^3$	$2.6 \times 10^6 \pm 4.2 \times 10^5$	ND	ND	ND	ND
CFZ + INH		10 ± 3	1/2 + 1/2	0.075 + 0.006	1	Additive
CFZ + RMP		$1.95 \times 10^2 \pm 7$	1/4 + 1/4	0.0375 + 0.001	0.5	Synergistic
CFZ + EMB		$3.5 \times 10^4 \pm 3.9 \times 10^3$	NA	NA	NA	NA
EMB + INH		$6.1 \times 10^4 \pm 1.5 \times 10^3$	NA	NA	NA	NA
EMB + RMP		$1.7 \times 10^3 \pm 1.8 \times 10^2$	1 + 1	2.4 + 0.006	2	Indifferent
RMP + INH		13 ± 5	1/4 + 1/4	0.001 + 0.003	0.5	Synergistic
CFZ + INH + EMB		$3.4 \times 10^2 \pm 8$	1/3 + 1/3 + 1/3	0.05 + 0.004 + 1.2	1	Additive
CFZ + RMP + EMB		72 ± 7	1/3 + 1/3 + 1/3	0.05 + 0.001 + 1.2	1	Additive
CFZ + RMP + INH		26 ± 17	1/3 + 1/3 + 1/3	0.05 + 0.001 + 0.004	1	Synergistic
RMP + INH + EMB		$2.9 \times 10^2 \pm 21$	1/12 + 1/12 + 1/12	0.0004 + 0.001 + 0.5	0.25	Synergistic
CFZ + RMP + INH + EMB		7.2 ± 4.0	1/4 + 1/4 + 1/4 + 1/4	0.038 + 0.001 + 0.003 + 1.2	1.0	Additive

CBC, combined bactericidal concentration; FBC, fractional bactericidal concentration; FBCI, fractional bactericidal concentration index; NA, not achieved; ND, not done.

Table 7
The fractional bactericidal concentration indices of clofazimine in combination with the primary anti-TB agents against biofilm-forming *Mycobacterium tuberculosis*. The results are of three separate experiments performed in duplicate with one representative replicate performed in triplicate

Antibiotic combinations	cfu/mL ± SD (W0)	cfu/mL ± SD (W5)	Ratios of individual antibiotics at FBC values	CBC of individual antibiotics (μg/mL)	FBCI	Antibiotic interaction effects
CONTROL	$1.7 \times 10^4 \pm 1.5 \times 10^3$	$1.17 \times 10^7 \pm 1.6 \times 10^6$	ND	ND	ND	ND
CFZ + INH		32 ± 4	1/4 + 1/4	0.03 + 0.01	0.5	Synergistic
CFZ + RMP		$1.4 \times 10^2 \pm 58$	1/8 + 1/8	0.03 + 0.03	0.25	Synergistic
CFZ + EMB		$5.4 \times 10^2 \pm 14$	1/4 + 1/4	0.03 + 2.0	0.5	Synergistic
INH + EMB		45 ± 8	1/2 + 1/2	0.03 + 4.0	1.0	Additive
RMP + EMB		20 ± 11	1/2 + 1/2	0.06 + 4	1.0	Additive
RMP + INH		$1.1 \times 10^2 \pm 6$	1/4 + 1/4	0.03 + 0.015	0.5	Synergistic
CFZ + INH + EMB		$5 \times 10^2 \pm 17$	1/6 + 1/6 + 1/6	0.02 + 0.003 + 1.3	0.5	Synergistic
CFZ + RMP +EMB		$1 \times 10^2 \pm 46$	1/12 + 1/12 + 1/12	0.01 + 0.01 + 0.6	0.25	Synergistic
CFZ + RMP + INH		32 ± 1.5	1/12 + 1/12 + 1/12	0.01 + 0.01 + 0.005	0.25	Synergistic
RMP + INH + EMB		1 ± 0	1/12 + 1/12 + 1/12	0.01 + 0.006 + 0.6	0.25	Synergistic
CFZ + RMP + INH + EMB		1 ± 0	1/32 + 1/32 + 1/32 + 1/32	0.004 + 0.003 + 0.001 + 0.25	0.125	Synergistic

CBC, combined bactericidal concentration; FBC, fractional bactericidal concentration; FBCI, fractional bactericidal concentration index; ND, not done; W0/W5, week 0/week 5.

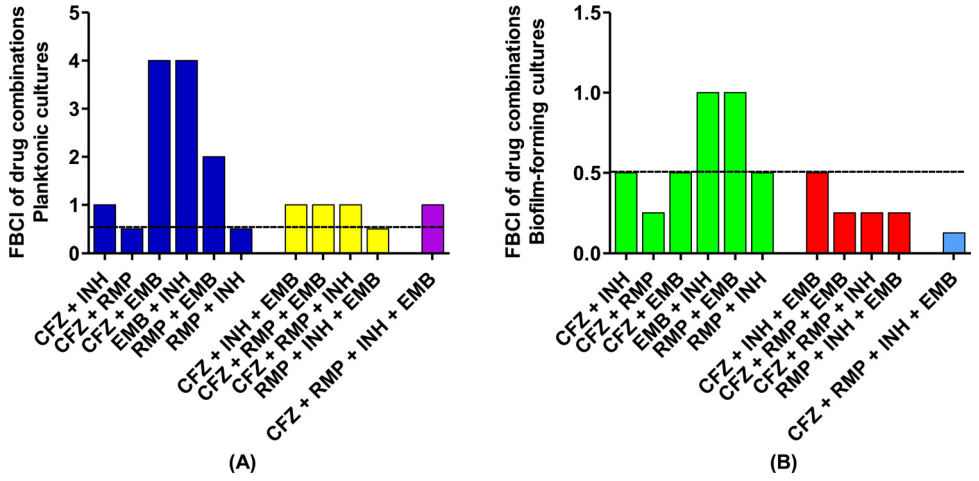

Fig. 2. Bactericidal interactions of clofazimine (CFZ) in combination with the primary antibiotics against (A) planktonic and (B) biofilm-forming *Mycobacterium tuberculosis* using the fractional bactericidal concentration indices (FBCIs). The graph was plotted using the final FBCI values shown in Tables 2 and 3. Synergistic bactericidal effect representing a FBCI value ≤0.5 is shown by the horizontal dashed line (–).

combination assays were determined from 2x MIC of each agent in a set.

However, in the case of the biofilm cultures, all three of the CFZ-containing two-drug combinations showed synergistic bactericidal effects, with the highest synergistic activity shown in combination with RMP. For the primary drugs in the absence of CFZ, synergistic bactericidal activity was exhibited by the combination of RMP and INH as noted with the planktonic bacteria.

3.3.2.2. Three-drug combinations. For planktonic cultures, none of the three CFZ-containing three-drug combinations demonstrated synergistic bactericidal effects on the bacteria. However, these three-drug combinations achieved an additive bactericidal effect against bacteria in planktonic culture. A synergistic bactericidal effect was, however, observed with the combination consisting of the three primary anti-TB drugs in the absence of CFZ.

In the case of the biofilm cultures, all four of the three-antibiotic combinations demonstrated synergistic bactericidal effects against biofilm-forming *M. tuberculosis*. Two of the three CFZ-containing combinations (CFZ+RMP+INH and CFZ+RMP+EMB) and the combination of the three primary antibiotics in the absence of CFZ demonstrated the highest, albeit comparable, synergistic activities (FBCI = 0.25, for all).

3.3.2.3. Four-drug combinations. Interaction of CFZ with the three primary drugs in a four-drug combination achieved an additive bactericidal effect against planktonic bacteria. However, this antibiotic combination resulted in synergistic bactericidal activity against biofilm-forming bacteria. It was also the most potent of all the bactericidal combinations tested against biofilm-forming bacteria (FBCI = 0.125) (Table 4).

The CFZ-containing combinations that demonstrated the highest inhibitory and bactericidal effects in both planktonic and biofilm-forming cultures are summarised in Table 4.

4. Discussion

Anti-mycobacterial chemotherapy administered to DS-TB patients is effective in eliminating the AR organisms, while it has limited effect against the SR and NR organisms [2]. This limited activity against SR and NR organisms contributes to treatment failure in DS-TB patients, which in turn leads to the emergence of drug resistance, highlighting the need for the development of an effective chemotherapeutic regimen for treatment of these patients [2,28].

The benefit of addition of CFZ in DS-TB chemotherapy has been demonstrated in a murine model of experimental TB, resulting in shorter treatment length from six to four months, as well as a delay in bacterial regrowth in infected organs, suggesting a delay in relapse development [12,29]. This contention has been supported by clinical studies involving MDR-TB patients treated with a CFZ-containing regimen that reported a delay in relapse within 24 months following treatment cessation [30]. Despite these in vivo effects, the clinical utility of addition of CFZ to the standard chemotherapeutic regimen of DS-TB patients has not been described. The theoretical rationale for this therapeutic strategy was investigated in the current study by determining the effects of combining CFZ with three primary anti-TB drugs, RMP, INH and EMB, against the AR and SR variants of *M. tuberculosis* H37Rv cultured in planktonic and biofilm-forming environments, respectively, using the Loewe Additivity Model [20–22]. This model, which allows for the simultaneous evaluation of the interaction of any number of antibiotics, enabled investigation of combinations of CFZ and the primary anti-TB agents used in chemotherapeutic regimen of DS-TB patients in two-, three- and four-drug combination sets against the AR and SR metabolic variants of *M. tuberculosis*.

In the context of AR phase organisms, we have previously demonstrated that CFZ alone has high growth inhibitory activity against these bacteria [31,32]. However, in the current study, we showed that its inhibitory activity was lower than that of RMP and INH individually, but higher than that of EMB. In combination with the three primary anti-TB antibiotics, the observed synergistic inhibitory effects on bacterial growth were either comparable to or less than the corresponding activities of combinations of the primary agents alone, especially those containing both RMP and INH.

With regard to lethal activities, CFZ demonstrated low bactericidal activity against AR bacteria, being lower than the primary anti-TB agents, RMP and INH, and comparable to that of the lowest bactericidal antibiotic, EMB. The low bactericidal effect of CFZ has been associated with the necessity for prolonged exposure of the bacteria to the antimicrobial agent [30,33], as well as to high antibiotic concentrations [16]. These effects were also evident when CFZ was used in combination with the primary anti-TB agents, demonstrating only low-level synergism with respect to bactericidal activity against AR bacteria in a two-drug combination with RMP. As with inhibitory activity, the most effective bactericidal combinations were those consisting of primary anti-TB agents, especially those containing RMP and INH together.

In the case of the SR bacteria, on the other hand, CFZ possessed high antimicrobial activity against these organisms, showing both high growth inhibitory and bactericidal activities [16]. Surprisingly, the concentrations of CFZ at which these antimicrobial effects on SR organisms were observed were similar to those that had minimal effect on AR organisms. This distinction with respect to the differential susceptibility of AR and SR organisms to CFZ may relate to augmentation of the antimicrobial activity of this agent due to the presence of oxygen radicals, which are highly generated during biofilm formation [24,34]. These findings contrasted with the decreased activities of RMP and INH against SR organisms, being achievable at higher concentrations of these antibiotics. This may be related to the mechanisms of antimicrobial action of these antibiotics, which target metabolic systems that operate optimally in AR bacteria, including protein synthesis in the case of RMP [35] and cell wall biosynthesis by INH and EMB [36], which are attenuated during the transition of *M. tuberculosis* from AR to SR organisms. We do concede, however, that lower activity of primary anti-TB agents in biofilm-forming cultures experiments may possibly relate to instability of these antibiotics over the five-week incubation period.

Likewise, in contrast to the moderate synergistic effects, both inhibitory and bactericidal, against AR planktonic organisms, the CFZ/primary anti-TB drug combinations were considerably more effective against the SR organisms with respect to both bacteriostasis and bactericidal activities. In this context, combining CFZ with the primary anti-TB agents resulted in levels of synergism that were higher than any of the combinations of the primary anti-TB agents in the absence of CFZ, with the highest activities observed in the CFZ-containing combinations with RMP and INH. As mentioned earlier, RMP and INH are used during the continuation phase of TB chemotherapy, seemingly targeting SR and NR bacteria, against which they appear to have limited therapeutic activity, with many patients developing relapse [2,28]. Although speculative, the therapeutic potential of CFZ may be most prominent during the continuation phase of anti-TB therapy.

Despite their effective in vitro antimicrobial activities, the therapeutic efficacy of CFZ and the primary anti-TB agents in the clinical setting of TB lesions, which consist of varying types of tuberculous granulomas, may be affected by various pharmacokinetic properties such as drug distribution, microenvironmental conditions and the half-lives of the antibiotics. The proposed locations and different metabolic phenotypes of *M. tuberculosis* in the granuloma lesions, as well as the influence of the pharmacokinetic profiles of the test antibiotics, are depicted in Fig. 3. Intracellular AR and SR organisms are located in the cellular peripheral layer, while the non-cellular central hypoxic, anaerobic region harbours extracellular SR and NR bacteria. In these environments, CFZ has demonstrated a high level of penetration and accumulation in the cellular rim due to its high lipophilicity and long half-life in tissues (~70 days), but low accumulation in the caseum and non-cellular aqueous regions [7,37,38]. The lipophilic antibiotic, RMP, has shown high accumulation in both regions, while, INH, a hydrophilic agent, has demonstrated high penetration in both regions but poor accumulation in caseous lesions due to rapid clearance [37,38]. These differences in drug distribution suggest that the addition of CFZ to RMP- and INH-containing anti-TB drug regimens may augment therapeutic efficacy against the various metabolic phenotypes of *M. tuberculosis*. In this setting, the high accumulation of CFZ and both primary anti-TB agents, RMP and INH, in cellular regions leads to disruption of the granuloma membrane, with

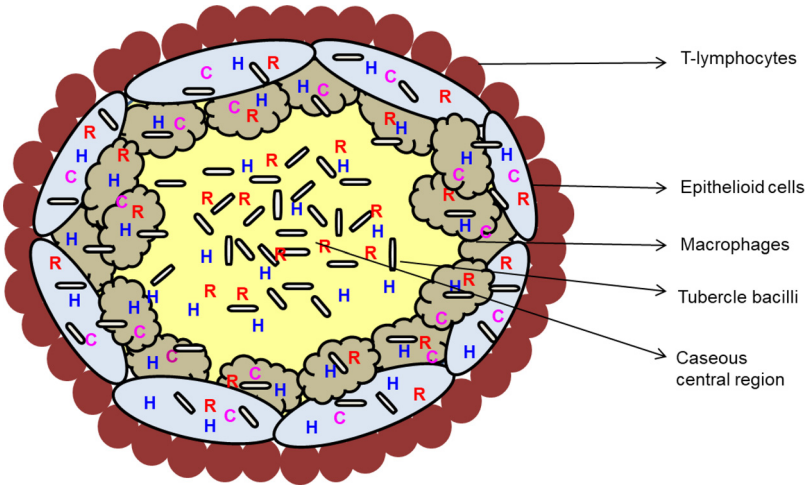

Fig. 3. The working model showing distribution of clofazimine (CFZ), rifampicin (RMP) and isoniazid (INH) in the granuloma lesion of tuberculosis showing the peripheral lining of the lipid-rich macrophages and epithelioid cells, T-lymphocytes and the aqueous necrotic centre containing non-replicating (NR) organisms. The antibiotic distribution shows CFZ and RMP and INH accumulating in the lipid-rich macrophages and epithelioid cells, while RMP and INH are concentrated in the aqueous central foci. The letters C, R and H denote clofazimine, rifampicin and isoniazid, respectively.

the synergistic activity of these antibiotics resulting in killing of the AR and SR organisms, while accumulation of the primary antibiotics INH and RMP may act synergistically against the SR organisms residing in the caseum granuloma [39].

5. Conclusion

The findings of this study have revealed that combining CFZ with primary anti-TB drugs, especially RMP, results in synergistic suppression and elimination of the growth of AR bacteria, albeit less so than that observed with CFZ-free combinations of the primary drugs, specifically RMP and INH. More importantly, however, CFZ demonstrated significant augmentative interactions with RMP and INH against SR organisms found in biofilm-forming cultures. These findings demonstrate the potential benefit of addition of CFZ to standard anti-TB chemotherapy, which may promote increased efficacy in eliminating SR bacteria. This is likely to be achieved through the bactericidal activity of CFZ against these organisms, possibly shortening treatment and/or preventing disease relapse, due to reactivation and slow growth of moderately drug-tolerant SR persister bacterial populations. Nevertheless, in order to provide conclusive information on inclusion of CFZ in the chemotherapy of DS-TB patients, future studies involving the inclusion of PZA are necessary.

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Competing interests

None declared.

Ethical approval

Ethical approval was received from the Faculty of Health Sciences Research Ethics Committee of University of Pretoria, South Africa [Approval number 300/2013].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.03.008.

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