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Drug Discovery and Resistance

Effect of various drugs on differentially detectable persisters of *Mycobacterium tuberculosis* generated by long-term lipid diet

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ABSTRACT

Persisters of *Mycobacterium tuberculosis* (Mtb) that fail to form colonies on agar media when de-stressed are termed as differentially detectable (DD) persisters. Since in the host, Mtb primarily survives by utilizing lipids, we used a long-term lipid diet model to induce DD persisters of *M. tuberculosis*. Persisters were induced by replacing the dextrose-containing medium with one containing fatty acids instead of dextrose (FAM). After 2, 4 or 6 weeks, CFU and most probable number assays were performed; the difference between the two gave an estimate of DD persisters. Since rifampicin has been shown to induce formation of DD persisters in vitro, one set of FAM cultures were also given short-term rifampicin stress after 2, 4 or 6 weeks. Fraction of DD persisters increased with time and rifampicin treatment enhanced the effect of fatty acids, at 2 and 4 weeks. At six weeks, even in the absence of rifampicin, ~95% population were DD persisters. The DD persisters were vulnerable to drugs interfering with bacterial respiration such as thioridazine, bedaquiline and clofazimine. The study indicates potential formation of DD persisters of Mtb in a lipid-rich microenvironment in the host even before antibiotic therapy.

1. Introduction

Infection with *Mycobacterium tuberculosis* (Mtb) is tough to be eliminated from the host because of the remarkable ability of the bacilli to become persisters that are phenotypically drug tolerant [1,2]. Bacterial persisters are a transiently antibiotic-tolerant subpopulation that are often slow-replicating or non-replicating, and are able to resume growth up on removal of a lethal stress [3]. Persisters are therefore responsible for lengthy treatments, relapse and chronic infections [4]. In TB patients undergoing treatment for drug susceptible TB, a small drug tolerant population of bacilli in the lesions has been considered responsible for the decreased rate of killing by anti-tubercular agents after an initial fast killing [5]. In vitro, Mtb persisters occur in low numbers during early growth phase and increase sharply to contribute ~1% of the population in the stationary phase [6]. Transcriptomes of in vitro as well as in vivo persisters show that they are slow growing or growth arrested, in a downregulated biosynthetic and metabolic state [5,6]. Mtb persisters are thought to be heterogenous populations consisting of nonreplicating bacilli, produced by varying stress conditions encountered in the host. Properties of the persisters therefore are defined by the particular stress condition that may affect the acid fast staining, ability to grow on solid media and dependence on resuscitation promoting factors [4]. Nevertheless, a property that is common to all persister populations is the phenotypic drug tolerance. Hence it becomes essential to test the bactericidal activity of newly identified anti-tubercular drugs on non replicating Mtb persisters. Various methods employed to generate nonreplicating Mtb in vitro include exposing the bacilli to hypoxia [7], nutrient starvation [8], multiple stress [9] or fatty acids as sole carbon source [10]. A streptomycin-dependent Mtb strain has also been demonstrated suitable for screening molecules against dormant Mtb [11]. The non-replicating bacilli resume growth upon plating out on agar media without the stress factor (s). Newly identified anti-tubercular compounds are tested against these 'colony forming' persisters of Mtb, notably in well established models employing hypoxia or nutrient starvation. However, the stress might also convert a certain population of the bacteria to viable but 'non-colony forming' persisters which go undetected on the re-growth experiment on agar media. This type of persisters are estimated by most probable number (MPN) assays, based on the fact that they are capable of resuming division when diluted in liquid media, and hence named recently as "differentially detectable or DD" persisters [12].

Presence of DD persisters of Mtb has been reported in sputa from

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active tuberculosis cases [13,14]. Smear positive but culture (Löwenstein -Jensen medium) negative sputa that test positive for Mtb DNA by molecular assays [15], may indicate presence of DD persisters. However, it is not necessary that a drug bactericidal to colony forming persisters is bactericidal to DD persisters as well. For instance, rifampicin is bactericidal to nonreplicating, colony forming persisters formed in a hypoxic culture [7]. However, a later study showed that when Mtb in late stationary-phase were treated with a high dose of rifampin, despite a 5-log killing, nearly 3 logs of bacteria remained viable by MPN assay, in other words, remained DD [16]. This in vitro observation can be related to the in vivo situation after chemotherapy. Hence, it is important to include both CFU assay and MPN assay in such models. Occurrence of DD persisters in various in vitro models of Mtb dormancy reported so far make use of hypoxia [17], drug stress [17,18], potassium starvation [19], gradual acidification [20] and most recently, sequential stress of nutrient starvation and rifampicin treatment [12]. On the contrary, DD persisters were not obtained in certain models, for instance, those using nutrient starvation or multiple stress in presence of butyrate [12].

Both old and new studies indicate that Mtb in the infected host prefer to use fatty acids and cholesterol as carbon source [21,22]. An elegant study conducted in tuberculous sputa showed the invariable presence of Mtb containing lipid bodies, which significantly correlated with a transcriptome signature and growth indices for nonreplicating Mtb [23]. Lipid-rich macrophages (foamy macrophages) of the tubercular granuloma [24] and adipocytes [25,26] are thought to be reservoirs used by Mtb for long-term persistence within the host. Hence, in the present study we used a fatty acid mixture as sole carbon source to induce nonreplicating persistence and to get an enriched population of DD persisters of Mtb. Further we tested a few drugs of different mechanisms of action, on the persisters.

2. Materials and methods

2.1. Antimicrobials and other chemicals

Moxifloxacin and bedaquiline were purchased from Cayman Chemical (MI, USA) while rifampicin, clofazimine and thioridazine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions (5 mg/ml) for all compounds were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. Sodium palmitate, sodium oleate and potassium linoleate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Bacterial culture

Mycobacterium tuberculosis H37Rv was grown in 100 ml Middlebrook 7H9(MB7H9) (Becton Dickinson, Sparks, MD) containing 0.2% glycerol, 0.05% tween-80 supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) at 37 °C up to OD_{600} ~ 1.0. Culture was washed twice in MB7H9 base medium and resuspended in 100 ml of MB7H9 base medium containing tyloxapol (0.05%). Twenty millilitres of the culture was aliquoted separately while remaining 80 ml was supplemented with albumin (0.5%), NaCl (0.085%), sodium palmitate (0.004%), sodium oleate (0.004%) and potassium linoleate (0.004%). This fatty acid mixture supplemented medium is hereafter called fatty acid medium (FAM). Both the aliquoted cultures were incubated statically at 37 °C/5% CO_2. Two millilitres of sample were collected from each culture at different time points (2, 4 and 6 weeks). Out of each 2 ml culture, rifampicin was added to 1 ml (100 μM final conc.) and was further subjected for CFU and MPN assays after five days.

2.3. Antibiotic sensitivity testing

The FAM culture at 6 weeks of incubation was sampled for CFU and MPN assays (Day 0). The remaining culture was either directly tested with various antibiotics or given a five-days pre-treatment with 100 μM rifampicin/DMSO (vehicle control). The rifampicin/DMSO pre-treated cultures were washed twice with MB7H9 base medium and re-suspended in FAM. Aliquots were subjected to CFU and MPN estimation. One millilitre-aliquots of the pre-treated or untreated FAM cultures in microfuge tubes were treated with drug solutions and incubated at 37 °C for 7 days. Bedaquiline (BDQ), rifampicin (RIF), moxifloxacin (MXF), and clofazimine (CFZ) were tested at 0.5 and 5 μg/ml whereas thioridazine (TRZ) was tested at 5 and 50 μg/ml along with DMSO as vehicle control. After incubation, bacilli were harvested, and washed twice with MB7H9 base medium and subjected to CFU and MPN assays.

2.4. MPN assay

MPN assay was performed in 96-well plates, by diluting culture in MB7H9 + OADC + glycerol + tyloxapol medium as pentuplicates, according to the method described by Saito et al 2017 [12]. Plates were incubated at 37 °C and 5% CO_2, without agitation and were observed every week for any visible growth. Data was entered in to the MPN calculator excel sheet, as provided by Jarvis et al 2010 [27].

2.5. CFU plating

Pentuplicates of each dilution made for MPN assay were pooled, before plating out fifty micro liters on 7H11 agar + OADC + glycerol. Plates were sealed by adhesive tape to avoid evaporation and incubated at 37 °C for 4–8 weeks.

2.6. Data analysis

Statistical significance of data when required were determined by one way ANOVA with Dunnett's multiple comparison test, using GraphPad Prism version 5.01 (San Diego, CA, USA).

3. Results

3.1. Generation of DD persisters of Mtb in a long-term lipid diet dormancy model

It has been demonstrated that Mtb enters non-replicating phase exhibiting phenotypic drug tolerance when grown in presence of long chain fatty acids as the sole carbon source [10]. Here we used a similar 'lipid-only' diet for generating persisters. We hypothesized that a certain population in this culture may also turn DD persisters with time.

Since the growth rate is slower in presence of fatty acids than in presence of dextrose [10], we first grew the bacilli till stationary phase (~9 $\log_{10}$ CFU/ml) in the standard dextrose containing medium (MB7H9 + OADC + glycerol + tween80). The bacilli were harvested and resuspended at density of ~9 $\log_{10}$ CFU/ml in either basal medium (MB7H9 base only) or medium containing a mixture of three even-length fatty acids-palmitate, oleate and linoleate-as the sole carbon source (FAM). These cultures were incubated statically at 37 °C up to 6 weeks. At 2, 4 and 6 weeks, CFUs and most probable number (MPN) were estimated for both cultures directly or after a 5-day treatment with 100 μM RIF. The experiment design is shown schematically in Fig. 1. The additional RIF treatment was included since it has been recently demonstrated that RIF stress generated DD persisters of Mtb in nutrient starved cultures [12]. DD persisters were estimated by subtracting the CFUs from MPN.

The results (Fig. 2) clearly show the role of fatty acid diet and/or RIF stress in DD persister formation in vitro. In the absence of fatty acids as the sole carbon source or rifampicin stress, even after 6 weeks, the DD persister population remained low (2%), comparable to what has been reported for stationary phase cultures in Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase [6]. In FAM, without the RIF stress, DD persister population increased

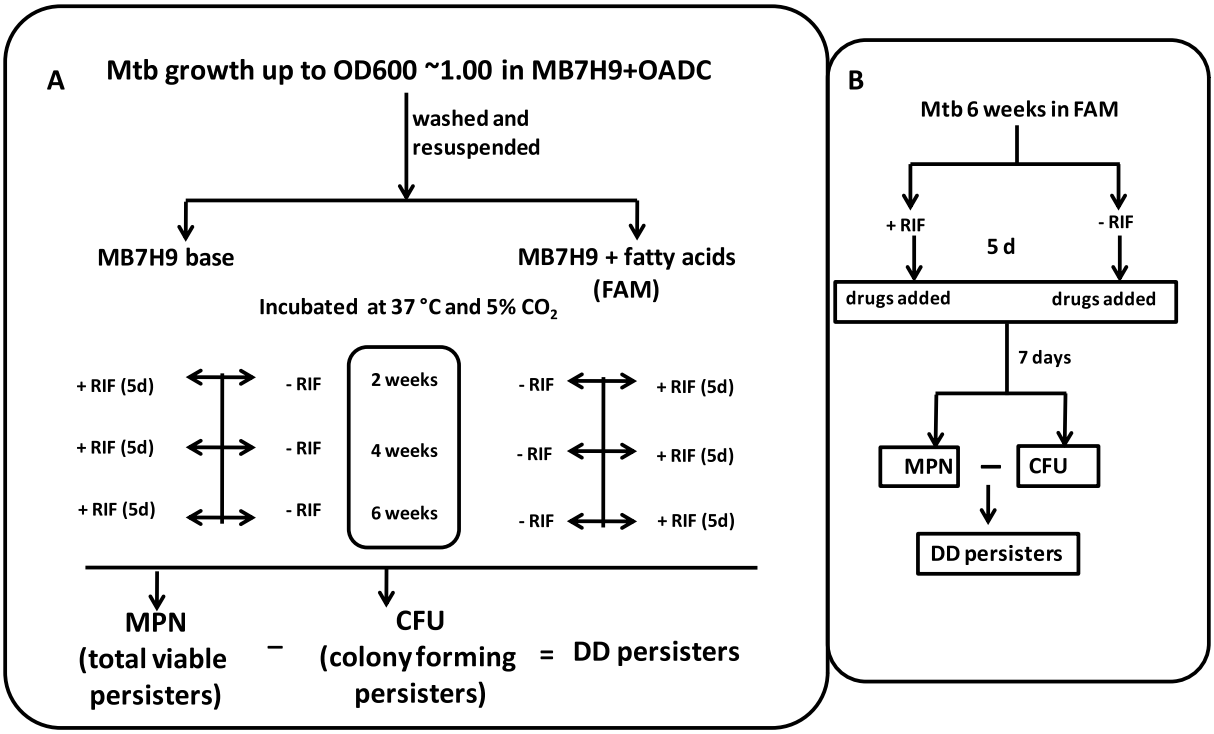

Fig. 1. Experimental design used in this study for developing DD persister model (A) and antibiotic testing in the model (B). Mtb grown to OD_{600} = 1 in Middlebrook 7H9 + OADC broth were harvested, washed and resuspended in Middlebrook 7H9 basal medium or FAM [Middlebrook 7H9 + tyloxapol (0.05%), albumin (0.5%), NaCl (0.085%), palmitate (0.004%), oleate (0.004%) and linoleic acid (0.004%)]. Cultures were incubated statically at 37 °C and 5% CO_2. At weeks 2, 4 or 6, one set of FAM & basal medium cultures were treated with 100 μM RIF or DMSO (vehicle) for 5 days. A second set of cultures were left untreated. CFUs and MPN assays were performed for each set of culture. Differentially detectable (DD) persisters were estimated by subtracting CFUs (colony forming persisters) from MPN (total viable persisters). Antibiotic testing was performed in 6 weeks FAM culture either after a brief rifampicin stress or without. Either culture was exposed to single drugs for seven days before performing CFU and MPN assays. Details of the procedures are given in Materials and Methods.

drastically from 2% at 2 weeks to 95% at 6 weeks. Notably, a short-term RIF stress in FAM accelerated the DD persister formation, for instance even at 2 weeks the DD persister population was 14%. However, a longer duration (6 weeks) in FAM was essential to convert > 90% of the population to DD persisters by RIF stress. RIF stress in the absence of fatty acids significantly enhanced DD persister formation, but later (after 4 weeks) than for FAM. Moreover, even after 6 weeks in the fatty acid-free basal medium, RIF could generate only 63% DD persisters. Therefore, the results demonstrate that fatty acid diet promotes Mtb DD persister formation in vitro and a longer duration in fatty acids can convert majority of the bacterial population to DD persisters. Even though a short RIF stress do accelerate the DD persister formation in presence of fatty acids, a long-term survival in fatty acids is necessary to convert > 90% of the population. Unlike the bacilli in basal medium, those in FAM appeared losing the acid fast property with time (Supplementary Fig. S1). Bacilli in basal medium failed to stain with nile red, a neutral lipid stain (data not shown), while those in FAM were stained (Supplementary Fig. S2).

3.2. Effect of various drugs on colony forming persisters in long-term lipid diet model

Six weeks-FAM cultures, either after RIF stress or without, were exposed to single drugs (RIF, MXF, BDQ, TRZ, or CFZ) of different modes of action. The MICs as determined using resazurin microplate assay [28] were 0.25 μg/mL (RIF & BDQ), 0.5 μg/mL (MXF & CFZ) and

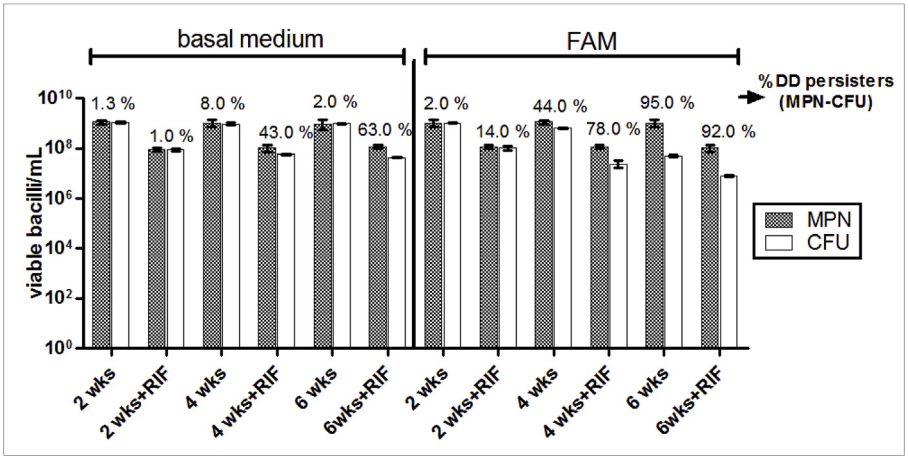

Fig. 2. Effect of fatty acid carbon source and/rifampicin stress in the generation of DD persisters of Mtb. Bars represent show CFUs and MPN/ml. The difference between the two values was estimated as DD persisters. Percentage value indicated above each group of bars represents DD persisters in that sample. Error bars show standard deviation of data from three independent experiments.

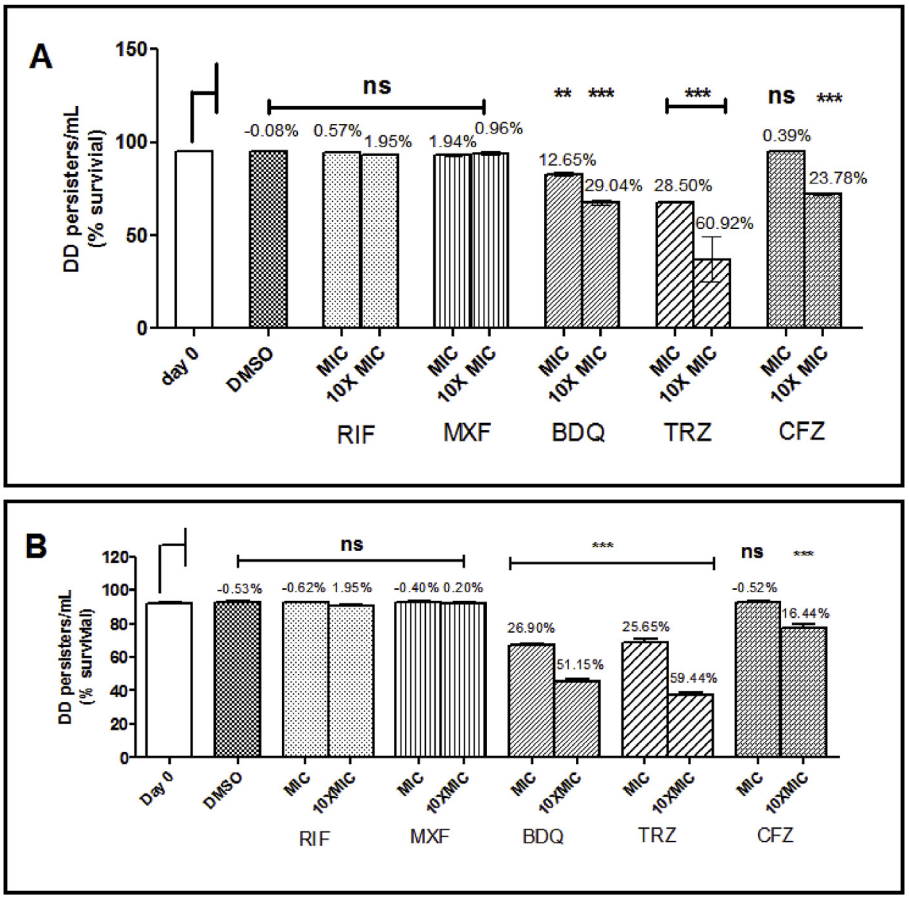

Fig. 3. Bactericidal activity of various drugs on colony forming persisters in long-term lipid diet model. Drugs were tested in a 6 weeks-FAM culture either after a brief RIF stress or without. Either culture was exposed to rifampicin (RIF), moxifloxacin (MXF), bedaquiline (BDQ), thioridazine (TRZ) or clofazimine (CFZ) at their respective MIC and 10 × MIC for seven days. Colony forming persisters as $\log_{10}$ CFU/mL before (day 0) and after drug/DMSO treatment in FAM medium without RIF stress (A) and with prior RIF stress (B). Percentage killing is indicated above each bar. Error bars show standard deviation of data from three independent experiments. Statistical significance of the data assessed by one way ANOVA with Dunnett's multiple comparison test is indicated as: ns, no significant difference; **, $p < 0.001$; ***, $p < 0.0001$.

5 μg/mL (TRZ). Each drug was tested at its MIC and 10 × MIC for 7 days. CFU assay was performed to estimate the bactericidal action of the drugs on colony forming persisters. For FAM cultures that did not receive a prior RIF stress (Fig. 3A), TRZ showed approx. 0.5 $\log_{10}$ (~70% killing) and 2.7 $\log_{10}$ (~99% killing) reduction at MIC and 10 × MIC respectively. BDQ showed 0.8 $\log_{10}$ (~84% killing) and 1.6 $\log_{10}$ (~97% killing) reduction at MIC and 10 × MIC respectively. RIF showed similar activity as BDQ. CFZ showed moderate (~66%) bactericidal activity at 10 × MIC. MXF was slightly bactericidal at 10 × MIC. All drugs except RIF, showed similar bactericidal activity for FAM cultures which received prior RIF stress (Fig. 3B). The reduced bactericidal activity of RIF suggests that a prior exposure to high concentration of RIF may increase the RIF tolerance of the persisters. In summary, TRZ was the most bactericidal on colony forming Mtb persisters in long-term lipid diet model, followed by bedaquiline and rifampicin.

3.3. Effect of various drugs on DD persisters in long-term lipid diet model

MPN assay was performed to estimate the total viable bacilli in FAM cultures on day 0 (6 weeks) and after treating with various 7 days drugs/vehicle. Percentage of surviving DD persisters was calculated after subtracting out CFUs from MPN. At 6 weeks, the FAM culture not given RIF stress, had ~95% DD persisters, which was unchanged by vehicle (DMSO), or even 10 × MICs of MXF or RIF (Fig. 4A). Significant reduction in % DD persisters was caused by BDQ, TRZ (MIC and 10 × MIC) and CFZ (10 × MIC). TRZ at 10 × MIC caused nearly 60% reduction in DD persisters, which was the highest bactericidal activity for all drugs tested. It is evident that DD persisters are more drug tolerant than the colony forming persisters under the same culture conditions. The trend was similar for FAM cultures that were given a prior RIF stress (Fig. 4B). In summary, DD persisters are relatively more drug tolerant than the colony forming counterparts in the long-term lipid diet model. TRZ, BDQ and CFZ worked most effectively against the DD persisters. Rifampicin shows no bactericidal activity on DD persisters in FAM, whether or not given a prior RIF stress.

4. Discussion

Utilization of host-derived lipids by pathogenic mycobacteria for persistence has been already investigated [24]. In fact, interaction between lipid bodies and pathogen-carrying phagosomes has been observed for many intracellular pathogens with implications on the pathogen survival in the host cell [29]. Since this is an inevitable microenvironment for the pathogen irrespective of anti-tubercular treatment, we chose this factor for generating DD persisters. In a lipid-induced dormancy model, Rodríguez et al., grew Mtb in medium having even-length long-chain fatty acids as the sole carbon source since they are the most abundant in human body [10]. Mtb in fatty acids grew at a slower rate compared to dextrose containing medium and upon reaching stationary phase (two weeks old culture) showed dormancy phenotype [10]. We used the same composition of fatty acid mixture, but at four times higher concentration to ensure that the carbon source is not depleted until six weeks. Rodríguez et al., grew the cultures with agitation, while in our set up the stationary phase cultures in FAM were incubated statically. However, since the flasks were opened and contents mixed at two weeks - intervals for sampling, a hypoxic condition was not introduced. The long-term lipid diet for Mtb not only induced phenotypic drug tolerance, but also converted ~95% of the persisters to non-colony forming entities, termed differentially detectable (DD) population. Our data shows that a longer incubation in fatty acids is required to convert majority of the persisters to DD persisters. Hence the major difference between the previously reported lipid-diet model [10] and the present model is the generation and enrichment of DD

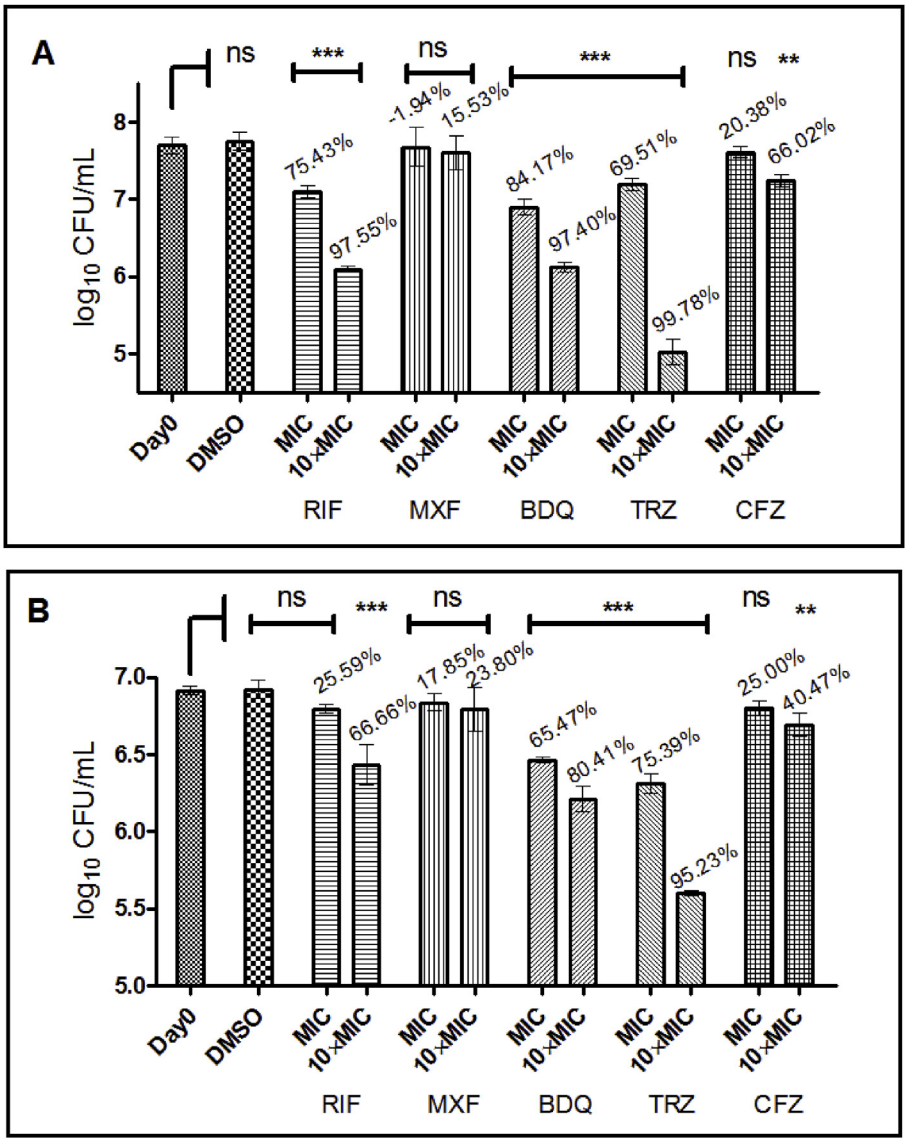

Fig. 4. Bactericidal activity of various drugs against DD persisters in long-term lipid diet model. Drugs were tested in a 6 weeks-FAM culture either after a brief RIF stress or without. Either culture was exposed to rifampicin (RIF), moxifloxacin (MXF), bedaquiline (BDQ), thioridazine (TRZ) or clofazimine (CFZ) at their respective MIC and 10 × MIC for seven days. Percentage of viable DD persisters/ml of the culture before (day 0) and after drug/DMSO treatment in FAM medium without RIF stress (A) and with prior RIF stress (B). Percentage killing is indicated above each bar. Error bars show standard deviation of data from three independent experiments. Statistical significance of the data assessed by one way ANOVA with Dunnett's multiple comparison test is indicated as: ns, no significant difference; **, $p < 0.001$; ***, $p < 0.0001$.

persister population in the latter.

In a recent study, Saito et al., used the MPN assay based on a replicate series of limiting dilutions (MPN–LD assay) to estimate the total viable Mtb, including the non colony forming Mtb persisters from in vitro culture [12]. In this method, non colony forming persisters are revived in a nutritionally rich liquid medium, which is MB7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (OADC) and glycerol. They used sequential stress wherein a two weeks starved (first stress) Mtb cultures in phosphate buffered saline was treated with high dose of rifampicin (second stress) for five days to convert > 90% of the population to DD persisters. We used the MPN-LD assay protocol as described by Saito et al. to estimate DD persisters in FAM at various time intervals. We compared the DD persister formation in FAM culture to that in FAM culture sequentially stressed with high dose RIF. However, sequential RIF stress to a culture in MB7H9 basal medium (without any carbon source) did not enrich the DD persister population significantly unless the culture was at least four weeks old. Even for a six week-old culture in basal medium, sequential RIF stress generated only ~60% DD persisters. Thus, a complete nutrient starvation stress followed by brief RIF stress [12] is suitable for generating DD persisters in a relatively short time. On the other hand, in case of cultures surviving on fatty acids as sole carbon source (FAM cultures), eventhough RIF stress accelerated the generation of DD persisters, the DD persister population crossed the > 90% mark only at six weeks, irrespective of the RIF stress. However, our observations in long-term lipid diet model show that it is possible to convert majority of the bacterial population in an in vitro culture to DD persisters without giving antibiotic stress. This also suggests that Mtb may turn DD persisters during prolonged persistence in a lipid environment in the host, even before the start of anti-TB therapy. Hence, the long-term lipid diet model without RIF stress, though time consuming, can be used to generate DD persisters on a lipid diet, as experienced by the bacilli in the human host, even in the absence of antibiotic stress.

RIF, regarded as a sterilizing drug in standard anti-TB regimens is bactericidal to nonreplicating Mtb generated under hypoxic condition [7], but is poorly permeable into nutrient-starved nonreplicating Mtb and hence ineffective [30]. An excellent study demonstrated that RIF may appear sterilizing Mtb persisters if the DD Mtb are not estimated [16]. We observed that RIF was effective on colony forming Mtb persisters on a sole lipid diet, while being totally ineffective on the DD persister population, even accelerating their formation. Moxifloxacin targets type II DNA topoisomerase (DNA gyrase) of Mtb, with MIC for Mtb 0.5 μg/mL. Moxifloxacin had been shown bactericidal to rifampin-tolerant Mtb persisters in vitro [31], acid phase Mtb and nonreplicating Mtb strain 18b in streptomycin starvation [32]. Nutrient starved Mtb is phenotypically tolerant to moxifloxacin [12,30]. Our results show that

both colony forming as well as DD persisters generated in long-term lipid diet model are tolerant to moxifloxacin even at 5 μg/mL. Rodríguez et al. showed that Mtb grown on fatty acids for short-term were more tolerant than that grown on dextrose, to a combination of rifampin, moxifloxacin, metronidazole and amikacin [10]. However, the tolerance was found to decrease when exposure time to the drugs was increased. However DD persisters were not considered in this study.

BDQ, TRZ and CFZ target the electron transport chain of mycobacteria [33]. All these three drugs were bactericidal to DD persisters generated by the long-term lipid diet. All three drugs showed better efficacy on colony forming persisters than on DD persisters. TRZ was the most effective, closely followed by BDQ. Rao et al. found BDQ and TRZ highly potent, MXF and RIF moderately efficient while isoniazid completely ineffective against nonreplicating Mtb generated by Wayne model [34]. BDQ targets the F1Fo-ATP synthase ATP synthase [35]. ATP synthesis by oxidative phosphorylation is crucial for survival of both actively replicating and nonreplicating Mtb [34]. BDQ is ineffective on nutrient-starved persisters [36]. We observed that BDQ was moderately bactericidal to both colony forming and DD persisters of Mtb in the long-term lipid diet model. TRZ belonging to the phenothiazine class of neuroleptic drugs has been found to inhibit Mtb clinical isolates irrespective of their drug resistance profile [37]. TRZ has been shown to kill drug sensitive and resistant strains of Mtb inside macrophages at concentration as low as 0.01 μg/ml and 0.1 μg/ml, those are well below that is achievable in serum of patients taking this drug [38]. Phenothiazines inhibit type II NADH dehydrogenase (NDH2) of mycobacteria [39]. Evidences suggest that NDH2 of Mtb can initiate the electron transport chain (ETC) under both aerobic and anaerobic conditions [34,39]. TRZ and chlorpromazine were also reported to inhibit efflux pumps in mycobacteria [40]. Majority of the efflux pumps in mycobacteria are dependent on PMF or ATP and hence dependent on the normal functioning of ETC [41]. Thus hampering of ETC by TRZ could simultaneously inhibit the efflux pump system in mycobacteria. TRZ sterilizes semi-dormant as well as nonreplicating Mtb in different models exposing the bacilli to hypoxia, nutrient starvation, or acidic pH [30,34,42]. An elegant in vitro study on the bactericidal as well as pharmacokinetic-pharmacodynamic parameters of thioridazine, demonstrated that less toxic congeners of thioridazine could be more effective than pyrazinamide in reducing the anti-TB treatment duration [42]. CFZ is a riminophenazine drug reported to have MIC 0.1 μg/ml against Mtb H37Rv, recommended to treat drug resistant TB [43]. Differential transcriptional response of Mtb to various drugs demonstrated that CFZ clustered with respiratory modulators such as phenothiazines, cyanide and azide [44]. CFZ kills Mtb through producing reactive oxygen species, mediated by NDH2 [45].

In conclusion, the observations from the present study imply that long-term persistence in a lipid diet with in host tissues may convert a certain population of Mtb to drug tolerant non-colony forming persisters/DD persisters. However, the fact that the differentially detectable persisters can be tolerant even to drugs like rifampicin and moxifloxacin, that are bactericidal to certain forms of persisters, is a matter of concern. However, these extremely tolerant persisters can potentially be eliminated by agents that can hit vulnerable targets or pathways or processes. The electron transport chain seems a vulnerable target in persisters as shown by other recent studies as well this study.

Transparency declarations

None to declare.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.02.007.

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