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Cholesterol-dependent activity of dapsone against non-replicating persistent mycobacteria

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Abstract

One-third of the world's population is estimated to be latently infected with *Mycobacterium tuberculosis*. This reservoir of bacteria is largely resistant to antimicrobial treatment that often only targets actively replicating mycobacteria, with current treatment for latent infection revolving around inhibiting the resuscitation event rather than preventing or treating latent infection. As a result, antimicrobials that target latent infection often have little to no activity *in vivo*. Here we report a method of *in vitro* analysis of physiologically relevant non-replicating persistence (NRP) utilizing cholesterol as the sole carbon source, alongside hypoxia as a driver of *Mycobacterium bovis* BCG into the NRP state. Using the minimal cholesterol media NRP assay, we observed an increased state of *in vitro* resistance to front-line anti-tubercular compounds. However, following a phenotypic screen of an approved-drug library, we identified dapsone as a bactericidal active molecule against cholesterol-dependent NRP *M. bovis* BCG. Through an overexpression trial of probable antimicrobial target enzymes, we further identified FolP2, a non-functional dihydropteroate synthase homologue, as the likely target of dapsone under cholesterol-NRP due to a significant increase in bacterial resistance when overexpressed. These results highlight the possible reason for little *in vivo* activity seen for current front-line anti-NRP drugs, and we introduce a new methodology for future drug screening as well as a potential role for dapsone inclusion within the current treatment regime.

INTRODUCTION

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB) and is responsible for over 10 million new infections and 1.4 million deaths per year [1]. The most abundant *Mtb* infection is latent-TB infection (LTBI), with an estimated 2 billion people worldwide infected and, with a 5–10% lifetime risk of reactivation, around 1–4 million people will die as a result of LTBI [2, 3]. LTBI is driven by the ability of mycobacteria to enter into a non-replicating persistent (NRP) state where they are resistant to therapy and can act as a reservoir of active infection [4]. Chemotherapeutically targeting LTBI without inducing reactivation will dramatically reduce the mortality of TB as the world's leading bacteria-borne infectious disease.

Following inhalation of *Mtb*-contaminated droplet nuclei, the immune system of an immunocompetent individual suppresses the bacterial infection at the site of infection by forming a granuloma [4–6]. Despite being an obligate aerobe, *Mtb* bacilli persist in an NRP, dormant state inside hypoxic tissue for many years, with the ability to reactivate at any time [4–7]. NRP-*Mtb* is resistant to most TB chemotherapeutic agents and plays a significant role in clinical TB persistence, as well as contributing to multi-drug resistance (MDR), extensive drug resistance (XDR) and total drug resistance (TDR)-TB [8]. We do not yet fully understand the process by which NRP-*Mtb* loses sensitivity to anti-TB drugs and is able to persist in the NRP state.

During LTBI, *Mtb* can occupy many different microenvironments. Important features of these microenvironments are cholesterol and cholesterol esters, which can act as one of the critical carbon sources during latent infection [9–11]. Genome-wide microarray techniques used to track the transcriptional response to low oxygen tension environments have shown that metabolic flux is adapted to scavenge host-derived cholesterol in the granuloma [9–11]. ATP synthesis has also been shown to be essential for viability in NRP [12]. Despite our understanding of these essential processes, few NRP-active compounds have been identified to date, and none has been incorporated into TB treatment regimens. This high attrition rate is probably the

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Abbreviations: ADC, albumin–dextrose–catalase; DHPS, dihydropteroate synthase; DHPS, dihydropteroate synthase; LTBI, latent-TB infection; *Mb*BCG, *Mycobacterium bovis* BCG Pasteur; NRP, non-replicating persistence; OADC, oleic acid–albumin–dextrose–catalase; TB, tuberculosis.

result of screening for NRP-actives using mycobacteria in oleic-acid-rich media, a condition that is neither physiologically nor metabolically relevant.

An understanding of NRP mechanisms and discovery of drugs that can kill NRP mycobacteria is essential and has never been more relevant in the fight against TB. In this study, we utilized the model organism *Mycobacterium bovis* BCG Pasteur (*Mb*BCG) to assess the drug susceptibility differences between mycobacteria in hypoxia-induced rich media NRP and hypoxic minimal cholesterol-based media NRP. We have developed a reproducible method for culturing and drug-testing NRP-*Mb*BCG that is a significant improvement on other models, requiring less equipment, lower culture volumes, and increased throughput and data output whilst reducing the overall experimental time [13]. Furthermore, our model offers a physiologically relevant approach, where the cholesterol in the growth medium emulates the available carbon source for sustaining growth of *Mtb* in the granuloma during LTBI. Our results show a clear loss of antimicrobial activity for a range of front-line anti-TB drugs when assessed by hypoxic minimal cholesterol media NRP assays. Additionally, through phenotypic antimicrobial screening, we have identified dapsone as an active compound against NRP-*Mb*BCG when the bacteria are exposed to the hypoxic minimal cholesterol media assay and also delineated that FolP2 is the enzyme target of dapsone through an overexpression trial.

METHODS

Growth of *M. bovis* BCG

M. bovis BCG Pasteur 1173P2 was grown at 37 °C under a number of conditions with varying media, depending upon the assay being performed. All aerobic studies were performed at 37 °C either statically with a 5% CO_2 supplement or shaken at 180 rpm. All anaerobic studies were conducted in an anaerobic chamber (10% CO_2, 10% H_2 and 80% N_2) at 37 °C. For the rich media assay, *Mb*BCG was grown in Middlebrook 7H9 media base broth (Sigma-Aldrich) supplemented with 1% (w/v) glycerol, 10% (v/v) albumin–dextrose–catalase (ADC) supplement and 0.05% (w/v) Tween 80. For growth in the minimal cholesterol media assay, *Mb*BCG was grown in Middlebrook 7H9 media broth base (Sigma-Aldrich) supplemented with 0.01% (w/v) cholesterol and 0.05% (w/v) tyloxapol. *Mb*BCG was grown aerobically to exponential phase (OD_{600nm} of 0.6) in both liquid media before use. On solid media, *Mb*BCG was aerobically grown on Middlebrook 7H11 agar with 10% (v/v) oleic acid–albumin–dextrose–catalase (OADC) supplement and 1% (w/v) glycerol.

Hypoxia-induced rich media NRP assay

This assay was performed as described previously [13]. Briefly, methylene blue (1.5 µg ml^{-1}) was added to mid-log phase *Mb*BCG and subjected to anaerobic conditions by being placed in an anaerobic cabinet in glass culture tubes leaving a head space ratio of 0.5 [14]. The media were not pre-reduced in the anaerobic cabinet so as to maintain a hypoxic down-shift in the culture. *Mycobacterium* species cannot survive direct entry into NRP stage II. Respiration of the mycobacterial cells allows the slow reduction of oxygen until the environment becomes anaerobic (> 0.06% O_2) [13]. NRP status was confirmed by a static growth curve and the decolourization of methylene blue (1.5 µg ml^{-1}). NRP cultures were then aliquoted into 100 µl volumes in 96-well microtitre plates containing associated antimicrobial compounds and sealed with oxygen-impermeable plate seals (Molecular Dimensions) and incubated under anaerobic conditions at 37 °C. After being hypoxically sealed, microtitre plates were briefly removed from the anaerobic cabinet and read every 24 h using an absorbance spectrophotometric plate reader at 570 nm (BioTek EL808) for 1000 h. The microtitre plates were placed back inside the anaerobic cabinet after each read. Methylene blue was used to ensure hypoxia was maintained during data collection.

Hypoxic minimal cholesterol media NRP assay

*Mb*BCG were grown to mid-log phase (OD_{600nm} of 0.6) in minimal cholesterol media. We set up 96-well microtitre plates by the same process as the rich media assay.

Colony counts of *Mb*BCG

After 1000 h under NRP, *Mb*BCG was resuscitated for determination of colony counts. One hundred microlitres of culture from each condition was 10-fold serially diluted and plated out onto OADC enriched 7H11 agar for colony counts (c.f.u. ml^{-1}). Plates were incubated at 37 °C with 5% CO_2 until growth was seen.

Generation of *Mb*BCG overexpression strains

The *folP*1 (Rv3608c) and *folP*2 (Rv1207) genes from *M. tuberculosis* H37Rv were amplified by PCR. The primers used were as follows (with restriction site underlined): *folP*1 forward primer AAAAAAGGATCCGTGAGTCCGGCGCCCGTG, *folP*1 reverse primer AAAAAAAAGCTTGCCATCGCGTTCTATCCT, *folP*2 forward primer AAAAAAGGATCCGTGCGTTCAACACCGCCG and *folP*2 reverse primer AAAAAAAAGCTTTGCGAGTCCTCTCACCGT (Eurofins Genomics). Amplicons were purified and ligated into pVV16 using *Bam*HI and *Hin*DIII restriction sites and the correct sequence was confirmed by DNA sequencing (Eurofins

Genomics). The pVV16-*folP*1 and pVV16-*folP*2 constructs, as well as pVV16, were inserted into electrocompetent *Mb*BCG by electroporation (2.5 kV, 25 µF and 1000 Ω).

Antimicrobial testing of *Mb*BCG FolP1- and FolP2-overexpressing strains

*Mb*BCG FolP1- and FolP2-overexpressing strains, along with the empty plasmid control (*M. bovis* BCG pVV16), were grown to mid log-phase in either ADC enriched 7H9 media or minimal cholesterol media with both media supplemented with kanamycin at 50 µg ml^{-1}. Strains were then placed in anaerobic conditions and hypoxically sealed to initiate hypoxic down-shift into the NRP state. On the point of entry to NRP stage I, all strains were challenged by a serial dilution of dapsone (DAP) (100, 50, 25, 12.5 and 0 µg ml^{-1}). They were then read every 24 h at 570 nm for 1000 h. After 1000 h had elapsed, strains were serially diluted and plated onto 7H11 agar and allowed to aerobically resuscitate to elucidate minimum bactericidal concentrations (MBCs).

RESULTS

In order to compare antibiotic susceptibility of hypoxia-induced NRP-*Mb*BCG cholesterol as the sole carbon source against rich media, we established a method for producing NRP-*Mb*BCG under each of these conditions. In both conditions, it took around 500 h before the inhibitory phenotype could be observed, and we therefore continued assays for at least 1000 h. The loss of isoniazid inhibitory activity in rich media and minimal cholesterol media demonstrates the successful transition of *Mb*BCG into NRP (Fig. 1a, b, respectively).

Metronidazole has previously been demonstrated to be effective against NRP-*Mb*BCG and NRP-*Mtb* in glycerol-containing media, but this inhibitory activity has not been successfully translated to the clinical setting. We hypothesized that this may be due to the lack of physiological relevance of glycerol-based bacterial growth media, as cholesterol has been shown to be the abundant carbon source of the *Mtb* granuloma. In rich (glycerol-containing) media, dose-dependant metronidazole inhibitory activity was clearly observed, but this activity was completely lost when cholesterol was used as the sole carbon source (Fig. 1c, d, respectively). We hypothesize that this could explain the lack of *in vivo* efficacy of metronidazole against NRP-*Mtb*, and further believe this result highlights the importance of our work in exploring physiologically relevant growth conditions for antibiotic susceptibility testing in order to ensure *in vitro* observations translate into *in vivo* efficacy.

Moxifloxacin has recently been proposed as an inhibitor of NRP-*Mtb* [14] and is currently being tested in animals in combination with pretomanid as a proposed new combination therapy for latent-TB infection. All previous work on moxifloxacin NRP-inhibitory activity has been conducted in glycerol-based rich media. We therefore felt it would be appropriate to conduct a parallel study of moxifloxacin NRP-*Mb*BCG activity in both glycerol-containing and cholesterol-containing media as we had conducted for metronidazole. We observed the same result as for metronidazole, wherein moxifloxacin activity was observed in glycerol-containing bacterial growth media, but activity was lost in media containing cholesterol as the sole carbon source (Fig. 1e, f, respectively).

Following a phenotypic screen of an approved-drug library, we discovered a compound that demonstrated the opposite phenotype, where dose-dependent inhibitory activity was observed against NRP-*Mb*BCG cultured with cholesterol as the sole carbon source, and this inhibitory activity was not observed in glycerol-containing media culture conditions (Fig. 1h, g, respectively). Dapsone is a drug that has been used as a highly effective anti-leprotic since 1945. It has well-defined *in vivo* inhibitory action against *Mycobacterium leprae*, the causative agent of leprosy. Dapsone also has been reported to be effective against different mycobacteria strains such as *M. avium* and *Mtb* [15–17]. Intended use against *Mtb* was studded recently where Poly lactic-co-glycolic acid (PLGA) nanoparticles were used as a delivery system of dapsone in combination with clofazimine [18]. These studies provide significant promise for this discovery to impact LTBI treatment in the future, as we hypothesize that activity observed against NRP-*Mb*BCG will translate into NRP-*Mtb*.

The growth curve data in Fig. 1 use optical density (OD) as a readout of antimicrobial activity, as this enables a high-throughput spectrophotometric-based measure of bacterial inhibition under NRP conditions. We wanted to confirm that the inhibitory activity observed with these compounds correlates with a reduction in bacterial viability in terms of colony forming units (c.f.u. ml^{-1}). We therefore conducted endpoint analysis of the dose responses to confirm that a reduction in OD correlated to inhibition and bactericidal activity. These data are depicted in Fig. 2 wherein endpoint c.f.u. analysis of each of the antibiotics that were investigated in Fig. 1 is shown (Fig. 2a-h). It is clear that isoniazid does not exhibit bactericidal activity in each of the growth conditions, with no change in c.f.u. ml^{-1} observed at any concentration (Fig. 2a, b). Conversely, metronidazole and moxifloxacin demonstrated bactericidal activity against NRP-*Mb*BCG in rich (glycerol-containing) bacterial growth media at 12.5 and 0.5 µg ml^{-1} respectively, but that bactericidal activity is lost when bacteria are grown under nutrient-limited conditions where cholesterol is the sole carbon source (Fig. 2c-f). Dapsone shows no bactericidal activity against NRP-*Mb*BCG when in rich media, but when the assay was performed using minimal cholesterol media dapsone demonstrated bactericidal activity at 25 µg ml^{-1} and was the only antimicrobial to show bactericidal activity in the hypoxic minimal cholesterol media NRP assay (Fig. 2g, h).

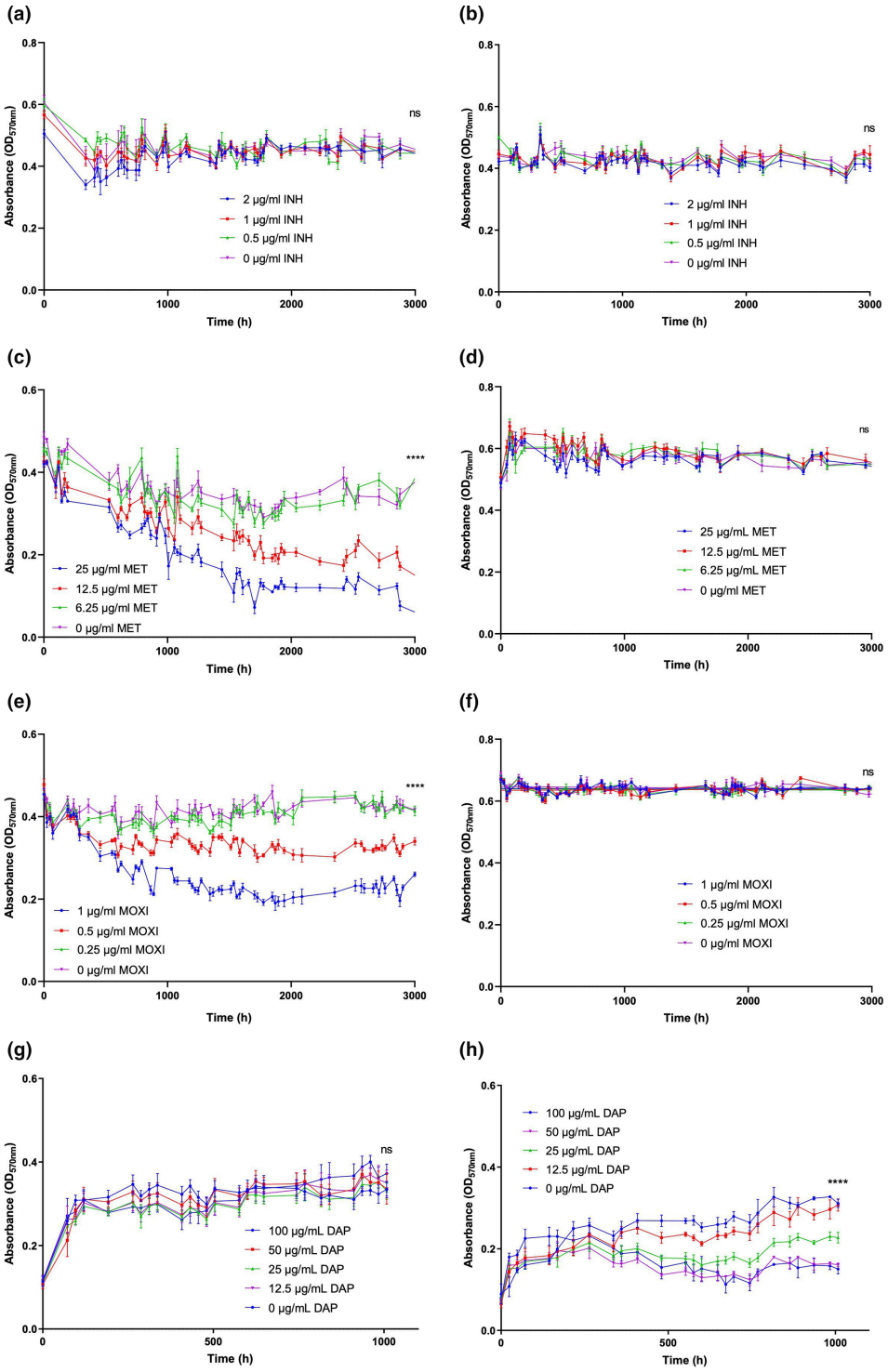

Fig. 1. Absorbance over time of cultures containing NRP-*Mycobacterium bovis* BCG and a dose response of the antibiotics isoniazid, metronidazole, moxifloxacin and mapsone in a hypoxia-induced rich media NRP assay (a, c, e and g, respectively) and in a hypoxic minimal cholesterol media NRP assay (b, d, f and h, respectively). (a,b) There was no significant difference between any concentration of isoniazid between the rich media and minimal cholesterol media. (c) Dose-dependent inhibition with metronidazole in the rich media ($P<0.0001$), whereas (d) minimal cholesterol media lack dose-dependent inhibition. (e) Dose-dependent inhibition with moxifloxacin in the rich media ($P0.0001$), whereas (f) minimal cholesterol media lack dose-dependent inhibition. (g) Rich media lack dose-dependent inhibition with dapsone, whereas (h) dose-dependent inhibition is observed in minimal cholesterol media. All experiments were conducted with $n=5$. All statistical ANOVA was conducted using Graphpad Prism 8.

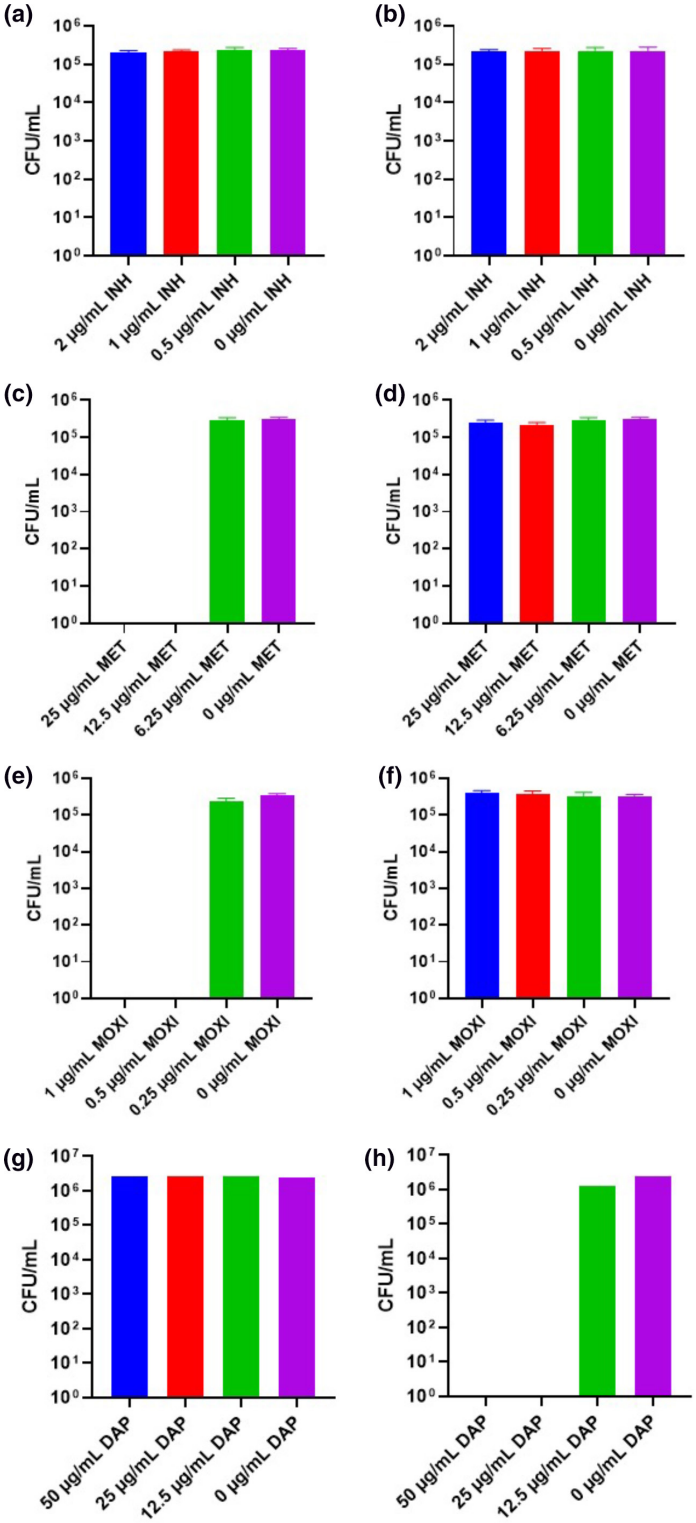

Fig. 2. Endpoint colony counts (c.f.u. ml^{-1}) of cultures containing NRP-*Mycobacterium bovis* BCG and a dose response of antibiotics isoniazid (INH), metronidazole (MET), moxifloxacin (MOXI) and dapsone (DAP) in the hypoxia-induced rich media NRP assay (a, c, e and g, respectively) and in the hypoxic minimal cholesterol media NRP assay (b, d, f and h, respectively). Isoniazid does not show activity in either media. (a, b) Metronidazole and moxifloxacin only show bactericidal activity in rich media (c, e), and that activity is not observed in growth conditions where cholesterol is the sole carbon source (d, f). Dapsone does not show activity in rich media (g), but gains bactericidal activity in cholesterol-based media (h). These results confirm the inhibitory activity observed in Fig. 1.

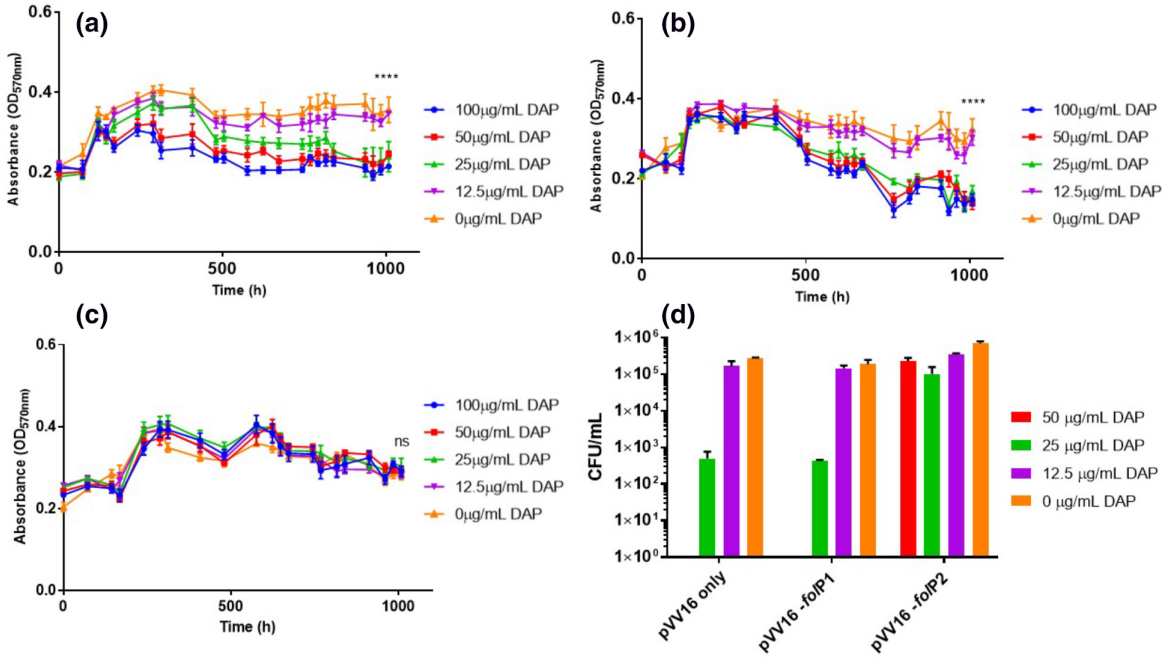

Fig. 3. Growth of *Mycobacterium bovis* BCG transformants in media with cholesterol as the sole carbon source in a concentration gradient of dapsone. (a) Empty vector control strain (*Mb*BCG- pVV16), (b) FolP1-overexpressing strain (*Mb*BCG- pVV16-*folP1*), and (c) FolP2-overexpressing strain (*Mb*BCG-pVV16-*folP*2). Dose-dependent inhibition can be observed in both (a) and (b), but the inhibitory action of dapsone was lost upon overexpression of FolP2 (c). (d) This inhibitory activity, as observed by a change in OD, directly correlates with inhibition in terms of c.f.u. ml^{-1}.

Having observed that dapsone demonstrated selective NRP-*Mb*BCG inhibitory activity only in cholesterol-based bacterial growth media and that that activity was lost under rich growth conditions, we set about validating the mechanism of action of dapsone under these conditions. Dapsone has previously been identified as an inhibitor of folic acid biosynthesis by inhibiting the synthesis of dihydrofolic acid through competition with para-amino-benzoate for the active site of dihydropteroate synthetase (*folP1* and *folP2* genes in *M. leprae*). Mutations in the *folP1* gene have been widely associated with dapsone resistance in *M. leprae* [19] and essentiality studies to determine which was the functioning dihydropteroate synthetase protein, FolP1 or FolP2, and the target of dapsone in *M. leprae*, concluded that FolP2 was non-essential, and therefore FolP1 was the target [20]. Both of these studies were conducted using bacterial growth media containing carbohydrates as nutrient sources. In order to establish the target protein of dapsone under growth conditions with cholesterol as the sole carbon source, we produced pVV16-*folP*1, pVV16-*folP*2 and pVV16 only (control) strains of *Mb*BCG and assessed their respective sensitivities to dapsone under NRP. With rich media, we did not observe any inhibition in any strain at any concentration, which was to be expected given the selective activity of dapsone, requiring cholesterol-limited metabolism, as previously described (Fig. 1g, h). However, under nutrient-limited conditions with cholesterol as the sole carbon source, we observed dose-dependent inhibition in both the empty vector control strain (*Mb*BCG-pVV16) and the FolP1-overexpressing strain (*Mb*BCG-pVV16-*folP*1), although the inhibitory action of dapsone was lost upon overexpression of FolP2 (*Mb*BCG-pVV16-*folP*2) (Fig. 3a, b and c respectively). Fig. 3(d) demonstrates that this inhibitory activity as observed by a change in OD directly correlates to inhibition in terms of colony counts (c.f.u. ml^{-1}). There is a reduction in c.f.u. ml^{-1} with dapsone at 25 μg ml^{-1} seen for both NRP-*Mb*BCG-pVV16 and NRP-*Mb*BCG-pVV16-*folP*1, rather than complete bactericidal activity that was observed previously (Fig. 2g), suggesting that the vector, which contains both kanamycin and hygromycin resistance cassettes, is providing some small protection to NRP-*Mb*BCG. However, NRP-*Mb*BCG-pVV16-*folP*2 shows resistance to dapsone to concentrations >100 μg ml^{-1}, which is much higher than the previous bactericidal concentration of 25 μg ml^{-1}.

We therefore hypothesize that FolP2 is the target protein of dapsone under nutrient-limited conditions, where cholesterol is the sole carbon source in the bacterial growth media. We further postulate that this corresponds to conditions *in vivo*, where host-derived cholesterols form the major nutrient source in the granuloma during LTBI.

DISCUSSION

It has been quarter of a century since the observation was first made that mycobacteria have the incredible ability to persist in a quiescent, non-replicating, antibiotic-tolerant state for years, and it is this unique characteristic that *Mtb* owes its success

and longevity as a human pathogen to [21]. This ability to asymptomatically (latently) reside within an infected host for years before reactivating at an opportune moment to cause an active and often drug-resistant secondary infection is the reason why all efforts to eradicate TB have thus far proven unsuccessful. Previous *in vitro* models of NRP mycobacteria have utilized either hypoxic conditions or nutrient deprivation as the main entry trigger to NRP [22–24]. Here, we sought to combine one of the restricted nutrient sources within the granuloma with the limited oxygen availability to produce a hypoxic minimal cholesterol media NRP assay, whereby cholesterol is the only carbon source available [9–11]. *Mb*BCG is a live-attenuated model of *Mtb* which is widely used due to the reduced safety concern in the laboratory. Furthermore, it has a highly conserved genome sequence (99.9%) with *Mtb*. However, limitations should be noted including metabolic differences between *Mtb* and *Mb*BCG [25] such as the inability of *M. bovis* to generate energy from glycolytic intermediates [26]. Nevertheless, by comparing the antimicrobial susceptibilities of this assay to a more traditional hypoxia-induced rich media NRP assay, we identified that resistance to compounds that require actively growing bacteria for activity, such as isoniazid, was maintained (Figs 1a, b and 2a, b). However, antimicrobials that have been shown to be effective against bacteria within the NRP state such as metronidazole and moxifloxacin lost their activity against NRP-*Mb*BCG in the hypoxic minimal cholesterol media NRP assay (Figs 1c-f and 2c-f,). This phenomenon has been observed previously for metronidazole between hypoxic and nutrient-deprived NRP models [23, 27, 28]. However, moxifloxacin has been shown to be active against NRP mycobacteria in rich media assays, and as such is a promising component of the anti-NRP regime [14]. The observation of increased resistance to moxifloxacin in the hypoxic minimal cholesterol media NRP assay highlights a worrying change of drug susceptibility phenotype, based solely on one type of nutrient available in the granuloma.

After extensive antimicrobial screening using the hypoxic minimal cholesterol media NRP assay, dapsone was identified as an active compound against NRP-*Mb*BCG. In rich media, no inhibitory or bactericidal activity was observed, but when the carbon source was restricted to cholesterol, dapsone displayed bactericidal activity down to 25 μg ml^{-1} (Figs 1g, h and 2g, h). Furthermore, the fact that dapsone has not previously been identified as an inhibitor of NRP-*Mtb,* based on previous studies using bacterial growth media that do not accurately represent conditions *in vivo,* further highlights the critical importance of exploring physiologically relevant *in vitro* culture conditions for antibiotic discovery.

In order to ascertain the target of dapsone, overexpression of *Mtb*FolP1, which is the primary dihydropteroate synthase (DHPS) within the essential folic acid pathway, and the non-functional homologue *Mtb*FolP2 revealed an alteration in bactericidal activity of the antimicrobial. Surprisingly, overexpression of FolP1 did not lead to an altered drug resistance, but overexpression of FolP2 increased the resistance of the NRP-*Mb*BCG to dapsone activity (Fig. 3). This observation clearly indicates that FolP2 has a function beyond that of a non-functional DHPS homologue as previously thought. Prior research investigating the role of FolP2 to determine whether it functioned as a DHPS has been conducted, where it was shown that due to conformational differences between both FolP2 and FolP1, FolP2 could not effectively bind the substrate, 6-hydrox ymethyl-7,8-dihydropterin-pyrophosphate (H2PtPP), but interestingly could bind to dapsone [29]. Therefore, despite the fact that FolP2 shares structural similarities to FolP1, the lack of binding affinity of the folate substrate means that FolP2 is unlikely to be part of the folic acid biosynthesis pathway. Identification of the natural substrate of FolP2 will undoubtedly shed light on the true function of the enzyme within the NRP state.

In conclusion, this research highlights an assay for NRP mycobacteria with increased physiological relevance for antimicrobial screening, which is significant due to the unique drug susceptibility profiles yielded. Furthermore, our results cast doubt on the efficacy of the current TB drug pipeline. The identification of dapsone activity against NRP-*Mb*BCG within this assay is important not only for treatment of LTBI but also for future TB drug development. Finally, we have highlighted an important role of FolP2 within NRP, which could have significant impact as a future drug target for anti-tubercular agents targeting latent TB disease.

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Author contribution
S.E.R.G.: Conceptualization, Formal analysis, Methodology. J.H.: Conceptualization, Formal analysis, Methodology. A.M.: Writing – review & editing, Formal analysis, Data curation. J.A.G.C.: Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – original draft.

Conflicts of interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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