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Effects of tafenoquine against active, dormant and resistant *Mycobacterium tuberculosis*

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ABSTRACT

Antimalarial drugs have been suggested as promising scaffolds with anti-tubercular activities. In this work, we demonstrated, for the first time, the effectiveness of tafenoquine against mycobacteria. Firstly, tafenoquine inhibited the growth of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* with lower MICs values as compared to other antimalarial drugs, such as mefloquine, chloroquine, and primaquine. Importantly, tafenoquine was active against three multi-drug resistant strains of *M. tuberculosis* with MIC values similar to pan-sensitive strains, suggesting that tafenoquine is capable of evading the major mechanisms of resistance found in drug-resistant clinical isolates of *M. tuberculosis*. Importantly, tafenoquine displayed a synergistic effect when combined with mefloquine. In addition, tafenoquine displayed an improved activity compared to the groups treated with both isoniazid and rifampicin in the six-week nutrient starved *M. tuberculosis* cultures. This finding suggests that further investigations of tafenoquine against dormant mycobacteria are worth pursuing. Moreover, different concentrations of tafenoquine ranging from 1.25 to 80 μM displayed different effects against *M. tuberculosis*, from moderate (reduction of a 1.8 log CFU/mL) to potent bactericidal (reduction of a 4.2 log CFU/mL) activities. Tafenoquine may represent a hit for further drug optimization and for future clinical development as a new anti-mycobacterial agent, especially in cases of resistant and/or dormant forms of tuberculosis.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the leading infectious disease killer and one of the main causes of deaths overall [1]. Despite some progress in the pipeline for new drug candidates and regimens, there is still an urgent need for the identification of new drugs to treat TB [2]. Repurposing of drugs has been considered an interesting alternative to overcome global TB epidemic, especially to kill drug-resistant forms of the disease [2]. In this regard, the anti-mycobacterial activities of antimalarial drugs such as primaquine [3], chloroquine [4] and mefloquine [5–7] have been reported.

Chloroquine was shown to increase the activity of standard drugs such as isoniazid, pyrazinamide, and streptomycin [4], and primaquine was suggested as a promising scaffold with anti-tubercular effect [3]. Mefloquine was reported to be active against *Mycobacterium avium* [5], and displayed promising effects in macrophage and murine models of *M. tuberculosis* infection [6]. Of importance, mefloquine was suggested as an alternative to treat multi-drug resistant (MDR) strains of TB [7].

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The drug tafenoquine, an 8-aminoquinoline analogue of primaquine, is currently used to treat *Plasmodium vivax* malaria, and its promising activity has been demonstrated against other pathogenic organisms, such as *Leishmania donovani* [8] and *Trypanosoma brucei* [9]. No study investigated its potential against mycobacteria to date. In this work, we assessed the effectiveness of tafenoquine against mycobacteria, comparing to the activities of other antimalarial and anti-tubercular drugs.

2. Materials and methods

2.1. Determination of minimum inhibitory concentrations (MICs)

MICs were determined by using the resazurin reduction microplate assay (REMA) as a growth indicator, as previously reported [10]. All drugs were purchased from Sigma-Aldrich. Mefloquine and tafenoquine were solubilized in DMSO at a concentration of 3.2 mM, and then diluted in Middlebrook 7H9 broth (Difco) plus ADC enrichment (albumin, dextrose, catalase; Becton Dickinson) to reach a concentration of 0.16 mM. Chloroquine and primaquine were solubilized in 7H9 to reach concentrations of 20.9 and 21.9 mM, respectively. Serial two-fold dilutions were performed in 96-well U-bottom polystyrene microplates at concentration ranges of 80–0.6 μM for mefloquine and tafenoquine, and 10–0.08 mM for chloroquine and primaquine. The final concentration of DMSO in all wells was 2.5%. Mycobacterial suspensions were cultivated and diluted in 7H9 medium at an optical density (OD_{595nm}) of 0.001 for *M. smegmatis* and 0.006 for *M. tuberculosis*, and 100 μL were added to each well. Following incubation at 37 °C for 24 h for *M. smegmatis*, or 7 days for *M. tuberculosis*, 30 μL of a sterile resazurin solution (0.02%) were added to the plates and the results were evaluated after 24 h for *M. smegmatis*, or 48 h for *M. tuberculosis* [10,11]. MICs were considered as the lowest drug concentration that prevented a color change from blue (resazurin) to pink (resorufin).

2.2. Drug combinations

Combinations of tafenoquine with isoniazid, rifampicin, ethambutol, moxifloxacin, streptomycin and mefloquine were performed by a checkerboard assay in a two-drug association scheme, by using the REMA colorimetric method as a growth indicator, as previously reported [11]. Briefly, drugs were diluted in 7H9 + ADC to obtain concentration ranges in microplates of 30–0.47 μM for isoniazid, 0.25–0.004 μM for rifampicin, 60–0.94 μM for ethambutol, 1.5–0.023 μM for moxifloxacin, 8–0.125 μM for streptomycin, 160–2.5 μM for mefloquine, and 80–1.25 μM for tafenoquine. Tafenoquine was diluted vertically (rows B to H) while combined drugs were diluted horizontally (columns 2 to 8). The concentration of DMSO in all wells was maintained in 2.5%. The *M. tuberculosis* H37Ra inoculum, the incubation conditions of the microplates and the readout of results were carried out as described before for MICs determination. The fractional inhibitory concentration index (FICI) was calculated, in which values below 0.5 indicate a synergism between the compounds, in between 0.5 and 4 indicate an indifferent influence (drugs act independently), and above 4 suggest an antagonistic effect [11].

2.3. Efficacy against dormant mycobacteria

Mycobacterial dormant cultures were prepared by using the nutrient starvation model, as previously described [12]. Briefly, *M. tuberculosis* H37Ra was grown in sterile Middlebrook 7H9, containing 10% OADC enrichment (oleic acid, albumin, dextrose, catalase; Becton Dickinson), 0.2% glycerol, and 0.025% Tween-80. After reaching log phase, cultures were pelleted and washed twice with sterile PBS. The pellet was then resuspended in PBS in sealed bottles and incubated at 37 °C for 6 weeks [12]. The six-week-starved and a log phase culture of *M. tuberculosis* were then treated for 7 days with tafenoquine or mefloquine in concentrations corresponding to their MIC values, 10 or 20 μM, respectively. Isoniazid and rifampicin were used as controls in the concentration of 10 μM. DMSO (2.5%) was present in all groups including the untreated control wells. Samples were serially diluted and plated on Middlebrook 7H10 Agar (Difco) supplemented with 10% OADC enrichment. Bacterial colonies were counted after incubation of plates for three weeks at 37 °C. This experiment was performed in triplicate, and the results are expressed as the log mean numbers (±standard deviation) of colony forming units (CFU) per mL. Data were evaluated by one-way analysis of variance (ANOVA), followed by Bonferroni's post-test, using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

2.4. Evaluation of different concentrations of tafenoquine and data modeling

In parallel, we investigated the effects of different concentrations of tafenoquine in a time kill experiment, as previously described [13]. *M. tuberculosis* H37Ra time kill curves were plotted upon incubation with the following tafenoquine concentrations: 1.25 μM (0.125 x MIC), 2.5 μM (0.25 x MIC), 5 μM (0.5 x MIC, 10 μM (1 x MIC), 20 μM (2 x MIC), 40 μM (4 x MIC), and 80 μM (8 x MIC). An additional group without drug and containing only the vehicle plus medium (2.5% DMSO in Middlebrook 7H9 plus 10% ADC enrichment) was included as a growth control. All groups were inoculated with approximately 10^5 CFU/mL of *M. tuberculosis* H37Ra, in a final volume of 10 mL, and incubated for 14 days at 37 °C with shaking at 96 rotations per minute. At defined time intervals (1, 3, 7, 10, and 14 days), 100 μL was taken from each tube, diluted in sterile 0.9% NaCl solution, and plated on Middlebrook 7H10 (Difco) supplemented with OADC for CFU counting. Agar plates were incubated at 37 °C and the number of viable colonies were determined after 21 days. The time-kill curves were modeled by nonlinear regression using the sigmoidal maximum-effect (E_{max}) with the aid of the software Scientist v. 3.0 (MicroMath, Salt Lake, UT, USA). In the E_{max}-model, tafenoquine bactericidal effect over time (dN/dt) is described by EC_{50} which is the concentration of the drug necessary to achieve 50% of the maximum killing effect (k_{max}), C is tafenoquine constant concentration in each experiment, k is *M. tuberculosis* H37Ra generation rate constant in the absence of the drug, and γ is the Hill slope:

$$\frac{dN}{dt} = \left(k - \frac{k_{max} \cdot C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}\right) \cdot N$$

The control curve was modeled first, and the generation rate constant (0.029 d^{-1}) determined was fixed for the individual modeling of the effect of each constant drug concentration.

3. Results and discussion

3.1. Tafenoquine is active against laboratory strains of M. tuberculosis and Mycobacterium smegmatis

We first determined the MICs for primaquine, chloroquine, mefloquine and tafenoquine in *Mycobacterium smegmatis* mc^2155 and *Mycobacterium tuberculosis* H37Ra. As shown in Table 1, tafenoquine and mefloquine inhibited the growth of *M. smegmatis* and *M. tuberculosis* H37Ra with the lowest MIC values. The MIC of 20 μM for mefloquine corresponds to 8 μg/mL, which is similar to previously described values [6,7]. The MIC value of 10 μM for tafenoquine corresponds to approximately 6 μg/mL. In addition, mefloquine and tafenoquine were also active against *M. tuberculosis* H37Rv, a pan-susceptible virulent laboratory strain (Table 1). *M. smegmatis* has been considered as a preliminary model of MDR strains susceptibility [14]. Consistent with this suggestion, we have observed augmented MIC values against *M. smegmatis* of more than 1,000 times for rifampicin and more than 60 times for isoniazid, comparing to *M. tuberculosis* H37Ra (Table 1).

Table 1
Activity of antimalarial drugs against *M. smegmatis*, *M. tuberculosis* clinical isolates and laboratorial strains.

Drug	MIC in μM | MIC in μg/ml [a]					
	M. smegmatis mc^{2}155	*M. tuberculosis* H37Ra	*M. tuberculosis* H37Rv	PT-2[b]	PT-12[c]	PT-20[d]
Chloroquine	320 | 165	2,612 | 1,347				
Mefloquine	20 | 8.3	20 | 8.3	20 | 8.3	20 | 8.3	20 | 8.3	20 | 8.3
Primaquine	620 | 282	1,372 | 625				
Tafenoquine	10 | 5.8	10 | 5.8	20 | 11.6	20 | 11.6	10 | 5.8	10 | 5.8
Ethambutol	2 | 0.6	8 | 2.2				
Isoniazid	125 | 17	1.9 | 0.3	0.6 | < 0.1	80 | 11	40 | 5.5	80 | 11
Moxifloxacin	0.1 | < 0.1	0.2 | < 0.1				
Rifampicin	31 | 26	0.03 | 0.02	<0.1 | <0.1	>80 | > 66	>80 | > 66	>80 | > 66
Streptomycin	0.1 | < 0.1	0.2 | 0.1				

[a] MIC values reported here were observed in three or four independent experiments.
[b] Drug-resistant PT-2 clinical isolate holds mutations in *inh*A (S94A) and *rpoB* (S531L) genes, and in the promoter sequence of *inhA*, [C(-15)T].
[c] Drug-resistant PT-12 clinical isolate holds mutations in *kat*G (S315T) and *rpoB* (S531L) genes.
[d] Drug-resistant PT-20 clinical isolate holds mutations in *kat*G (S315T) and *rpoB* (S531L) genes.

3.2. Tafenoquine is active against drug-resistant clinical isolates of *M. tuberculosis*

Mefloquine and tafenoquine were therefore selected to further test against virulent and resistant strains of *M. tuberculosis*. Three MDR clinical isolates (PT-2, PT-12, PT-20) were evaluated. The clinical isolates used in this work have been previously characterized by Perdigão et al. (2014) [15]. All three clinical isolates carry the same mutation (S531L) in the *rpo*B gene, responsible for causing resistance to rifampicin. PT-2 carries a mutation [C(-15)T] in the promoter sequence of the *inh*A (Rv1484) gene and also harbours a mutation (S94A) in the *inh*A gene, the molecular target for isoniazid. PT-12 and PT-20 carry the mutation (S315T) in the *kat*G (Rv1908c) gene, which is the most frequent mutation found in isoniazid-resistant strains. Importantly, tafenoquine and mefloquine inhibited the growth of MDR strains of *M. tuberculosis* with MIC values of 10 or 20 μM (Table 1). These findings suggest that mefloquine and tafenoquine are capable of evading the major mechanisms of resistance to isoniazid and rifampicin found in resistant clinical isolates of *M. tuberculosis*, such as mutations in either *kat*G or *rpo*B genes.

3.3. Tafenoquine displays a synergistic effect in combination with mefloquine

The effects of the combination of tafenoquine with the clinically used anti-TB drugs isoniazid, rifampicin, ethambutol, moxifloxacin, streptomycin and mefloquine were assessed. As shown in Supplementary Table 1, tafenoquine showed an indifferent effect when combined with isoniazid, rifampicin, ethambutol, moxifloxacin, and streptomycin (FICI values between 1 and 2). In fact, tafenoquine displayed a synergistic effect in the presence of mefloquine (FICI values of 0.5 in three independent experiments). Both MIC of tafenoquine in the presence of mefloquine and MIC of mefloquine in the presence of tafenoquine were improved 4-fold, in all three experiments (Supplementary Table 1). Of note, no combination presented antagonistic interactions in our experiments. The lack of antagonism and the observation of indifference are in accordance with results published for mefloquine combinations with standard anti-TB drugs [6].

3.4. Tafenoquine effectively reduced CFU counts from dormant bacteria

We have also determined the activity of tafenoquine and mefloquine in a dormancy model for *M. tuberculosis*. The non-starved log phase cultures of *M. tuberculosis* treated with tafenoquine, mefloquine, isoniazid, or rifampicin, for 7 days, had their bacterial loads significantly reduced (Fig. 1A), when compared to untreated control (***$P < 0.001$ for all treatments). Reductions in CFU counts ranged from 3.6 to 4.6 $\log_{10}$ for all treated groups. However, incubation with isoniazid or

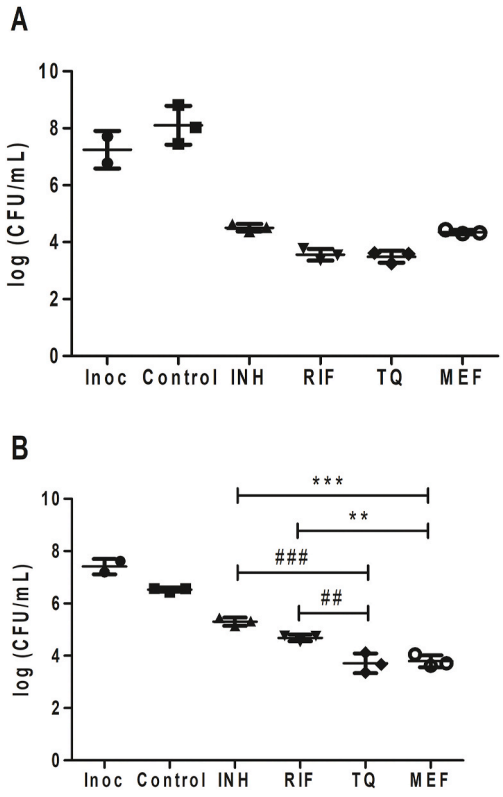

Fig. 1. Activity of tafenoquine (TQ) and mefloquine (MEF) against non-starved log phase (A) and nutrient starved (B) *Mycobacterium tuberculosis*. Inoc represents the inoculum, mycobacterial suspensions before treatments were added. Control group was treated with the vehicle, 7H9 medium with 2.5% DMSO. INH, isoniazid; RIF, rifampicin. ***$P < 0.001$, **$P < 0.01$ compared to MEF-treated group; ###$P < 0.001$, ##$P < 0.01$ compared to TQ-treated group. Data were evaluated by ANOVA, followed by Bonferroni post-test, using GraphPad Prism 5.0.

rifampicin caused a decrease of 1.2 and 1.8 $\log_{10}$, respectively, in the CFU/mL loads from the nutrient starved *M. tuberculosis* cultures (Fig. 1B). The reduced effectiveness of isoniazid and rifampicin found in the model of six-week nutrient starved *M. tuberculosis* are in accordance with previous observations [12]. Of importance, treatment with tafenoquine resulted in an improved potency compared to the groups treated with both isoniazid ($P < 0.001$) and rifampicin ($P < 0.01$) against the nutrient starved bacteria. Similarly, significant differences in the bacterial loads were observed between mefloquine-treated group and both isoniazid- ($P < 0.001$) and rifampicin-treated ($P < 0.01$) groups

(Fig. 1B). These observations allow us to suggest a satisfactory activity of tafenoquine and mefloquine in this well-established model of dormant mycobacteria.

3.5. Effects of different concentrations of tafenoquine in a time kill experiment

As shown in Fig. 2A, the seven concentrations of tafenoquine evaluated (ranging from 1.25 to 80 μM) displayed different effects against *M. tuberculosis*, and growth control increased approximately 3 log CFU/mL, during 14 days. In the first 3 days of incubations, groups treated with the highest concentrations of tafenoquine (80, 40, and 20 μM) showed a mean decrease of 3 log CFU/mL, compared to the initial inoculum. Of note, between days 7 and 10, treatments were found to be more effective, especially at day 10 where the reductions ranged from 3.4 to 4.2 log CFU/mL for tafenoquine concentrations equal or higher than 5 μM (0.5 x MIC), comparing to the mean initial inoculum of $10^{5.2}$ CFU/mL. Importantly, these observations allow us to suggest that treatment with concentrations equal or higher than 5 μM (0.5 x MIC) display potent and satisfactory bactericidal effects. In addition, the concentrations of 2.5 (0.25 x MIC) and 1.25 μM (0.125 x MIC) resulted in modest to moderate activities, showing mean reductions of, respectively, 2.9 and 1.8 log CFU/mL at day 14 compared to the initial inoculum, and these promising effects seem to be time dependent (Fig. 2A). These findings are clinically relevant since tafenoquine was active against *M. tuberculosis* in concentrations (1,25 μM, which corresponds to 727 ng/mL) found in human plasma, after 3 consecutive daily oral doses of 400 mg [16]. As can be seen on Fig. 2B, the E_{max} model adequately described the time-kill data with a model selection criteria (MSC) between 1.62 and 3.34. The average parameters determined by the model were a k_{max} of 0.17 ± 0.03 d^{-1}, an EC_{50} of 1.43 ± 0.34 μM and a Hill slope of 1.16 ± 0.18.

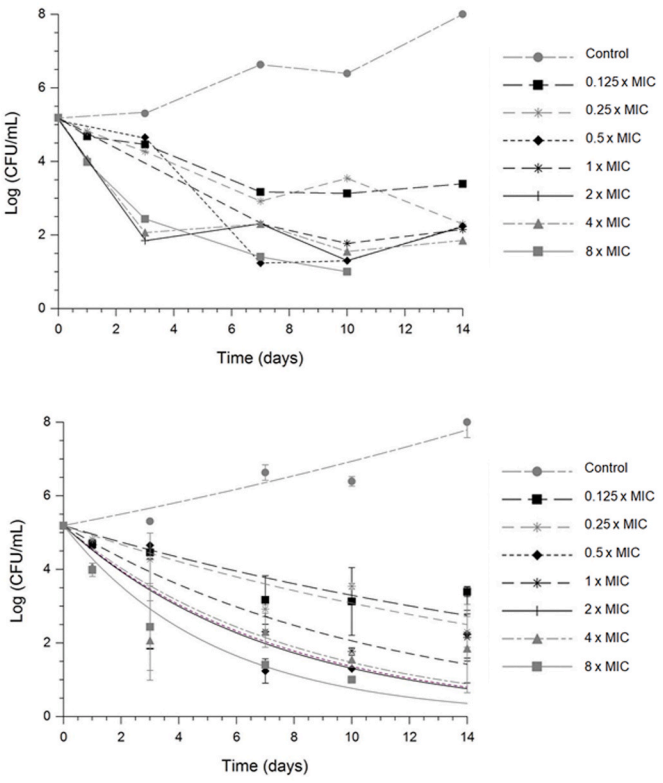

Fig. 2. Time-kill kinetics for different concentrations of tafenoquine (upper panel). Control group was treated with the vehicle, 7H9 medium with 2.5% DMSO; Time-kill curves fitted by the E_{max} model where the symbols represent the experimental data (±SD) and the lines are modeled (lower panel) using Scientist® v.3.0. Each point represents the mean of duplicate values.

4. Conclusion

In this work we show, for the first time, the activity of tafenoquine against drug sensitive and drug resistant *M. tuberculosis*. Additionally, both tafenoquine and mefloquine showed promising results against dormant bacteria, allowing us to suggest that tafenoquine and mefloquine might represent hits for further drug optimization and for future clinical development of new anti-mycobacterial agents. Further experiments will seek to address if tafenoquine or mefloquine could be administered in combination with clinically available drugs, especially in the case of resistant TB forms.

Author statement

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CRediT authorship contribution statement

Maria Gabriella S. Sidrônio: Methodology, Investigation, Writing – review & editing. **Ana Paula O.T. Castelo Branco:** Investigation. **Bruno L. Abbadi:** Methodology, Investigation, Writing – review & editing. **Fernanda Macchi:** Investigation. **Maiele D. Silveira:** Investigation. **Graziela de A. Lock:** Investigation. **Teresa Dalla Costa:** Investigation, Data curation, Writing – review & editing. **Demétrius M. de Araújo:** Resources, Writing – review & editing. **Samuel Cibulski:** Writing – review & editing. **Cristiano V. Bizarro:** Resources. **Pablo Machado:** Writing – review & editing, Funding acquisition. **Luiz Augusto Basso:** Resources, Writing – review & editing, Funding acquisition. **Valnês S. Rodrigues-Junior:** Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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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.2021.102089.

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