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Drug Discovery and Resistance

Repurposing artemisinin as an anti-mycobacterial agent in synergy with rifampicin

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ABSTRACT

The current anti-TB treatment consists of a prolonged multi-drug therapy. Interventional strategies are required to reduce the chemotherapeutic load. In this regard, we have previously identified a synergistic interaction between hydroperoxides and rifampicin. This strategy has been extended here to repurpose a new drug against TB. A hydrophobic antimalarial drug, artemisinin, with an unstable endoperoxide bridge structure, has been investigated as a potential candidate. In combination with rifampicin, artemisinin was found to be synergistic against *M. bovis* BCG and *M. tuberculosis* H37Ra. Furthermore, artemisinin was observed to induce peroxides in a time and concentration dependent manner and the levels of the peroxides were significantly higher in cells treated with the drug pair. Coupled with rapid disintegration of the membrane, this enhanced the clearance of the bacterial culture *in vitro*. On the other hand, formation of the peroxides was significantly reduced in the presence of ascorbic acid, an antioxidant. This translated to a loss of the synergistic effect of the combination, indicating the importance of peroxide formation in the mode of action of artemisinin. Interestingly, artemisinin also had a synergistic interaction with isoniazid, amikacin and ethambutol and an additive interaction with moxifloxacin, other drugs commonly used against TB.

1. Introduction

The emergence and spread of drug resistance has created a major setback in the treatment and eradication of tuberculosis. Targeting resistance by developing new drugs is an expensive process in terms of both time as well as money. In contrast, repurposing existing drugs, whereby drugs that are in use against one disease are used effectively against a new disease, offers a more efficient solution; saving millions in the cost of discovery and approval of the drug [1]. This strategy has been widely explored against a number of diseases with many successful applications. Moxifloxacin, a second line drug used for the treatment of multi-drug resistant strains of TB [2] is one such example of a repurposed drug. Moxifloxacin is a broad range antibiotic, which was originally used against drug-resistant pneumococci [3].

Identification of clinically effective drug candidates can be enhanced by prior knowledge of the cellular targets. In this context, a recent study from our group demonstrated that the combination of hydroperoxides and rifampicin can synergistically inhibit different strains of mycobacteria, including drug-resistant strains. Lipid peroxidation was shown to be important to achieve synergy with rifampicin [4]. In this work, we utilized this concept to identify an effective hydrophobic-ROS inducing drug against mycobacteria. Artemisinin, an anti-malarial drug [5], was observed to be a promising candidate. Artemisinin has a unique 1, 2, 4 trioxane ring in its structure (Fig. 1) and this endoperoxide bridge has been proposed to be involved in clearance of the malarial parasite from the host. The iron from the heme group present in the red-blood cells of the host, reacts with the endoperoxide bridge and leads to the generation of reactive radicals. This carbon-centered free radical then reacts with the essential proteins of the parasite, eventually killing the parasite [6]. The involvement of the reactive species has also been confirmed by studies which exhibit a loss of the antimalarial property of artemisinin in the presence of anti-oxidants and an increase in the activity in the presence of pro-oxidants [7,8]. Moreover, due to the hydrophobicity of the drug, it is retained near the membrane of the cells and can induce lipid peroxidation [9].

Recently, artemisinin has been reported to have an inhibitory effect on *M. tuberculosis*. In a study by Choi *et al.* [10], both artemisinin and its derivative artesunate were observed to be effective against TB *in vitro* as well as in a rat-infected model. Moreover, artemisinin was also predicted as an inhibitor of the DosRST regulon, which is associated with the transit of mycobacteria into a non-replicating persistent (NRP) state under hypoxic conditions. The NRP state, against which most of the

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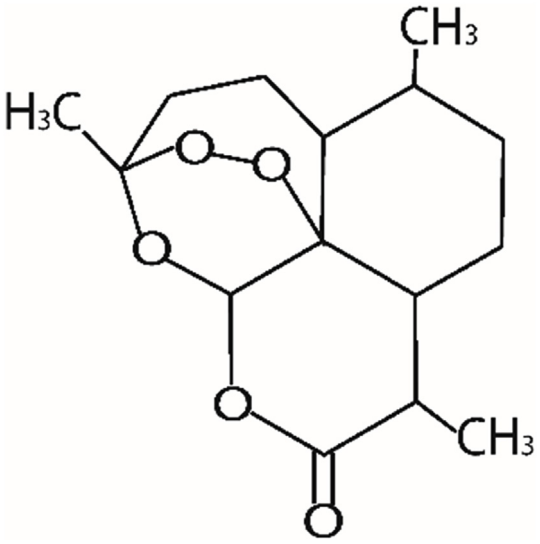

Fig. 1. Structure of artemisinin. The 1,2,4- trioxane ring is hypothesized to be the active site of the drug.

antibiotics are ineffective, is the most important survival strategy employed by mycobacteria [11]. In another study, a mycobactin-artemisinin conjugate was designed, which exhibited activity against sensitive as well as drug-resistant strains of mycobacteria at a much lower concentration. Here, mycobactin, a siderophore, assimilates iron (Fe), which reacts with artemisinin and promotes the initiation of the radical reaction [12].

These studies have, to some extent, established the role of artemisinin as a potent anti-mycobacterial agent. However, treatment for tuberculosis requires combination therapy with multiple drugs, due to several factors, including development of resistance against single drugs and bacterial persistence [13]. In this study, we evaluate the effect of artemisinin in combination with rifampicin, one of the most successful anti-Tb drug in use that is also active against persister cells [14]. Based on earlier work from our lab, we hypothesize that artemisinin, with its ability to cause lipid peroxidation, can have a synergistic action in combination with rifampicin. We also shed light on the mechanism of action of artemisinin alone and in combination. Further, we screen the effect of the combination of artemisinin with other first line anti-TB drugs, isoniazid and ethambutol, and two of the second line drugs, moxifloxacin and amikacin, which are routinely used in the therapy against drug-resistant TB.

2. Materials and methods

2.1. Bacterial strains and growth conditions

M. bovis BCG [15] and *M. tuberculosis* H37Ra [16], were used for all experimental studies. Both the strains were handled in a Biosafety Level-2 facility. For liquid culture, all cells were grown in Middlebrook 7H9 broth (HiMedia) supplemented with 0.44% glycerol (Sigma–Aldrich), 0.15% Tween-80 (Sigma–Aldrich), and 10 %v/v ADN solution. The cultures were maintained at 37 °C with aeration at 200 rpm. For growth on solid media, cells were plated on Middlebrook 7H11 agar plates containing 10 %v/v ADN and incubated at 37 °C for at least 21 days. ADN solution comprises of 5 gm albumin, 2 gm dextrose and 0.85 gm NaCl in 100 mL autoclaved water. This solution was filter sterilized prior to its use.

2.2. Drug preparation

Artemisinin, Isoniazid, resazurin, and DMSO were obtained from Sigma-Aldrich. Rifampicin, ethambutol, moxifloxacin, amikacin and ascorbic acid were obtained from HiMedia Laboratories (India). Artemisinin stock of 10000 μg/mL and rifampicin stock of 5000 μg/mL were prepared in DMSO. For rifampicin, the working stock was diluted in water, which was always prepared fresh. Both the drug stocks were kept away from direct light (prepared in amber tubes) and stored at −20 °C. Isoniazid, ethambutol and amikacin stocks were prepared in autoclaved water. Moxifloxacin stock was prepared in 0.1 N NaOH. The resazurin stock of 0.01 %v/v was also prepared fresh in autoclaved water. The stock of antioxidant, ascorbic acid [17], was prepared in autoclaved water at 50000 μg/mL.

2.3. Checkerboard assay

The minimum inhibitory concentration (MIC) of drug(s) was detected using the checkerboard assay [15]. Cells were grown till the mid-log phase, that is 0.5 Optical Density$_{600\ nm}$ (OD), which corresponds to ~10^7 cells/mL. These cells were then diluted 10 times to obtain 10^6 cells/mL of which 200 μL was dispensed in each well of a 96 well plate. Subsequently, the required concentration of the drugs was added in each well. A growth control with no drug was also included in the setup. The plates were then incubated at 37 °C for a period of 4 days in the case of *M. bovis* BCG and 7 days in the case of *M. tuberculosis* H37Ra strain. Humidity in the incubator was maintained at 80% to avoid significant evaporation. Post incubation, MIC was detected by adding 30 μL of 0.01 %v/v of resazurin in each well. This setup was again incubated at 37 °C for 4 days for complete conversion of resazurin to resorufin. The lowest concentration, at which the blue colour of resazurin was sustained even after 4 days of incubation, was identified as the MIC [18]. For isoniazid, ethambutol and moxifloxacin, due to interference in resazurin conversion, MIC was detected by streaking 10 μL of cells on LB agar plates and evaluating growth after 21 days for BCG and 5–6 weeks for H37Ra. MIC is defined as the lowest concentration at which no growth was observed.

2.4. Fractional inhibitory concentration (FIC) index

To quantify the interaction between two drugs, FIC index was used. It is defined as follows

$$\text{FIC Index} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

The interaction is defined based on the guidelines by Odds [19], where, the interaction will be considered synergistic for FIC index values ≤ 0.5, it is additive for values between 0.5 and 4 and antagonistic for FIC ≥ 4.

2.5. Kill kinetics

6 well plates were used to setup this assay. 3 mL of 10^6 cells/mL of *M. bovis* BCG was added in each well with the required concentration of the drugs. Incubation was done at 37 °C and 80% humidity for 5 days. CFU/mL was estimated at different time points as mentioned in the figure legends. Percent viability was calculated considering 100% viability at day 0.

2.6. Detection of peroxides by FOX-II assay

Peroxides were detected by treating 500 μL of approximately 10^6 cells/mL with the desired drugs for 6, 12 and 24 h. Post treatment, the cells were centrifuged at 4000 rpm at 4 °C for 10 min and re-suspended in 500 μL of phosphate buffer saline (PBS). 20 μL of the re-suspended cells were taken in a fresh 96 well plate. To this 180 μL of

FOX-II colour reagent [4] was added and incubated overnight for the reaction to complete. In brief, the colour reagent was prepared with methanol (90%), H_2SO_4 (25 mM), $FeSO_4{\cdot}7H_2O$ (250 μM) and Xylenol orange (100 μM). In the presence of peroxides, a purple coloured end product was formed which was quantified by measuring the absorbance at 560 nm [4]. The data is plotted as the normalized peroxide levels calculated as follows:

$$\text{Normalized lipid peroxides} = \frac{Condition(A_{560nm})}{Control(A_{560nm})}$$

2.7. Estimation of membrane integrity by CRYO-FEG-SEM imaging

CRYO-FEG-SEM imaging was performed to qualitatively observe the effect of drugs on membrane integrity. 1 mL of ~10^6 cells/mL was treated with the desired drugs for 6,12 and 24 h. Post treatment, the cells were fixed with 4% paraformaldehyde in phosphate buffer saline (pH 7.4) for 30 min for complete sterilization of the culture. This was required prior to removing the cells from the biosafety containment level 2 [20]. Further, the cells were washed at least 3 times with autoclaved water to remove the presence of salts. For imaging, JSM-7600F platform was used. Multiple images were captured for each condition.

2.8. Estimation of membrane integrity by TEM imaging

The effect on membrane integrity was observed under JEOL-JEM 2100F platform. 1 mL of ~10^6 cells/mL was treated with desired drugs at 37 °C for 24 h. Post treatment, the culture was centrifuged at 4000 rpm for 10 min and the cells were fixed with 4% paraformaldehyde in phosphate buffer saline (pH 7.4) for 30 min. The cells were centrifuged and suspended in autoclaved water. A drop of cells was placed on a formvar coated copper grid and observed under TEM.

2.9. Statistical analysis

All the experiments were performed with at least three biological replicates. Statistical analysis for intergroup comparisons was carried out using single factor ANOVA, whenever more than two treatment groups were compared. Significance was established if F was greater than F_{crit} (at 5% significance level). Further, significance between two treatment groups was quantified using the student's t-test with equal variances. $*p < 0.05$, $**p < 0.005$, ns – not significant.

3. Results

3.1. Effect of the combination of artemisinin and rifampicin by checkerboard assay

The combinatorial effect of artemisinin and rifampicin was evaluated on the vaccine strain, *M. bovis* BCG using checkerboard assay. MIC for the drugs was detected using resazurin. This assay is based on the reduction of resazurin (blue) into resorufin (pink) by live, metabolically active cells. The respective MIC's for each drug individually as well as in combination have been mentioned in Table 1. The FIC index has been calculated as explained in the methods section. A synergistic interaction was observed against the BCG strain with as low as 25 μg/mL artemisinin exhibiting growth inhibition in combination with 0.0075 μg/mL of rifampicin.

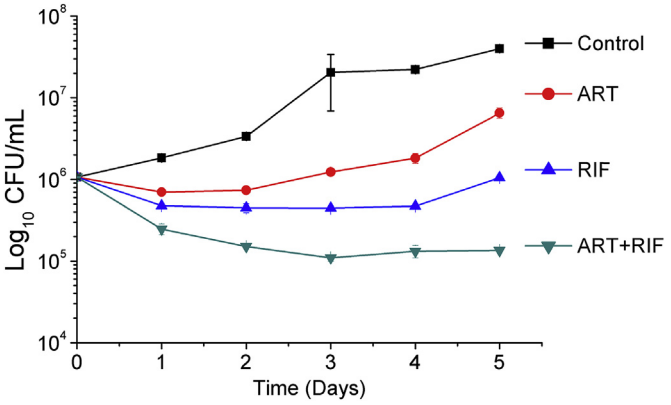

Fig. 2. Effect on the cell viability of *M. bovis* BCG cells on treatment with 25 μg/mL of artemisinin, 0.0075 μg/mL of rifampicin and its combination. Data is plotted as CFU/mL determined for each day up to 5 days of treatment.

The effect of this combination of 25 μg/mL artemisinin and 0.0075 μg/mL of rifampicin was also evaluated by determining CFU/mL of the cultures for each day of treatment (Fig. 2). Cells treated with 25 μg/mL artemisinin exhibited an increase in viability with time. Treatment with 0.0075 μg/mL rifampicin exhibited growth inhibition till 4 days of treatment followed by an increase in viability of the cells by 5th day. However, on treatment with the combination of the two drugs, approximately 90% of the viability was lost within 1 day of treatment and the effect was maintained till at least 5 days of treatment.

3.2. Kill kinetics

We next evaluated the time-kill kinetics of the two drugs alone and in combination against *M. bovis* BCG strain. Kill kinetics is an important asset of the pre-clinical studies, as it provides evidence of the rate of the sterilizing activity of the drugs against the bacterium [21]. To determine kill kinetics, artemisinin concentration was fixed at the MIC concentration (200 μg/mL), as higher concentration would not be practical. Rifampicin concentration was chosen to be at 1× MIC (0.03 μg/mL), 2.5× MIC (0.075 μg/mL) and 5× MIC (0.15 μg/mL). CFU was estimated each day, until five days, to determine whether the drugs can lead to complete clearance of the culture.

As shown in Fig. 3, on treatment with 200 μg/mL of artemisinin, the rate of decrease of CFU was very low, with less than one-log fold decrease in viability even after 5 days of treatment. At 0.03 μg/mL (1× MIC) of rifampicin, there was a significant reduction in the viability of the culture. However, complete sterilization was not observed. At 0.075 μg/mL rifampicin, viability decreased by more than 4-log fold after 5 days of treatment, whereas with 0.15 μg/mL of rifampicin, a similar effect was achieved within 3 days of treatment. In all the cases, in combination with 200 μg/mL artemisinin, there was a significant increase in the rate of reduction of the CFU. For the combination of 200 μg/mL of artemisinin and 0.03 μg/mL of rifampicin, an increased rate of loss of viability was observed from day 2 onwards. For higher rifampicin concentrations of 0.075 and 0.15 μg/mL, around 90% viability was lost within 12 h of treatment with the combination, as compared to 50% loss of viability on rifampicin treatment alone.

Table 1
MIC's of artemisinin and rifampicin against *M. bovis* BCG.

Organism	Artemisinin (μg/mL)	Rifampicin (μg/mL)	MIC in combination		FIC index
			Artemisinin (μg/mL)	Rifampicin (μg/mL)	
M. bovis BCG	200	0.03	25	0.0075	0.375

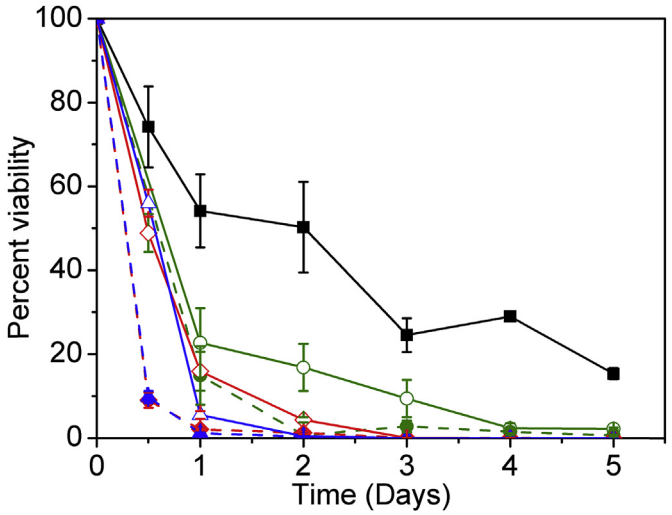

Fig. 3. Kill kinetics of *M. bovis* BCG cells on treatment with artemisinin and rifampicin. The concentrations evaluated were 200 μg/mL of artemisinin alone (Black squares) as well as in combination with rifampicin at 0.03 μg/mL (1 × MIC, represented with green colour), 0.075 μg/mL (2.5 × MIC, represented with red colour), and 0.15 μg/mL (5 × MIC, represented with blue colour) concentrations. Solid line with open symbol represents the single drug exposure whereas the dotted line with solid symbols represents the combination of rifampicin with 200 μg/mL of artemisinin. The data is plotted as percent viability with time. The CFU/mL at 0 h (~10^6 CFU/mL) is considered as 100% viability. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

We next investigate the mechanism behind the synergistic action of artemisinin with rifampicin. The MIC concentrations of the drugs were selected for this study to minimize any significant loss of viability that would limit the cell number required for the assays. Post 24 h of treatment, a significant difference in the cell viability was observed for cultures treated with the combination. Thus, to effectively capture the mechanism behind the enhanced inhibition, all the assays were performed at 24 h of drug treatment.

3.3. Lipid peroxidation by artemisinin

Artemisinin generates carbon-centered free radicals in the presence of Fe^{2+} ions [22]. Due to the hydrophobicity of the drug, these radicals can target membrane lipids, thereby inducing lipid peroxidation. Here, we have quantified the levels of peroxides in *M. bovis* BCG strain using the FOX-II assay. Fig. 4A shows the colorimetric assessment of LPO after 1 day (24 h) of drug treatment. In the presence of peroxides, the orange-coloured dye gets oxidized to a purple-coloured complex. Fig. 4B shows the normalized absorbance values at 560 nm. Increasing concentration of artemisinin resulted in an increase in the peroxide levels. Rifampicin treatment did not induce any peroxide formation. On treatment with a combination of 100 μg/mL of artemisinin plus 0.03 μg/mL of rifampicin, peroxide levels were similar to that of 100 μg/mL of artemisinin alone. However, the peroxides were significantly higher in the cells treated with the combination of 200 μg/mL of artemisinin plus 0.03 μg/mL of rifampicin.

The production of peroxides on treatment with the combination of artemisinin and rifampicin was observed to be time-dependent (Fig. 4C). After 6 h of treatment with the drugs, we could not detect any significant effect on the peroxide levels as compared to the untreated control. After 12 h of treatment, a significant increase in the peroxide levels was detected. However, the levels were lower than those observed after 24 h of treatment.

3.4. Loss of membrane integrity by artemisinin

Peroxidation of membrane lipids leads to membrane disintegration and can eventually cause cell death [23]. The relation of LPO and its effect on membrane integrity (Fig. 5) was studied on *M. bovis* BCG cells after 6, 12 and 24 h of treatment with artemisinin and rifampicin alone and in combination. Representative images have been presented in Fig. 5. Further, Table 2 lists the percentage of damaged cells as determined by analysis of all the images. No significant damage to the cell membrane was observed when cells were treated with 200 μg/mL of artemisinin for 6 h. This correlates with the absence of cellular peroxides. After 12 h of treatment, with an increase in the level of peroxides, a consequent increase in the damaged cell population was observed. Nevertheless, the frequency of damaged cells was significantly lower than that observed after 24 h of treatment, where, almost 35% of the cells were detected with damaged membrane. Rifampicin alone, which did not lead to any peroxide formation, did not induce any significant membrane damage with only 14% of population observed with damaged membrane even after 24 h of treatment. In contrast, on treatment with the combination of 200 μg/mL artemisinin and 0.03 μg/mL rifampicin, a majority of the cell population (75%) had degenerated membrane.

In brief, the CRYO-FEG-SEM data suggests that an increase in the peroxide levels correlates with an increase in the damaged cell population and subsequent inhibitory effect. Moreover, the data also suggests that artemisinin at a concentration of 200 μg/mL (detected as MIC by REMA assay) can induce membrane damage. However, the combined treatment of artemisinin and rifampicin induces the damage in a larger cell population, which eventually leads to sterilization of the culture. This effect corroborates the results observed by the kill kinetics study.

We next validated the observed membrane damage by imaging *M. bovis* BCG cells under TEM. It has been shown previously that membrane damage corresponds to a loss of the cytoplasmic content of the cells [24,25]. As a result, cells appear to be significantly less dense when imaged under TEM. As shown in Fig. 6, untreated control cells appear as dark rod shaped bacilli. On treatment with 200 μg/mL artemisinin, we observed a distribution of cells, where some cells appeared as intact, dark, and rod-shaped and others exhibited a lower density indicating membrane damage. For the combined treatment of 200 μg/mL artemisinin and 0.03 μg/mL rifampicin, majority of the cells had a distinct loss of cytoplasmic content and membrane damage validating the results we observed under CRYO-FEG-SEM.

3.5. Effect of antioxidants on cells treated with artemisinin

To confirm the importance of reactive oxygen species in inducing bacterial clearance, *M. bovis* BCG cells were treated with an antioxidant, ascorbic acid. If ROS is the primary mechanism of action of artemisinin, the inhibitory effect of the drug will be lost on addition of the anti-oxidant [17]. A sub-inhibitory concentration of ascorbic acid (200 μg/mL) was chosen for this study and the effect on the peroxide levels as well as on the bacterial viability was monitored. As observed in Fig. 7A, in the presence of ascorbic acid, the peroxide levels were significantly lower in the cells treated with 200 μg/mL of artemisinin alone as well as a combination of artemisinin plus 0.03 μg/mL of rifampicin. This effect was evident within one day of treatment with the antioxidant.

Further, the reduction in the levels of peroxides also correlated with a decrease in the inhibitory effect of artemisinin alone and the synergistic effects of the combination of artemisinin and rifampicin (Fig. 7B). In the presence of ascorbic acid, the time-kill kinetics of artemisinin plus rifampicin was similar to that of rifampicin alone. Moreover, this effect was maintained till at least 5 days of treatment. This significant loss of the inhibitory effect of artemisinin in the presence of an anti-oxidant strongly supports the involvement of ROS production as a plausible mechanism of action of artemisinin against

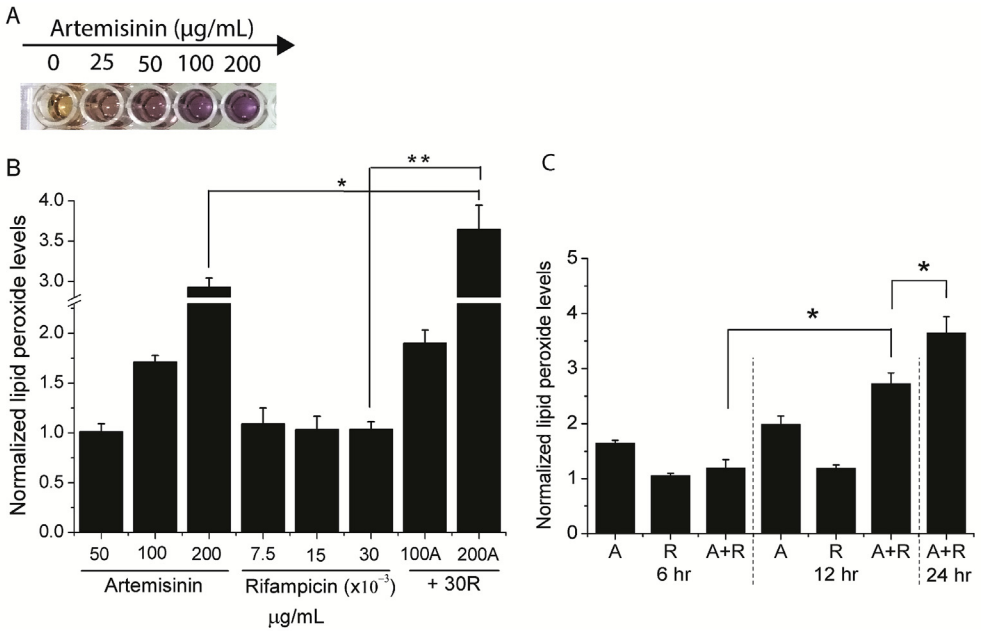

Fig. 4. Fox-II assay for the detection of peroxides in *M. bovis* BCG cells on treatment with artemisinin and rifampicin alone and in combination. (A) Treatment with 25, 50, 100 and 200 μg/mL of artemisinin for 24 h. In the presence of peroxides, the orange coloured dye gets oxidized into a violet product. A gradual increase in the violet product was observed with increase in the artemisinin concentration. (B) Normalized levels of lipid peroxides detected after treatment with increasing concentrations of artemisinin (50, 100, 200 μg/mL), increasing concentration of rifampicin (0.0075, 0.015, 0.03 μg/mL) and the combination of 100 μg/mL of artemisinin plus 0.03 μg/mL of rifampicin and 200 μg/mL of artemisinin plus 0.03 μg/mL of rifampicin (shown as 100A/200A + 30R). Cells were treated for 24 h. (C) Peroxide levels determined after 6, 12 and 24 h of treatment with artemisinin (200 μg/mL), rifampicin (0.03 μg/mL) and its combination. * represents p-value < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

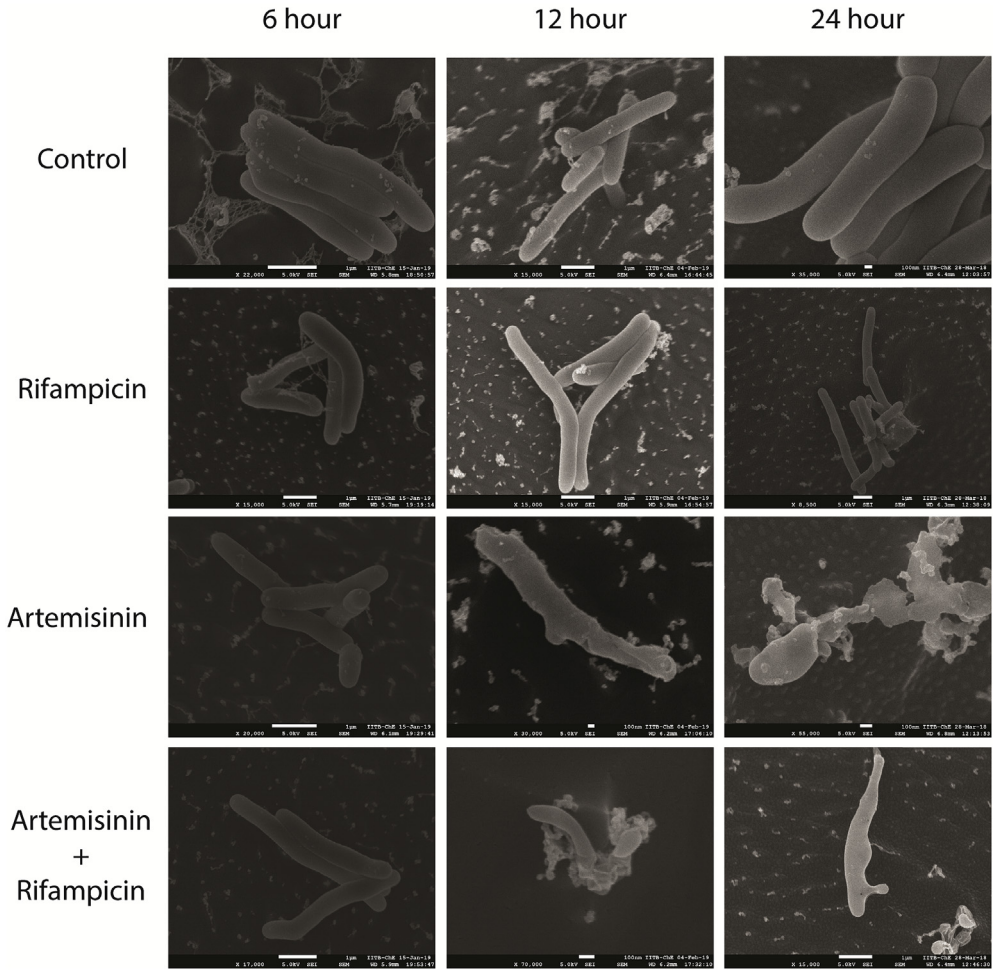

Fig. 5. CRYO-FEG-SEM images of *M. bovis* BCG captured after 6, 12 and 24 h treatment with rifampicin (0.03 μg/mL) and artemisinin (200 μg/mL) alone and in combination. Untreated cells have been shown as Control. Multiple frames were captured for all the conditions.

Table 2
Percentage of damaged cells after 6, 12 and 24 h of treatment with the conditions as observed by CRYO-FEG-SEM.

Condition	Percent Damaged Cells		
	6 h	12 h	24 h
Control (Untreated)	0.00	8.70	8.86
Rifampicin (0.03 μg/mL)	0.00	3.03	13.92
Artemisinin (200 μg/mL)	3.45	16.67	35.44
Artemisinin + rifampicin (200 μg/mL + 0.03 μg/mL)	6.38	27.63	75.00

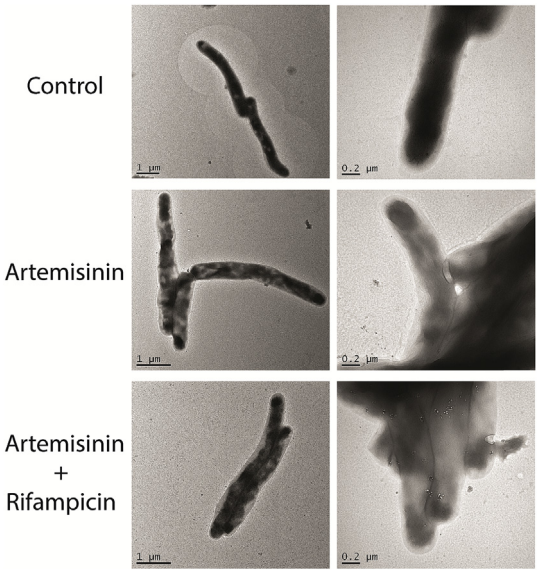

Fig. 6. Transmission electron microscopy (TEM) image of *M. bovis* BCG cells on treatment with 200 μg/mL artemisinin alone and in combination with 0.03 μg/mL of rifampicin. The cells were treated for 24 h.

mycobacteria. It is to be noted that 200 μg/mL of ascorbic acid alone had no significant effect on the cell viability.

3.6. Effect of artemisinin in combination with other anti-TB drugs against *M. bovis* BCG

To further support the use of artemisinin as an anti-mycobacterial therapy, we evaluated the interaction of artemisinin with other first-line anti-TB drugs, isoniazid and ethambutol and also with second-line drugs, amikacin [26] and moxifloxacin [2]. These second-line drugs are commonly used in the TB drug regimen, especially against the drug-resistant strains. The MICs of the drugs individually as well as in combination with artemisinin were measured using checkerboard assay. The respective MIC's for all the other drugs against *M. bovis* BCG have been mentioned in Table 3. Amikacin demonstrated a strong synergistic interaction with artemisinin, with a FIC Index of 0.38. For Isoniazid and ethambutol also, FIC index was observed to be 0.5, suggesting a synergistic interaction. For moxifloxacin, the interaction was observed to be additive with a FIC index of 0.75.

3.7. Effect of the drug combinations against *M. tuberculosis* H37Ra

With the interesting results observed on *M. bovis* BCG strain, we next screened the effect of the drug combination on *M. tuberculosis* H37Ra. This strain is closely related to the pathogenic *M. tuberculosis* H37Rv strain as it is derived from the same parent strain H37 [27,28]. The MICs of the drugs were measured using the checkerboard assay and have been mentioned in Table 4. The synergistic effect of artemisinin

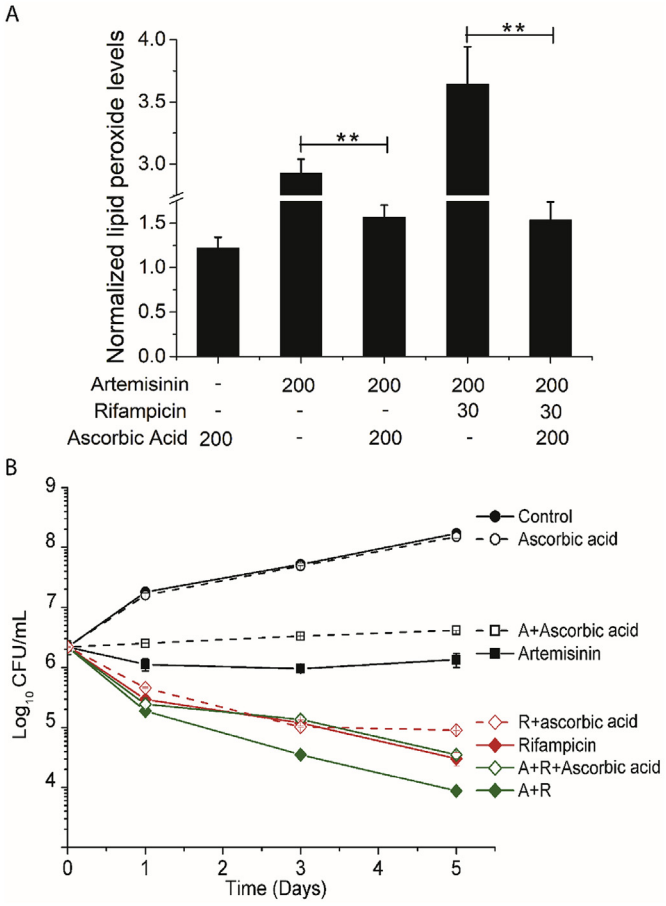

Fig. 7. Effect of an antioxidant on the inhibitory effect of artemisinin on *M. bovis* BCG cells. (A) Estimation of lipid peroxides detected after 1 day of treatment. (B) Kill kinetics plotted as CFU/mL with time. The concentrations screened were 200 μg/mL of ascorbic acid, 200 μg/mL of artemisinin, 0.03 μg/mL of rifampicin and their combinations. A+ ascorbic acid represents the combination of 200 μg/mL artemisinin and 200 μg/mL ascorbic acid. R+ ascorbic acid represents the combination of 0.03 μg/mL rifampicin and 200 μg/mL ascorbic acid. A + R represents the combination of 200 μg/mL of artemisinin and 0.03 μg/mL rifampicin. The standard deviation amongst biological replicates was very low. Distinct error bars are not visible as they are shorter than the symbols used in the graph.

Table 3
MIC's of artemisinin, isoniazid, ethambutol, amikacin and moxifloxacin against *M. bovis* BCG as detected by checkerboard assay.

Drug	Concentration of Artemisinin (μg/mL)	Concentration of Drug (μg/mL)	MIC in combination (μg/mL)		FIC index
			Artemisinin	Drug	
Isoniazid	200	0.5	50	0.125	0.5
Ethambutol	200	4	50	1	0.5
Amikacin	200	1	25	0.25	0.38
Moxifloxacin	200	0.12	50	0.06	0.75

with rifampicin and isoniazid was also maintained against this strain with a FIC index of 0.5 and 0.375, respectively. For all other drugs, The FIC index was observed to be between 0.5 and 1, suggesting an additive interaction between the drugs.

4. Discussion

In the current scenario, where millions of people are still affected by tuberculosis, especially the resistant strains, the treatment of the

Table 4
MIC's of artemisinin, rifampicin, isoniazid, ethambutol, amikacin and moxifloxacin against *M. tuberculosis* H37Ra as detected by checkerboard assay.

Drug	Concentration of Artemisinin (μg/mL)	Concentration of Drug (μg/mL)	MIC in combination (μg/mL)		FIC index
			Artemisinin	Drug	
Rifampicin	100	0.03	25	0.0075	0.5
Isoniazid	100	4	25	0.5	0.375
Ethambutol	100	5	50	1.25	0.75
Amikacin	100	1	25	0.5	0.75
Moxifloxacin	100	0.25	50	0.06	0.74

disease has become a major concern. There is an urgent need to discover better and efficacious drugs against mycobacteria. The field of drug repurposing provides a fast approach in identifying clinically relevant drugs that can be used against the pathogen.

Artemisinin [29] and its derivatives [30] are one of the most effective therapeutics against malaria. With a very low systemic toxicity, these compounds are promising candidates to be explored for repurposing against other diseases [31]. Artemisinin has been shown to exhibit anti-tumor properties [32] as well as inhibitory activity against many pathogenic viruses [33], protozoans [34] and fungi [35]. Artemisinin has also been shown to exhibit anti-mycobacterial activity against both replicating [10] and non-replicating persistent (NRP) mycobacteria [11]. However, artemisinin is best administered as a combination therapy, to reduce the rate of development of resistance [36] and overcome the short plasma half-life. In this regard, synergy between combination drugs would further enhance the efficacy of artemisinin, especially against various resistant bacteria. In this work, we have evaluated the effect of artemisinin in combination with a first line anti-TB drug, rifampicin, and have also determined its mode of action against replicating mycobacteria.

We observe that artemisinin and rifampicin function as a synergistic drug pair against mycobacteria with the combination having a FIC index ≤ 0.5. As an anti-malarial drug, generation of carbon-centered free radicals that induce lipid peroxidation has been perceived to be one of the mechanisms by which artemisinin acts against the parasite [37]. On similar lines, artemisinin was found to cause lipid peroxidation in *M. bovis* BCG within 12 h of treatment with the drug. The levels of the peroxides were significantly higher when the bacteria were treated with a combination of artemisinin and rifampicin compared to artemisinin alone. This subsequently led to extensive damage to the cellular membrane. Moreover, the presence of ascorbic acid was able to reduce both the peroxide levels and the inhibitory effects of artemisinin, supporting the role of ROS in its activity. Interestingly, the synergistic effect of the drug combination was observed until 3 days post treatment. Free radicals are extremely reactive and have a short half-life. Hence, we hypothesize that the radical formation and propagation has a strong effect on a short time-scale. With time, the radicals may get neutralized and thereby the drug has no further effect on enhancing the bactericidal activity of rifampicin.

In our previous study, we had demonstrated a synergistic action of cumene hydroperoxide (CHP) and rifampicin against several Mycobacteria species, including *M. bovis* BCG and sensitive and drug-resistant strains of *M. smegmatis*. Rifampicin, an inhibitor of bacterial transcription, was shown to downregulate the oxidative-stress response genes *ahpC* and *ohr* [38]. The synergistic action of CHP, an organic peroxide, and rifampicin was hypothesized to be due to the inability of cells to neutralize the ROS generated by CHP in the presence of rifampicin. We believe a similar phenomenon to be at play for the combined drug treatment of artemisinin and rifampicin. Rifampicin would inhibit the expression of the antioxidant enzymes of *M. bovis* BCG cells and thus prevent the neutralization of the reactive species. The ensuing propagation of radicals would further instigate membrane damage [4], leading to higher uptake of rifampicin and ultimately inducing rapid clearance of the bacteria. Thus, rifampicin and artemisinin work in tandem to enhance each other's mode of action leading to a synergistic effect against Mycobacterium.

We have primarily focused on replicating mycobacteria in this work. Interestingly, artemisinin has been shown to be effective against NRP bacteria also, with concentrations as low as 3 μg/mL causing more than 50% loss in viability of hypoxic cells. Artemisinin was shown to directly inhibit the DosS and DosT histidine kinases, and cause widespread differential expression of mRNA in cells grown under oxygen limitations, suggesting additional molecular targets. Therefore, a transcriptomic study of replicating mycobacteria under treatment with artemisinin and rifampicin combination would shed further light on their mechanism of action.

Reducing the required concentration of artemisinin, especially against replicating mycobacteria, is the next major challenge towards translating this drug in a combination therapy [39]. Recent studies in the area of drug delivery, such as delivering artemisinin as a mycobactin-artemisinin conjugate has shown promising results with a significant reduction in IC50 of artemisinin [12]. Alternatively, transferrin-tagged artemisinin has also being explored to enhance the uptake of the drug in cancer cells [40,41]. Thus, combined with an improved delivery system, artemisinin and rifampicin could serve as an attractive combination therapy against tuberculosis.

In summary, we have shown that artemisinin can be successfully repurposed as an anti-mycobacterial agent. When subjected in combination with rifampicin, artemisinin can synergistically inhibit *Mycobacterium bovis* BCG as well as *Mycobacterium tuberculosis* H37Ra. The inhibitory concentration of the drug alone was very high, 100 μg/mL and 200 μg/mL for H37Ra and BCG strains respectively. However, in combination with rifampicin, the MIC of artemisinin reduced to 25 μg/mL, whereas MIC of rifampicin reduced by 4-fold against both the strains. The kill kinetics data also demonstrated that the combination of 200 μg/mL of artemisinin and rifampicin was able to kill the bacteria at a much faster rate than rifampicin alone. The faster clearance rate achieved by the combination of artemisinin and rifampicin can help shorten the current duration required for tuberculosis treatment. Interestingly, artemisinin also shows synergistic/additive interaction with other anti-TB drugs such as isoniazid, ethambutol, amikacin, and moxifloxacin. These drugs are commonly used against TB. The ability of artemisinin to induce hydrophobic free radicals, which promote membrane disintegration, is shown to be critical to the synergistic activity of artemisinin and rifampicin against mycobacteria.

Conflicts of interest

The authors have no conflicts of interest.

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Author contributions

YP and SM conceived the study and designed the experiments,

analyzed the data and wrote the paper. YP and NM performed the experiments and analyzed the data.

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Appendix A. Supplementary data

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