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DRUG DISCOVERY AND RESISTANCE

Chemotherapeutic efficacy of thioridazine as an adjunct drug in a murine model of latent tuberculosis

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SUMMARY

Thioridazine, a potent phenothiazine compound was evaluated for its chemotherapeutic efficacy against experiment model of tuberculosis. Thioridazine potentiated the activities of both isoniazid and rifampicin (>1 log CFU reduction) against the in vitro latent *Mycobacterium tuberculosis* bacilli. Further, a murine model of latent tuberculosis was used and the standard 9-month isoniazid and 4-month rifampicin regimen along with thioridazine as an adjunct drug were evaluated. Thioridazine led to an accelerated clearance of bacilli with both the regimen, thereby leading to completion of therapy much earlier than the standard end-point. In the case of 9-month isoniazid regimen, when thioridazine was used along with isoniazid as an adjunct drug, complete clearance was observed as early as 24 weeks as compared to the 36 week standard isoniazid monotherapy regimen. Also, in the 4-month rifampicin regimen, it was observed that the bacillary clearance was more robust when rifampicin was used along with thioridazine (>3 log CFU reduction) than rifampicin alone (>2 log CFU reduction). Our findings implicate that thioridazine, when used as an adjunct drug along with isoniazid or rifampicin has the potential to augment their chemotherapeutic efficacy against experimental latency.

1. Introduction

The global impact of tuberculosis (TB) is consistently increasing as recent statistics show that there were 1.4 million deaths due to TB in 2010 alone [1]. It has been a long time since new and potent drugs have been introduced against TB, owing to the huge investment required for research and development as well as long duration of clinical trials. Newer drugs with potent activity against *Mycobacterium tuberculosis* (*M. tuberculosis*) that can be effective against active as well as latent forms of the disease are urgently required. Although a number of promising candidates are in the current pipeline, their ultimate impact on TB control will be clear in future only. The factors which hinder eradication of TB include poor patient compliance, emergence of resistant forms and latent tuberculosis infection (LTBI). An estimated 2 billion individuals are believed to harbor latent TB [2], which often goes undiagnosed and such subjects form a huge reservoir for reactivation of disease later on. Therefore, in addition to our focus on resistant forms of TB, effective strategies are needed to diagnose and treat LTBI as well. Isoniazid has been the mainstay of LTBI treatment as it is known to prevent as well as treat this sub-clinical form of TB [3]. The currently recommended therapy for LTBI includes 9-month daily isoniazid or 4-month daily rifampicin/rifabutin regimen. Alternative regimens of combination of rifampin and pyrazinamide are also possible, but due to the reports of severe liver injury and deaths, Centers for Disease Control and Prevention (CDC) and American Thoracic Society (ATS) recommend that this combination should generally not be offered for the treatment of LTBI [4]. Despite being the drug of choice for LTBI treatment, isoniazid itself is associated with moderate to severe hepatotoxicity which can be fatal at times [5]. Besides, the cost-effectiveness of such long therapeutic schedules to manage LTBI has been rendered doubtful by recent surveys [6] and patient compliance is also reported to be very low (less than 50%) for such regimens [7]. An alternative, cost-effective and novel approach could be the use of known chemical entities against LTBI and one of such promising compounds are the neuroleptic phenothiazines. Phenothiazines have significant in vitro and in vivo activity against various clinical strains of *M. tuberculosis* [8,9]. Phenothiazines are known to have multiple mechanism of action against bacteria, most notable being the inhibitors of calcium-calmodulin binding [10], effects on cell membrane and lipid bilayer [11], inhibiting bacterial efflux pumps [12,13], targeting members of the sigma factor SigB regulon [14], type II NADH-ubiquinone dehydrogenase and the integral membrane succinate dehydrogenase [15], and interfering with DNA

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replication and repair processes [16]. Interestingly, phenothiazine analogs have been shown to specifically inhibit type II NADH dehydrogenase of *M. tuberculosis* [17,18] a key enzyme involved in the metabolic transition of *M. tuberculosis* to latent or dormant state. This information thus raised the speculation that phenothiazines may be effective in preventing the transition of *M. tuberculosis* from actively growing to slow or non-replicating stage during latent disease. Amongst phenothiazines, thioridazine has been the most potent agent against *M. tuberculosis*, being effective against various drug resistant strains [19,20] as well as showing ex-vivo killing efficacy against phagocytosed *M. tuberculosis* [21]. Based on this information, it is hypothesized that thioridazine could be effective against latent TB and we explored its efficacy in a murine model of latent TB. Our studies show that thioridazine is effective against a murine model of latent TB.

2. Materials and methods

Drug-susceptible *M. tuberculosis* $H_{37}R_v$ and *Mycobacterium bovis* BCG were mouse-passaged, stored at −80 °C and then subcultured in Sauton's fluid medium (Himedia, India). Middlebrook 7H11 agar and oleic acid–albumin–dextrose–catalase (OADC) enrichment were obtained from Difco-Becton-Dickinson, USA. Drugs rifampicin, isoniazid and thioridazine hydrochloride were procured from Sigma, USA.

2.1. In vitro model of latent M. tuberculosis

An in vitro model of latent *M. tuberculosis* as described by Wayne et al [22] was used. Briefly, *M. tuberculosis* $H_{37}R_V$ was grown in Sauton's fluid medium to mid-log phase under shaking conditions at 37 °C (optical density 0.4 to 0.6, at 600 nm). Cultures were subsequently diluted (1:100), inoculated in fresh media and transferred to screw-capped 250 ml conical-bottom flasks (headspace ratio = 0.5) having rubber septa. The cultures were incubated with the screw caps tightly closed at 37 °C under shaking conditions for 28 days. Control flasks were kept aerated and cotton-plugged. Methylene blue (1.5 mg/L) was added to aerated and sealed flasks as a visual indicator of oxygen depletion. At different time intervals, sample of cultures were taken out for colony forming unit (CFU) enumeration. The dormancy was confirmed by stable CFU counts over time and Ziehl-Neelsen staining. In addition, niacin assay was done to rule out the presence of mycobacteria other than *M. tuberculosis*.

After the establishment & confirmation of in vitro model of latency (after 28 days), the killing capacity of thioridazine along with isoniazid and rifampicin against dormant bacilli was assessed. Thioridazine, isoniazid and rifampicin at concentrations corresponding to their minimum inhibitory concentrations (MICs) and twice the MICs (MIC × 2) were added to the oxygen-deprived dormant bacilli and observed for growth inhibition for 7 days. The MICs (mg/L) used for isoniazid, rifampicin and thioridazine were 0.1, 0.2 and 10 respectively, based on our previous experiments. The MICs were calculated by broth dilution method. The minimum bactericidal concentration (MBC) of thioridazine was detected as 11 mg/L. After 7 days of incubation, cells were harvested and serial dilutions were plated on Middlebrook 7H11 agar plates supplemented with 10% OADC and incubated at 37 °C for 3–4 weeks for CFU enumeration.

2.2. Murine model of M. tuberculosis infection

All the experimental protocols involving the use of animals were approved by the Institute Animal Ethics Committee. A chronic low dose infection model of murine latency based on the findings of Zhang et al. [23] was used by us, with few modifications. Swiss-albino mice (3–4 weeks old, 20–25 g, either sex) were initially vaccinated by sub-cutaneous administration of 5×10^5 viable bacilli of *M. bovis* BCG. Six weeks later, mice were infected with a low dose of *M. tuberculosis* $H_{37}R_v$ wherein 10^2 viable bacilli were gently placed in the external nares of mice (10 μL in each) by using a fine pipette tip. Mice were euthanized at different time intervals and lung & spleen were isolated aseptically. The tissues were homogenized in phosphate buffered saline and serial dilutions of the homogenates were plated on Middlebrook 7H11 agar plates supplemented with 10% OADC for CFU enumeration to confirm a stable and contained *M. tuberculosis* infection.

2.3. Dose selection for thioridazine

The clinically used doses for thioridazine vary from 50 to 100 mg thrice daily to 800 mg/day, based on the severity of the symptoms [24]. Initially, a 13 mg/kg dose of thioridazine was selected for mice (corresponding to a 100 mg daily dose for a 70 kg human being, based on body surface area conversion), but experiments showed that it was not able to clear the bacilli completely. Thereafter, the subsequent experiments were performed at a dose of 26 mg/kg.

2.4. Chemotherapy

Six weeks post-infection, drug treatments were initiated with the latently infected mice. Two regimens were employed for LTBI treatment: "Regimen A" employing 9 months isoniazid therapy and "Regimen B" employing 4 months rifampicin therapy [4]. Mice were randomized into the following groups:

Regimen A : Group I = Untreated controls, Group II = Isoniazid alone and Group III = Isoniazid + thioridazine

Regimen B : Group I = Untreated controls, Group II = Rifampicin alone and Group III = Rifampicin + thioridazine

The drugs were given orally, daily (7/7) using a gavage. The drug doses used were isoniazid (25 mg/kg), rifampicin (10 mg/kg) [25] and thioridazine (26 mg/kg).

2.5. Assessment of drug efficacy

Animals were euthanized at different time intervals and lungs and spleen were removed aseptically. Whole organ homogenates were prepared in a tissue homogenizer under sterile conditions. Serial dilutions of the homogenates (undiluted, 1:10, 1:100, 1:1000 diluted) were plated on Middlebrook 7H11 agar plates supplemented with 10% OADC and incubated at 37 °C for 3–4 weeks for CFU enumeration. For differential identification of *M. bovis* BCG and *M. tuberculosis* $H_{37}R_v$ colonies, thiophene-2-carboxylic acid hydrazide (TCH) was used.

2.6. Statistical analysis

Group means were compared by performing unpaired *t*-tests and one-way analysis of variance (ANOVA) using SigmaPlot Software Version 11.0.0.77 (Systat, Dundas, Leadtool, GmBH, Germany)

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