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REGULAR ARTICLE

A novel approach to eradicate latent TB: Based on resuscitation promoting factors

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Abstract Tuberculosis (TB) is a growing unsolved health concern, as it is the main infectious cause of death in the globe. The ability of the pathogen to develop into a dormant state is the main obstacle in overcoming the disease. It seems that the development of compounds that can target latent TB is the key to eradicate this pathogen. In this regard, many researchers started to search for novel compounds that could inhibit the activity of molecules involved in the resuscitation of latent bacilli. The discovery of an extremely potent anti-dormancy factor, a resuscitation-promoting factor (Rpf) from *Micrococcus luteus*, shifted the idea towards developing potent inhibitors of Rpfs to establish latent TB and avoid reactivation of the sleeping pathogen. However, besides the advantages of this approach over the application of annoying long-term regimes of toxic antibiotics, such approaches that rely on silencing latent TB have many drawbacks that may question their application in human research. The major drawback of the current approaches is that they hide the latent TB rather than treating/eradicating it. Here, we propose a novel cost-effective approach that could effectively eradicate both active and latent TB in a short period of time without having any risk of reactivation.

Introduction

Tuberculosis is a growing global health concern

Mycobacterium tuberculosis (MTB) is one of the most successful bacterial parasites of humans, affecting over one-third of the population of the world as a latent infection without clinical symptoms [1]. The number of diagnosed cases reached 8.7 million in 2011 [2,3]. In this context, tuberculosis (TB) ranks as the leading cause of death from a single infectious agent before malaria and HIV, and it leads to approximately 2 million deaths every year [4]. Moreover, studies show that there is a

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close relationship between TB and developing other dangerous diseases such as lung cancer [5–7]. People who are immunocompromised, in particular the HIV cases, have the highest risk for developing active TB [8–10]. Full eradication of TB is hampered by the ability of the parasite to survive up to decades in a dormant state, initially in hypoxic tubercles in the lung, and to drive recurrent and resistant forms [11]. Dormancy/latency is a reversible physiological state of bacteria in which cells can remain for a long period of time without division [12,13]. Dormancy is a viable but non-culturable state that was first detected in *Vibrio cholerae* in 1982 [14]. Treatment of active TB in humans by a regimen combining four drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) leads to rapid killing of the bacteria during the first 2 months [15,16]. However, relapses and multidrug resistance (MDR) frequently occur if the therapy is not continued further for 4 months to kill the remaining bacilli [17]. A new generation of antibiotics developed but none gained satisfactory results in controlling disease [18]. Furthermore, none of the TB vaccines studied so far effectively hinders the development of pulmonary TB in tested animal models [19]. Besides, patient compliance, cost of drugs and increased frequency of drug-resistant MTB strains that require application of long-term toxic dosages of antibiotics have added to the urgent requirement for developing novel TB therapies.

Forms of TB: latent TB versus active TB

TB has two faces: the active symptomatic form and the dormant (latent) asymptomatic one. Diagnosis of the active form is easy because patient represents the symptomatic signs of TB and this form is susceptible to a standard antibiotic regime [1]. The dormant (latent) form is the asymptomatic form in which the bacilli has a state of non-dividing form and could persist against antibiotic regimes [16]. About 90% of TB cases develop a latent TB infection. MTB bacteria are captured by white blood cells in lesions named 'tubercles' or 'granulomas' [12]. The resident evil inside the tubercle stays in a live, non-culturable state, and the situation leads to a weakened immune response, allowing the latent evil to get rid of the lesion and reactivate into active TB [9]. It is assumed that resistant and recurrent forms of TB could be eradicated by targeting the molecules and pathways involved in the dormant state of the bacilli.

Approaches developed to combat latent TB

Considering the emergence of resistant forms of TB and understanding about the relation between the latent form and recurrent disease, a large body of papers (most of them consisting of genome-wide analyses and *in silico* studies) focussed on developing compounds that could block the activation of latent bacilli [2,20–32]. In this regard, Dennis J Murphy and James R Brown performed a meta-analysis of several published data sets from microarray experiments coupled with genome-wide insertional mutagenesis that modelled infection leading to a dormant state. Based on bioinformatic prediction, several genes including those involved in adenosine triphosphate (ATP) synthesis, carbon synthesis and enzymes of redox balance and respiration, sulphur transport and fixation and pantothenate, isoprene and nicotinamide adenine dinucleotide (NAD) biosynthesis were identified as gene targets against the dormant phase of MTB infections [2].

In another study, Vilcheze et al. searched for novel inhibitors of InhA through the new possible inhibitors of enoyl–acyl carrier protein (ACP) reductase and a library of 300 compounds inhibiting *Plasmodium falciparum* enoyl reductase. The authors identified four compounds that effectively killed TB bacilli under aerobic (active form) and anaerobic (latent form) conditions. Subsequently, *in vitro* examination of these compounds shows that only two compounds (CD39 and CD117) were effective against drug-susceptible and drug-resistant MTB [26].

In another genome-wide-screening-based study, Stanley et al. screened a whole cell with various libraries of small molecules as an approach for the detection and introduction of novel inhibitors of MTB. In this way, they found two novel inhibitors – a benzimidazole and a nitro-triazole – both target proteins required for cell wall biosynthesis in MTB [33]. In their study, Stanley et al. added two other inhibitors to the large list of anti-latent TB compounds.

Rpfs: promising molecules to target latent TB

The view of bacterial dormancy and resuscitation was revolutionised when the role of Rpf from *Micrococcus luteus* was revealed by Mukamolova et al. in 1998 [34–36]. In *M. luteus*, picomolar concentrations of Rpf are needed to revive and resuscitate the growth of dormant bacilli [18,37,38]. MTB contains five Rpf-like genes which products, RpfA–E, promote replica-

Table 1 Comparison between the features of proposed approach with the previous ones.

Method	Antibiotic therapy	Inhibitors of latent TB	RPFs + antibiotic therapy
Features			
Require toxic doses	+	+	–
Kill active bacilli	+	+	+
Kill dormant bacilli	–	–	+
Emergence of recurrent TB	+	+	–
Developing resistant forms	+	±	–
Capable to eradicate latent TB	–	–	+
Low doses of drug required	–	–	+
Safety(no reactivation of disease)	–	–	+
Easy and fast process for drug development	–	–	+
Short length of treatment	–	–	+
Require permanent use of drug	+	+	–
Cost effective	–	–	+

tion and resuscitation of mycobacterial cells [40]. RpfB, one of the five Rpfs secreted by TB bacilli, has a crucial role in the resuscitation and growth stimulation of latent MTB. It is likely that this domain has biological features and immunogenicity similar to that of the full-length Rpf [41]. Studies show that RpfB is the most hopeful candidate of the five Rpf-like proteins regarding its immunogenicity and protective efficacy and these features propose it as a novel subunit vaccine for prevention of TB [41,42]. Hence, the authors picked out the RpfB domain protein from MTB to test the proposed hypothesis.

As Rpfs are the extensively potent anti-dormancy factors known until now [14], the second series of researches focussed on searching for compounds with potent anti-Rpf activities.

In this way, Galina et al. were the first who used a new class of 2-nitrophenylthiocyanate (NPT) compounds to suppress the enzymatic activity of Rpfs *in vitro*. It was demonstrated that a synthesised family of NPTs significantly inhibited sprouting of fungal spores and possess similar structures to Rpfs; hence, they were considered as potent inhibitors of cell wall hydrolases and subsequent resuscitation of latent organisms [43,44]. In this study, Galina et al. introduced NPTs as the first example of low-molecular-weight compounds that exert inhibitory effects on the muralytic activities of Rpfs [43]. Recently characterised NPT compounds could be used as a promising scaffold for the generation of therapeutic agents to prevent reactivation of latent TB [39].

In another study, Fan et al. developed monoclonal antibodies against the MTB RpfB domain in mice. They successfully produced three specific and effective monoclonal antibodies (MAbs) against the RpfB domain that suppress the promoting

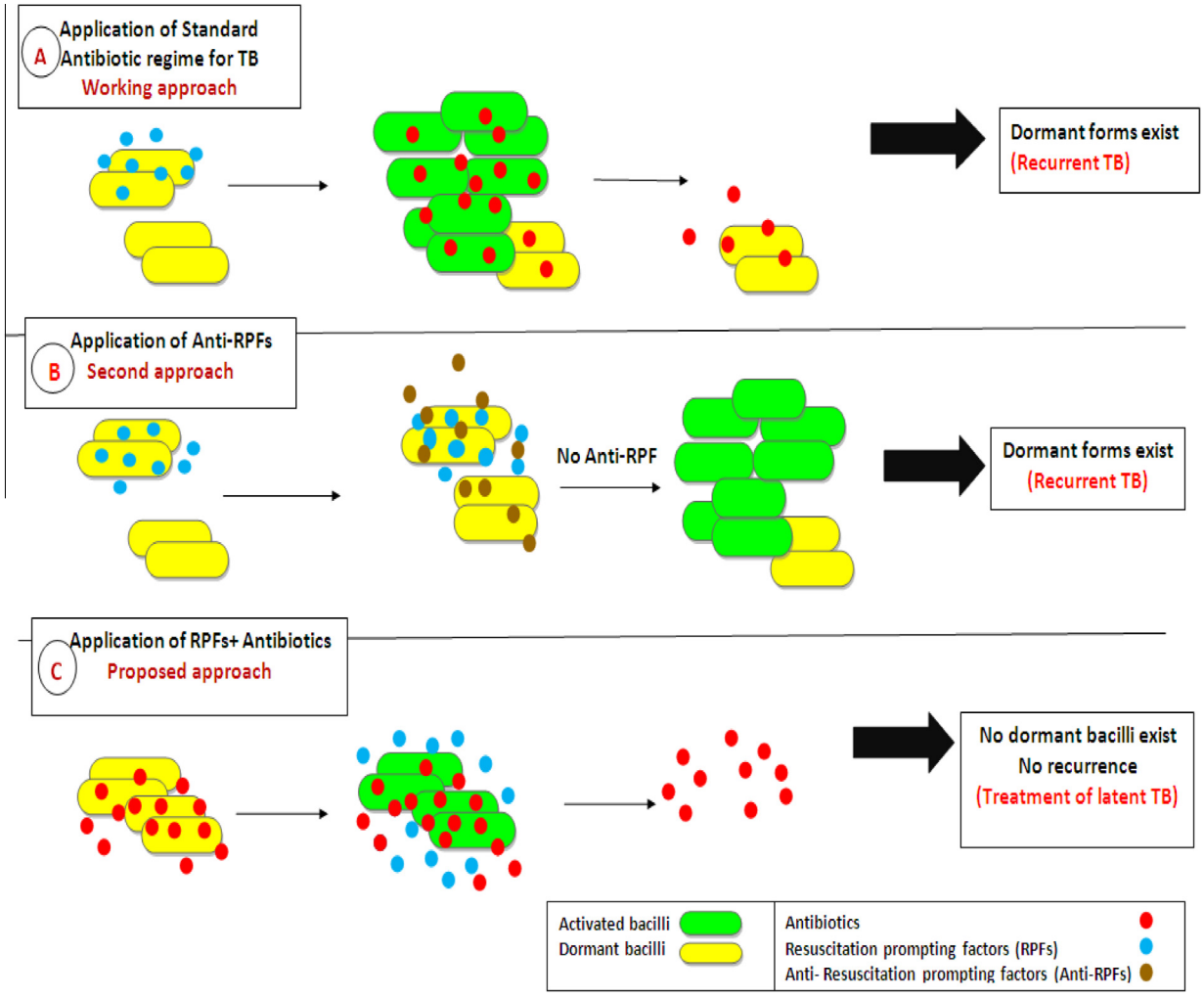

Figure 1 Comparison of Current approaches with our proposed approach for eradication of latent TB. (A) TB without treatment: In such condition without administration of anti TB drugs or inhibitors of RPF there is repeated periods of latent form of bacilli (asymptomatic phase of disease) and active form of bacilli (Symptomatic and acute phase of disease). Administration of standard TB antibiotics can kill all active form of bacilli but fail to eliminate the latent ones. So the recurrent form of TB occurs as a result of activation of dormant bacilli. (B) Current approaches based on silencing latent TB by deactivation of Rpfs. In this approach the dormant state of bacilli will be established by using inhibitors of Rpfs or other synthesized compound against the other factors involved in resuscitation of latent bacilli. This approach requires a permanent dose of inhibitors and the risk of reactivation of latent TB still remain. (C) Our proposed approach based on activation of RPFs: This method takes advantage of conversion of dormant bacilli into activated bacilli by RPFs. While activated form of bacilli are susceptible to antibiotics, so the high serum concentration of anti TB drugs will eliminate newly activated bacilli. Consequently this approach is capable to eliminate both active and dormant form of TB.

effect of the RpfB domain on the growth of MTB $H_{37}R_a$ strain and *M. luteus*, suggesting that MAbs produced against the RpfB domain might be able to suppress the reactivation of latent MTB *in vivo* [41]. In this study, Fan et al. produced MAbs rather than chemical compounds to inhibit the activity of Rpfs.

Although the above approaches eliminate using toxic intolerable antibiotic regimes, the main concern, which is the activation of the silent volcano of latent TB and the risk of reactivation (safety issue), still remains. These approaches are limited to *in vitro*/*in vivo* experiments under laboratory conditions and because of their safety issue none meet their clinical trial phase. Furthermore, these approaches are only applicable in the case of patients with confirmed TB; while such patients receive immunosuppressive drugs, these conditions put them at serious risk of reactivation of latent TB. On the other hand, the large number of cases with undiagnosed TB will be missed. These limitations push forward the idea of how to develop an approach that could eradicate latent TB rather than ignore it; hence, we thought about the optimisation of a reverse approach with the highest benefits for the patients. The main advantages of the proposed approach over other approaches are shown in Table 1.

The hypothesis

As discussed before, among the current strategies to avoid recurrence of TB, all concentrate on neutralising factors that are responsible for activation of latent TB. In contrast to previous approaches, which attempt to inhibit activation of Rpfs, we propose the reverse approach. We emphasise the activation of dormant bacilli within a defined time after the administration of a standard antibiotic regime for TB. Activation of Rpfs will kill all TB repertoires and so will eradicate any recurrence of TB in the treated subjects and will lower significantly the need for long-term expensive and toxic regimes in resistant forms. The main idea of our hypothesis is depicted in Fig. 1.

Evaluation of the hypothesis

Several mouse models have been developed for studying latent TB [45,46]. In this regard, we propose relatively resistant

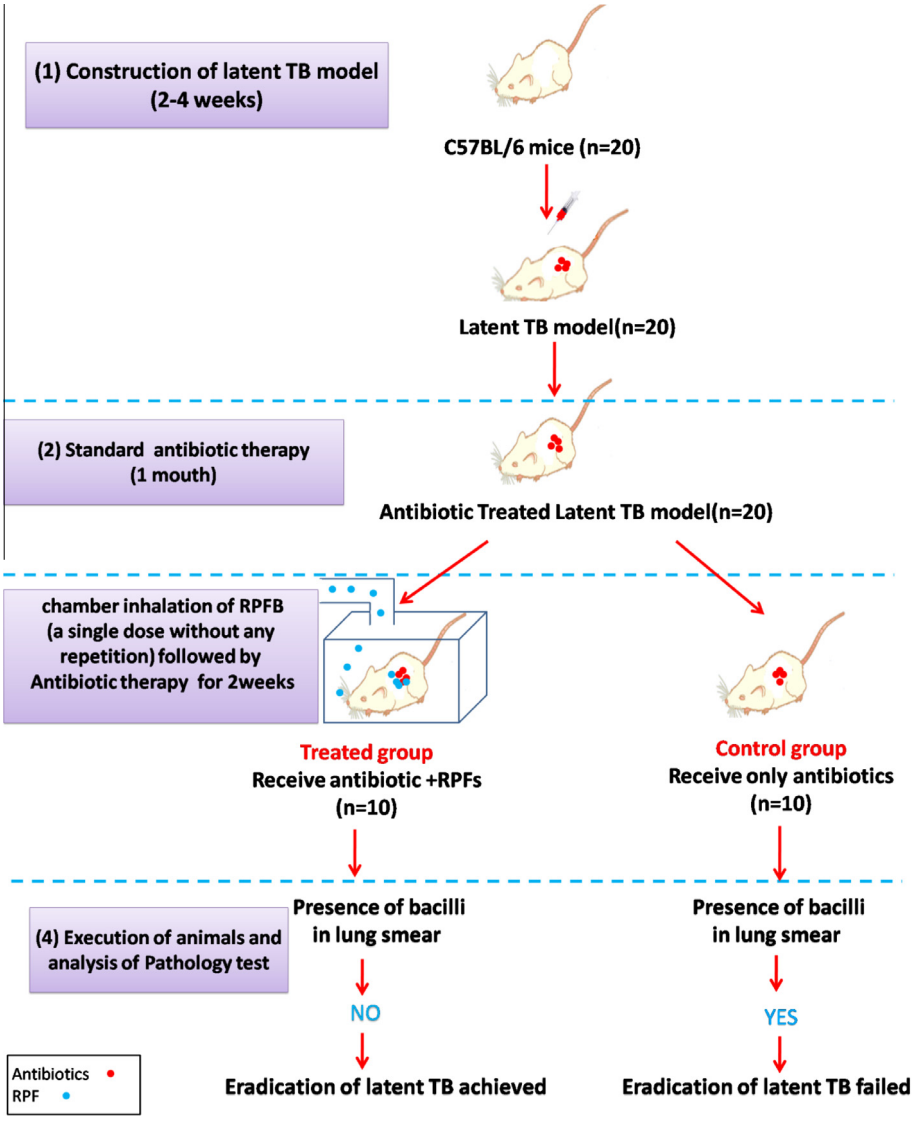

Figure 2 Different steps required for evaluation of proposed hypothesis.

mouse strains (such as C57BL/6) in which the immune system controls the growth of bacilli and provides a reliable latent model of TB [18,45]. As reviewed in reference 46, it takes approximately 1 month to develop a latent mouse model of TB. After this step, 20 latent TB mouse models will be divided in two equal groups as the control and experimental groups. Both groups will receive the same dose and regime of antibiotics (e.g., isoniazid, rifampicin, pyrazinamide and ethambutol). After 1 month when the serum level of antibiotics would reach the maximum concentration, the experimental group will receive a single dose of picomolar concentrations of RpfB domain protein through an inhalation chamber while at the same time the antibiotic therapy will be continued for both groups for another 2 weeks to ensure the stable high serum level of antibiotics. After 2 weeks, both control and experimental mice will be scarified and their lungs will be cultured to identify MTB bacilli. According to our hypothesis, it is predicted that control mice that received only antibiotics will be positive for the presence of TB bacilli. However, in experimental mice we expect a negative result because all dormant bacilli are activated by administration of Rpf and subsequently killed by exposure to high level of antibiotics; hence, the lung culture of treated mice should be TB negative. By this simple method that requires simple techniques such as pathology, one could easily evaluate the efficacy of proposed approach (Fig. 2).

Discussion and conclusion

TB still is a serious concern to human health affecting a large number of people throughout the world. In contrast to the accessible drugs applied for the treatment of TB, the long chemotherapeutic regimens frequently contribute towards the resistant and recurrent forms and this even requires toxic doses which are not tolerable by the patients [1–5]. Latency is a crucial characteristic of TB that enables the bacilli to survive in granulomatous lesions for decades and cause MDR and recurrent TB [9–12]. Therefore, strategies emerged to target and deactivate the latent form by developing various compounds against factors which are responsible for the latent state of the bacilli as discussed earlier [22–31]. Although these approaches seem to be a better choice over those cases which are resistant to standard antibiotic regimes, they have major drawbacks too. They were all conducted only under *in vitro*/*in vivo* conditions and they have not met animal or clinical trial studies. They do not cure latent TB but they just hide the existence of a latent TB. Considering Rpfs as the major factors responsible for resuscitation of dormant bacilli, the idea of using this protein as a potent tool against the pathogen itself emerged. Our proposed approach and the simple proposed protocol to test it in an animal model could easily and promptly undergo its clinical trial phase in a human population; and instead of an inhalation chamber, one could easily receive Rpfs through an aerosol inspiration similar to that of an asthma patient. The only concern about this approach is the time of administration of Rpf that will convert all latent bacilli into activated forms; but this concern is already solved by the presence of high serum level of antibiotics that are ready to clear host body of any activated antibiotic-susceptible bacilli. The proposed approach is simple, easy, fast, safe and cost-effective and as it can target the latent TB it leaves no chance for the reactivation of latent TB.

Conflict of interest statement

None declared.

Overview Box

What do we already know about the subject?
TB is one of the most prevalent infectious diseases worldwide. The major obstacle for full eradication of TB attributed to the dormant state of the bacilli. Routine combinatorial antibiotic therapy is only applicable to the active form of the disease, while dormant forms persist and provide the recurrent or resistant form of the disease.

What does your proposed theory add to the current knowledge available, and what benefits does it have?
All approaches focus on strategies that prevent the activation of dormant bacilli. This approach could not guarantee the full control of disease and at any time the dormant forms have a chance to reactivate. We propose the reverse approach that seems to be a more reliable strategy. According to our proposed strategy, by implementation of the active form of Rpfs and concomitant administration of antibiotics one can gain the best results – elimination of resistant TB.

Among numerous available studies, what special further study is proposed for testing the idea?
This approach is a novel idea and there is a simple protocol to test its feasibility. After predicted results obtained in animal models, the human clinical trial studies can be implemented too.

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