New Patentable Use of an Old Neuroleptic Compound Thiadizaine to Combat Tuberculosis: A Gene Regulation Perspective

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Received: March 28, 2011; Accepted: March 30, 2011; Revised: March 31, 2011

Abstract: Use of the old antipsychotic phenothiazine thiadizaine (THZ) for therapy of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) infection is now being seriously considered. It is reported that THZ primarily acts on enzymes involved in fatty acid metabolism and membrane proteins, particularly efflux pumps, as well as oxidoreductases and proteins involved in aerobic respiration that overlap with a number of conventional antituberculous drugs. It targets the products of the Rv3160c-Rv3161c operon, which are required for the detoxification of THZ, members of the sigma factor SigB regulon that play a crucial role in protecting the pathogen against cell envelope damage, and Rv2745c, a transcription factor that regulates ATP-dependent proteolysis. Some of these genes have been shown to be essential for the survival or persistence of Mycobacterium tuberculosis in the infected host. Since THZ targets multiple pathways, including those involved in cell wall processes and respiratory chain components, it may serve as a model for multi-target drug development, as well as constitute a highly potent addition to a combination of anti-tuberculous drug regimens. The discussion of some of the patents relevant to thiadizaine to combat tuberculosis is also included in the present manuscript.

Keywords: Cell envelope stress, drug targets, gene essentiality, Mycobacterium tuberculosis, Thiadizaine, respiratory chain components, sigma factor B.

INTRODUCTION

Tuberculosis (TB) takes a toll of more than 1.8 million deaths every year [1]. It is a major global health problem, particularly in India, China, Russia and South Africa. Of particular concern is the rapid increase in new cases of TB in South Africa, Namibia, Botswana and Zimbabwe [2] due to HIV co-infection [3]. The global emergence of multidrug-resistant (MDR) and now extensively drug-resistant (XDR) strains of Mycobacterium tuberculosis (M. tuberculosis) has made successful therapy more problematic, resulting in increased mortality [4]. The failure of the Bacillus-Calmette Guerin (BCG) vaccine [5] and lack of new effective chemotherapeutics have further complicated the situation.

The World Health Organization (WHO) has recommended a TB control strategy [6] consisting of conventional first-line drugs such as isoniazid (INH), rifampicin (RIF), ethambutol (ETH), and pyrazinamide (PZA), and the second-line drugs, including fluoroquinolones, streptomycin, cycloserine, capreomycin, ethionamide, PAS, thioacetazone, clofazimine and some macrolides [7], coupled to Directly Observed Treatment (DOT) programs that ensure medical adherence. Ongoing research into the development of new and effective drugs has yet to result in successful clinical trials [8, 9]. Furthermore, although many compounds show in vitro activity against M. tuberculosis, relatively few have activity at the site of infection [10]. Since many bacilli are located within pulmonary macrophages, if a drug is to be highly effective it must exhibit good intracellular penetration and concentration within the arrested phagosome where bacilli reside [11]. Thus, the threshold for an effective anti-TB drug is quite high [8].

An old antipsychotic phenothiazine, thiadizaine (THZ; patents US3879552, US4387086, US7544712) [12-14], has been shown to have in vitro [15] and ex vivo [16] activity against MDR and XDR strains of M. tuberculosis [17], to promote the destruction of intracellular XDR strains by non-killing macrophages [18], to cure mice of drug-susceptible [19] and resistant pulmonary TB infections [20] and to inhibit mycobacterial efflux pumps (Patents WO0179257, AU8009101, US2002177559) [18, 21, 22]. Recently, Amaral and colleagues have reported that THZ has been used to cure 10 of 12 XDR-TB patients in Buenos Aires, Argentina and the drug has been used as salvage therapy in XDR-TB patients in India [23].

1574-892X/11 $160.00+0.00 © 2011 Bentham Science Publishers
In this review, we focus on the regulation of key M. tuberculosis genes by THZ [24, 25], which lends support for new patentable uses of THZ for treating drug-resistant TB [23, 26]. Discussion is centered on the identification of possible antimycobacterial drug targets [27] by two different approaches: firstly, response of M. tuberculosis to novel anti-TB compounds (antimycobacterial non-antibiotics, Table 1) [15-20, 23, 28] coupled to computational genomics [24, 25] and secondly, gene essentiality [29-31], meaning study of those genes that are dispensable for M. tuberculosis survival in the host [32-37]. The potential drug targets of THZ compiled in this review may establish THZ as a "real" anti-TB compound that may contribute to the eradication of TB as a public health concern in the near future.

### Table 1. Anti-mycobacterial Non-antibiotics (Helper Compounds).

<table>
<thead>
<tr>
<th>MIC Range (mg/L)</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenothiazine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>10-12.5&lt;sup&gt;a&lt;/sup&gt;, 10&lt;sup&gt;b&lt;/sup&gt;, 0.9&lt;sup&gt;c&lt;/sup&gt;, 4-32&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Bactericidal, inhibits bacterial replication in cultured normal human macrophages, efflux pump inhibitor, effective against M. tuberculosis ex vivo (human macrophages)</td>
</tr>
<tr>
<td>Levomepromazine</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Promazine</td>
<td>&gt; 25&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Thoridazine</td>
<td>6-32&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Efflux pump inhibitor, effective against M. tuberculosis ex vivo (human macrophages), effective against M. tuberculosis in vivo (mice model), multi-target</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>4-32&lt;sup&gt;e&lt;/sup&gt;, &gt; 25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Bactericidal</td>
</tr>
<tr>
<td><strong>Antihistamine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promethazine</td>
<td>20&lt;sup&gt;f&lt;/sup&gt;, &gt; 25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>Methdilazine</td>
<td>5-15&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Bactericidal, synergistic with methyl-DOPA, effective against M. tuberculosis in vivo (mice model) synergistic with INH</td>
</tr>
<tr>
<td><strong>Antidepressant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desipramine</td>
<td>&gt; 25&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Incomplete phenothiazine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Anti-inflammatory</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>10&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Bactericidal, synergistic with streptomycin, effective against M. tuberculosis in vivo (mouse model)</td>
</tr>
<tr>
<td><strong>Antihypertensive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methyl-DOPA</td>
<td>15&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Bacteriostatic, synergistic with methadilazine, effective against M. tuberculosis in vivo (mouse model)</td>
</tr>
</tbody>
</table>

Reference for data: Cross reference reviews 28, 45-48
<sup>a</sup> Colony forming units; <sup>b</sup> incorporation of <sup>3</sup>H into lipids protein and DNA; <sup>c</sup> macrophage; <sup>d</sup> generation of <sup>14</sup>C in Bacitracin-450 system, 12h viable.
Antibiotics

Basic Research

Target Discovery

Lead Discovery

Target Identification

Lead Optimization

Preclinical Evaluation

Clinical Evaluation

1. Pyridones & quinolines
2. Thiolactomycin
3. KRQ 10018
4. PA 20013
5. Ethambutol analogs
6. MJI 98-I-81 & Analogs
7. Rifamidaz analogs

1. LL-385
2. SQ-109 (Pyrolo)
3. PNU 105489
4. AZD-5847
5. Rifampicin
6. Linezolid
7. TMC-207
8. OPC-67683
9. PA-824

Registration & post-clinical

Market

Non-antibiotics

Drug

Methylene Blue
Chlorpromazine
Thioridazine
Thioridazine derivatives

Year
1891
1953
1964
2004-2008

Fig. (1). An outline of global tuberculosis drug development pipeline.

Mechanism of action of drugs in clinical evaluation targeting *M. tuberculosis*: 

- **A** ATP synthase
- **B** Cell wall synthesis
- **C** DNA gyrase
- **D** Multiple targets
- **E** RNA polymerase

Reference for data: 8, 16-18, 24, 25

latter has the ability to enhance the bactericidal activity of macrophages that have ingested the microorganism [47-48].

The phenothiazine THZ is the most promising compound among reported antimycobacterial non-antibiotics (helper compounds; Table 1) [40-43]. In fact, the potential use of THZ for treatment of MDR-TB and XDR-cases is being considered seriously now [23, 26, 49, 50].

ANTI-TB ACTIVITY OF THZ - STRENGTH, WEAKNESS, OPPORTUNITY AND THREAT (SWOT) ANALYSIS

**Strength:** THZ (Patents WO05046694A1, US20070287702A1) [51] has broad-spectrum antibacterial activity (antimicrobial non-antibiotic) [15-20, 23], including inhibitory activity against various *Mycobacterium* sp. (MIC 6-12.5μg/ml) [15, 17, 50] and bactericidal activity (MBC 30μg/ml). THZ appears to be equally active against nutrient-starved, nonreplicating *M. tuberculosis* as it is during the logarithmic growth phase [52]. This is not the case for the frontline anti-TB drugs RIF or INH, which show markedly reduced activity against starved bacilli [53]. Synergistic activity between the phenothiazines and MIC doses of RIF and streptomycin, but not of INH [44], has been reported [46]. *In vitro* derived MIC values for THZ are significantly higher than the corresponding values in macrophages (reported as 0.23-3.6μg/ml and 0.1μg/ml [16, 18]), since the drug concentrates more than 100-fold in these cells [16, 18, 54]. No cytotoxic effects were observed in the macrophages at active concentrations of THZ [16]. Thus, clinically relevant doses of THZ might be highly active against intracellular *M. tuberculosis* without associated systemic toxicity. Finally, novel phenothiazine derivatives have been shown to inhibit nonreplicating *M. tuberculosis* [55, 56] at MIC values below those reported for actively growing bacilli, acting as 'macrophage modulators' (Patent US2003118541) [40-50, 52-54, 57]. THZ significantly reduced the number of colony forming units (CFU) retrieved from the lungs of mice infected with *M. tuberculosis* (10^6 CFU/ml, Ip) following one month of treatment at a daily dose of 0.5mg/day [19]. The drug targets the type II NADH dehydrogenase NDH2 and succinate dehydrogenase and disrupts aerobic respiration [24, 25] under microaerophilic conditions [52]. THZ significantly reduced the bacillary burden in a mouse model of drug-susceptible TB [19] and showed promising activity in a murine model of MDR-TB [20].

**Weakness:** In vivo information is lacking on the lead compound, particularly its bactericidal and sterilizing activities when used in combination with the standard anti-TB regimen, as well as its modulation of host responses.

**Opportunity:** Progress might be accelerated by conducting clinical trials with THZ on a global scale for the treatment of XDR-TB.

**Threat:** In some patients THZ is known to induce prolongation of the QTc interval [58], especially in those with mutations of the p450 cytochrome (caution note by Novartis), which has been associated very rarely with torsade
New Patentable Use of an Old Neuroleptic Compound Thiadizine
depointes or sudden death [58]. Therefore, patients should be monitored by electrocardiogram prior to and during treatment with THZ [39]. The risk of significant arrhythmia may be minimized by initiating treatment with relatively small doses of THZ (less than 50mg/day), which may be gradually escalated [60; personal communication]. Lastly, 12 clinically unresponsive XDR-TB patients were treated with THZ at 5mg/day plus three antibiotics to which the patients had not responded, and 10 were cured of their pulmonary TB within a few months [61]. Given the high mortality associated with XDR-TB, incremental dosing of THZ with cardiac monitoring appears warranted in this setting.

USE OF COMPUTATIONAL GENOMICS TO DETERMINE THE ACTIVITY OF THZ ON KEY GENES OF M. TUBERCULOSIS

Available data suggest that THZ modulates the expression of genes encoding membrane proteins, efflux pumps (emrE-encoded), oxidoreductases (nad encoded) and enzyme systems that are involved in fatty acid metabolism and aerobic respiration (Table 2). The Rv3165c-Rv3161c operon, a multi-drug transporter, the Rv3614c-Rv3615c-Rv3616c regulon and Rv2745c, a transcription factor that regulates ATP-dependent proteolysis, are highly induced in response to THZ. The alternative sigma factor σ^b confers baseline resistance to THZ, which once again underlines its importance in the physiology of the mycobacterial cell surface stress response (Table 2, Fig. 2). Based on global transcriptional analysis, the mode of action of THZ, unlike that of INH, appears to involve both cell and cell wall processes as well as respiratory chain components.

CELL WALL AND CELL PROCESSES

Sigma (σ) factors control gene expression in response to changes in an extracellular environment by influencing the promoter specificity of RNA polymerase [62]. Most subtypes have a key σ factor that modulates transcription of housekeeping genes and alternative σ factors that control responses to environmental stimuli, adaptation and virulence. Pathogenesis of TB is known to have multiple phases involving several adaptive bacterial virulence factors [63]. M. tuberculosis survives through these phases by the sequential expression of specific regulons controlled by one or more alternative σ factors [64]. The M. tuberculosis sigma factors σ^b, σ^r, and σ^E are well characterized. The expression of σ^b can be triggered by either σ^r or σ^E. σ^b is induced by heat, redox, nitrosative and acid stress [65-67] and phagocytosis [68,69]. σ^r regulates the transcription of 31 genes, including the σ^b, σ^r, groEL/ES and thioridoxin regulons [65]. σ^r is also induced upon uptake by macrophages [69], and upon treatment with hydrogen peroxide [70] and SDS [71]. σ^E regulates the expression of at least 23 genes, including σ^b, hsp, and hspX. σ^E is transcribed in either a σ^b [67,68] or MrpAB-dependent manner [72]. Thus, there exists a network of these three factors: σ^b, σ^r, and σ^E, with overlapping functions. The fact that the expression of σ^b is controlled by several different regulatory pathways suggests that this σ factor plays a crucial role in M. tuberculosis stress responses. Exposure of M. tuberculosis to THZ leads to upregulation of σ^b and 89 of 100 genes coexpressed with σ^b (coexpression coefficient 0.78601 to 0.40569; p < 0.0001). Interestingly, the top five of these 100 genes as ranked by coexpression coefficient, Rv2745c, Rv2744c, hsp, ideR, and Rv2694c, have been observed to be coexpressed with σ^b when the alternate M. tuberculosis sigma factors σ^b or σ^E were induced [65,67]. Expression of Rv2745c and its adjoining genes (Rv2744c and Rv2743c) was significantly induced at almost all time points, as was that of ideR (Rv2711), the iron-dependent repressor of siderophore and mycobactin biosynthesis, and hsp, which codes for a heat shock protein. Rv2745c is induced in a σ^b- and σ^E-dependent manner following disulphide or membrane damage stress [67,73].

M. tuberculosis σ^b expression is induced under various stress conditions, including exposure to sodium dodecyl sulfate (SDS) [74], and the σ^E regulon is required for the full response of M. tuberculosis to cell envelope stress [73]. Among 73 σ^E-regulated genes whose expression is induced by SDS, the expression of 57 of these genes is also induced by THZ treatment. Furthermore, 32 of 37 M. tuberculosis σ^b regulon genes induced by exposure to vancomycin [75], a glycopeptide antibiotic with cell wall activity, were also induced by THZ treatment [25]. These data support the hypothesis that the mechanism of action of THZ includes mycobacterial membrane damage [25].

Chlorpromazine, another phenothiazine non-antibiotic, has direct cell envelope damaging activity against Gram-positive and Gram-negative bacteria similar to that of betalactams [43] and σ^r-mediated stress responses are induced in S. aureus by diclofenac [76], another phenothiazine-like non-antibiotic [39,41,45]. It is reported that THZ affects transcription of genes involved in cell wall biosynthesis in methicillin-resistant S. aureus [77].

Corroborating the hypothesis that THZ damages the membrane of tubercle bacilli, recent studies showed that THZ modulates cell-envelope integrity using transmission electron microscopy [25]. Moreover, M. tuberculosis mutants deficient in the sigma factors σ^b or σ^E, each of which regulates σ^b expression, exhibited higher sensitivity to THZ, suggesting that σ^b is required for M. tuberculosis resistance to THZ. Finally, conditional overexpression of σ^b increased the survival of M. tuberculosis in the presence of THZ.

Rv2743c, encoding a conserved transmembrane protein, was found to be induced together with the upstream genes Rv2744c and Rv2745c following THZ exposure. This putative operon, whose transcription is under the control of σ^E [71-78], also was found to be upregulated in M. tuberculosis upon treatment with SDS. Rv2745c is predicted to be a transcriptional regulatory protein [67], whereas Rv2744c is highly homologous to the E. coli PspA (phage-shock protein A) [79]. In Escherichia coli, PspA is part of a cytoplasmic membrane protection system involved in suppressing proton leakage from damaged membranes [80]. In the presence of a specific surface stress signal (a decrease of the proton-motive force due to cytoplasmic membrane permeabilization), a transmembrane protein activates PspA, which releases the transcriptional factor PspF and associates with the internal surface of the cytoplasmic membrane, causing its stabilization [81].
Table 2. Activity of Thioridazine \(^{a,b}\) on Key Genes of M. tuberculosis and Essentiality. \(^{c,d}\)

<table>
<thead>
<tr>
<th>Nr number</th>
<th>Gene (^{a})</th>
<th>Description</th>
<th>Functional Category</th>
<th>TruSH (^{e})</th>
<th>DeADMA (^{d})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>In vitro (^{f})</td>
<td>Mouse (^{g})</td>
</tr>
<tr>
<td>Rv0170</td>
<td>mexD</td>
<td>MCE-family protein MCE1B</td>
<td>0</td>
<td>NR</td>
<td>R</td>
</tr>
<tr>
<td>Rv0251</td>
<td>hup</td>
<td>Hypothetical exported protein</td>
<td>3</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Rv1040</td>
<td>Rv1040c</td>
<td>PE family protein</td>
<td>6</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Rv1057</td>
<td></td>
<td>Conserved hypothetical</td>
<td>10</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Rv1403</td>
<td>Rv1403c</td>
<td>Putative methyltransferase</td>
<td>7</td>
<td>NR</td>
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<tr>
<td>Rv1404</td>
<td></td>
<td>Probable transcriptional regulatory protein</td>
<td>9</td>
<td>NR</td>
<td>NR</td>
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<tr>
<td>Rv1461</td>
<td></td>
<td>Conserved hypothetical protein</td>
<td>10</td>
<td>R</td>
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<td>Rv1463</td>
<td></td>
<td>Probable conserved ATP-binding protein</td>
<td>3</td>
<td>R</td>
<td>NR</td>
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<tr>
<td>Rv1466</td>
<td></td>
<td>Conserved hypothetical protein</td>
<td>10</td>
<td>R</td>
<td>-</td>
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<tr>
<td>Rv1497</td>
<td>diP/L</td>
<td>Probable Diaphanous LPL</td>
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<tr>
<td>Rv1854</td>
<td>ndh</td>
<td>Probable NADH dehydrogenase NDH</td>
<td>7</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Rv2053</td>
<td>Rv2053c</td>
<td>Probable membrane protein</td>
<td>3</td>
<td>NR</td>
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<tr>
<td>Rv2450</td>
<td>rpy/E</td>
<td>Probable respiratory-promoting factor RPF</td>
<td>3</td>
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<tr>
<td>Rv2483</td>
<td>rpsC</td>
<td>Probable membrane phospholipid</td>
<td>1</td>
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<td>R</td>
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<tr>
<td>Rv2759</td>
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<td>Sigma Factor B</td>
<td>2</td>
<td>R</td>
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<tr>
<td>Rv2771</td>
<td>idoR</td>
<td>Iron-dependent repressor and activator idr</td>
<td>9</td>
<td>-</td>
<td>NR</td>
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<tr>
<td>Rv2773</td>
<td>Rv2773c</td>
<td>Transmembrane alanine rich protein</td>
<td>3</td>
<td>NR</td>
<td>NR</td>
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<tr>
<td>Rv2744</td>
<td>35kDa/3g</td>
<td>Conserved 35 KDa alanine rich protein</td>
<td>10</td>
<td>NR</td>
<td>NR</td>
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<td>Rv2745</td>
<td>Rv2745c</td>
<td>Probable transcriptional regulatory protein</td>
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<td>NR</td>
<td>NR</td>
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<tr>
<td>Rv2930</td>
<td>fadA29</td>
<td>Probable FATTY-ACID-CoA ligase FADD29</td>
<td>1</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Rv3065</td>
<td>mrrE</td>
<td>Methyltransferase membrane protein MMR</td>
<td>3</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Rv3160</td>
<td>Rv3160c</td>
<td>Transcriptional regulatory protein</td>
<td>9</td>
<td>NR</td>
<td>-</td>
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<td>Rv3163</td>
<td>Rv3163c</td>
<td>Possible dimethylase</td>
<td>7</td>
<td>NR</td>
<td>NR</td>
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<td>Rv3162</td>
<td>Rv3162c</td>
<td>Possible integral membrane protein</td>
<td>3</td>
<td>-</td>
<td>NR</td>
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<tr>
<td>Rv3920</td>
<td>Rv3920c</td>
<td>Hypothetical protein similar to JAG protein</td>
<td>10</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

Reference for data: \(^{1,24,35,29,32,33,29,30,34,37}\)

R, required; NR, not required; \(^{a}\) no data available

DeADMA, Designer Arrays for Defined Mutant Analysis; TruSH, Transposon Site Hybridization

Although Rv2745c and Rv2743c do not share amino acid sequence similarity with the transcriptional regulator and the transmembrane protein, respectively, involved in the E. coli PspA system, it is tempting to speculate that they represent their functional analogues.

Phenothiazines primarily block the respiratory chain by inhibiting Ndh activity [54, 56, 82, 83]. A major role of the Rv2745c encoded protein may be to reinitiate energy metabolism and intracellular redox potential following stress. Evidence suggests that cell wall damage, induction of the Rv2745c gene as part of a \(\sigma^3/\sigma^5/\sigma^6\) transcriptional network, and energy metabolism, are closely linked [67, 84, 85].

Expression of Rv2745c is induced upon treatment of M. tuberculosis with vancomycin [75] and THZ, which may represent a transcriptional response to cell envelope damage analogous to SDS exposure. \(\sigma^3/\sigma^5/\sigma^6\)-mediated induction of Rv2745c following M. tuberculosis membrane damage appears to be specific to certain conditions or agents as this pathway is not activated by other envelope-damaging agents.
such as ethanol, INH, or ethambutol. Stress caused by damage to the *M. tuberculosis* cell wall may decrease the proton-motive force [75] and *Rv2745c* may help to stabilize the cell wall in response to this damage. Genes induced by *Rv2745c* encode cytochrome oxidase, mycothiol glycosyltransferase, ferridoxin C, an allosteric modulator of glucose-6-phosphate dehydrogenase, ribulose-phosphate epimerase, malate dehydrogenase, and various other dehydrogenases. These findings indicate that the cellular redox potential and energy stores are depleted following stress conditions, possibly due to membrane damage. Gene expression reprogramming by *Rv2745c* may represent an attempt by *M. tuberculosis* to restore this redox potential. This would explain the induction of *Rv2745c* in response to THZ, a drug that disrupts bacterial redox potential by inactivating NADH dehydrogenase [24, 56, 82, 83]. In addition, the expression of monoaquino synthase genes (*menA*, *menB*) is significantly repressed following *Rv2745c* overexpression. Menaquinone synthesis has been implicated in electron transport during nonreplicative persistence, although the significance of its repression by this regulator is not understood. Of particular interest had been the induction of *osaA*, which codes for trehalose phosphate synthase [84]. This gene is a member of the dominant biosynthetic pathway for trehalose, a disaccharide constituent of cell wall glycolipids. Trehalose plays an important role in cell wall biogenesis, and also acts as a defense mechanism for surface and oxidative stress. Due to its absence from mammalian cells and the central importance of the *M. tuberculosis* cell wall to the pathogen's virulence and survival in the infected host, trehalose biosynthesis and associated regulatory pathways appear to be promising targets of antitubercular drug development [84, 85]. Further, THZ regulates genes such as *Rv3200c*, *cipH*, *Rv3253c*, *mgfE*, *cpE*, *cfrP*, *mmpL6*, *narK2*, *Rv1037c*, *Rv1187*, *Rv1198*, *Rv1686c*, *Rv1793*, *Rv2346c*, *Rv3874*, *Rv1212c*, *Rv1218c*, *Rv1250*, *Rv1463*, *Rv2038c*, *Rv2326c*, *Rv3197*, *dppB*.

![Diagram of metabolic pathways](image)

Fig. (2). List of important ""*" possible targets of THZ

Importance based on: *; ATP-Binding Protein ABC transporter; *; Cation transport; *; Common with anti-TB drugs; *; Efflux; *; Hsp; *; NADH; *; Sigma factor

Reference for data: 24, 25
Increased activity of mycobacterial efflux pumps can prevent antibiotics from reaching their intended target, leading to a drug-resistant phenotype [90-93]. THZ has been shown to have efflux pump-inhibiting activity against mycobacteria both in vitro and ex vivo [18, 90, 94]. In particular, it appears to be an effective inhibitor of the intrinsic efflux pump system, which is thought to be responsible for intrinsic resistance to erythromycin [95].

The multidrug transport membrane protein Mmr, which is responsible for export of toxic compounds from the cell, confers resistance to multiple drugs, including tetraphenylphosphonium, erythromycin, ethidium bromide, acriflavine, safrinan O, and pyronin Y. *Mycobacterium tuberculosis* exposure to THZ results in significantly increased expression of the Rv3065-encoded EmrE multidrug transport membrane protein. Following one hour of THZ treatment (at both 1x and 4x MIC concentrations), the expression of only the emrE gene is induced, while 4 and 6 hours of drug exposure, the entire genomic loci containing this gene, Rv3064c to Rv3064c and Rv3065 to Rv3067 is induced. This region includes a putative DooR family transcription factor encoded by the Rv3065 gene and a gene belonging to the carbon starvation induced protein family, encoded by the Rv3063 gene. Another important gene kgfP, which encodes a major facilitator superfamility protein and paralogs of which are involved in nitrate uptake and nitrite efflux, was induced following 1 hour of THZ treatment [25]. Interestingly, chemical induction of the Rv2745c gene leads to increased expression of emrE [84].

REGULATION OF RESPIRATORY CHAIN COMPONENTS AND THE MODE OF ACTION OF PHENOTHIAZINES

It has been conjectured that THZ affects respiration directly [24, 82, 83]. The drug blocks NADH-dependent O₂ consumption by the *M. tuberculosis* membrane [24, 82, 83], thereby affecting respiratory and other intermediary metabolic activities of the cell. Among several gene clusters regulated by phenothiazines is one containing known components of the respiratory chain (GC149), including the alternative terminal oxidase encoded by the cydA and cydB genes. The phenothiazines have been shown to be potent inhibitors of the type II NADH-ubiquinone dehydrogenase as well as the integral membrane succinate dehydrogenase [82]. Inhibition of these enzymes leads to increased expression of relA, perhaps due to the expected decrease in charged tRNAs as a result of ATP depletion. THZ appears to induce the expression of ndh [24, 25], and that of other genes involved in energy metabolism, e.g. NADH dehydrogenases (nuoE, nuoF and nuoG), isocitrate dehydrogenase (icdI), NAD-dependent malate dehydrogenase (mez) and NADPH:quinone oxidoreductase (Rv0149). The decreased *M. tuberculosis* intracellular redox potential resulting from phenothiazine treatment, as evidenced by reduction of the intracellular NADH/NAD⁺ and menaquinol: menaquinone ratios, appears to be associated with induction of e⁺ expression [25].

**GENE ESSENTIALITY FOR THE SURVIVAL OF TUBERCLE BACILLI IN HOST**

High-throughput mutagenesis and genome-wide transcription analyses have been used to identify novel *M. tuberculosis* drug targets [31].

One such high-throughput technique that has been developed for subtractive identification of attenuated transposon (Tn) mutants based on microarray analysis is Transposon Site Hybridization (TraSH) [32, 33]. This technique has been used successfully to identify *M. tuberculosis* genes required for bacillary growth and survival in mouse spleens, and naïve and interferon γ-activated murine macrophages. In its original form, TraSH [32, 33] relied upon large undefined mutant pools, but the subtractive approach has produced related applications and refinements including collections of archived mutants that are deliberately pooled and then assessed simultaneously under a specific stress condition.

Another high-throughput approach is Designer Arrays for Defined Mutant Analysis (DeADMan), which is based on subtractive identification of archived, genotypically-defined *M. tuberculosis* Himar1 Tn mutants and has been used to identify *M. tuberculosis* genes required for growth and survival in mice [34], guinea pigs [35], and non-human primates (NHP) [37]. NHP experimentally infected with *M. tuberculosis* closely model the entire spectrum of human TB infection, ranging from latent TB infection to the various pathological features of active TB disease. Use of the NHP as a faithful model of human TB infection was pioneered by Walsh et al. [96] and Flynn et al. [97, 98]. *Mycobacterium tuberculosis* infection of NHP via the aerosol route has been used to further refine the model and its physiological relevance [37]. One of the advantages of DeADMan is that it allows simultaneous analysis of pools containing less than 100 defined mutants, thereby permitting lower inoculation titers, which is an important consideration when using the aerosol route for infection.

Knowledge of the mechanism by which a drug acts is critical for proper selection of drugs that inhibit a wide range of essential biochemical pathways and for minimizing the risk of selecting drug-resistant mutants.

Dutta et al. [25] studied the transcriptional profile of *M. tuberculosis* in response to different concentrations of THZ and exposure to altered growth conditions using whole-genome microarrays. Statistical interpretation of these data and grouping based on functional category yielded key genes responsive to THZ exposure Table 3, and revealed a distinct cluster of genes related to THZ activity on cell wall processes, which is distinct from its known activity on the *M. tuberculosis* respiratory chain during aerobic growth. The data from THZ-regulated genes were compared to historical data obtained from TraSH (in murine macrophages and mice) [32, 33] as well as DeADMan (in mice, guinea pigs, and NHP) [28-31] (Table 3). Although much information on essentiality of THZ-regulated genes is still lacking, this proof-of-principle shows the capacity of this system to provide important information on the likely mechanism of action of a drug. *Mycobacterium tuberculosis* is known to
adapt to a number of different microenvironmental conditions in the human host, regulating its growth and virulence based on local and systemic immune responses. Mycobacterium tuberculosis survival/persistence genes and proteins described herein may be used to screen candidate pharmacological agents/drugs and to identify those that can be used to treat or prevent mycobacterial infections. Agents that inhibit activity of these proteins potentially may be useful for inhibiting the growth and infectivity of mycobacteria in vivo. A recent report [27] reveals that 46 genes have been patented as anti-TB drug targets Table 3, and the number of patentable drugs may be in the hundreds. Of three genes reported to be differentially regulated by THZ, one (Rv2694) was found to be required for survival of tubercle bacilli in NHP lung by DeADMAn. In addition, the THZ-induced genes mceD and Rv2843 were found to be required for M. tuberculosis adaptation and survival in macrophages [33] and in mouse tissues, [32] suggesting promising new targets for THZ.

CURRENT & FUTURE DEVELOPMENTS

The recent emergence of MDR/XDR-TB has necessitated the discovery of novel and safe compounds with activity against M. tuberculosis, as well as the repurposing of older drugs that are already available on the market and economically accessible even in resource-limited areas of the world in which drug resistance is most prevalent. Despite the fact that THZ has some undesirable side-effects, its potential utility as an anti-mycobacterial drug cannot be ignored. THZ shows promising activity against clinical strains of M. tuberculosis, regardless of their antibiotic susceptibility. It has been used successfully to cure XDR-TB patients and to reduce transmission rates associated with inadequate treatment. THZ is relatively inexpensive (a few hundred dollars/kilogram) and beyond patent protection. Therefore, we believe that THZ should be considered as an alternative agent for the therapy of drug-resistant TB cases.

Pharmacogenomics employs the availability of full genome sequences, computer-aided analysis and high-throughput screening to identify probable anti-TB drug targets. Microarray analysis, along with biochemical findings and electron microscopy, has revealed THZ to have a mixed effect on pathways involved in both cell wall and cell processes and respiratory chain components. Further work may differentiate the effects of THZ in regard to survival of mutants via in vivo or in vitro models and to demonstrate genetic "essentiality" for a particular target. Studies aimed at understanding the reversal (patentsWO005046694, US20070287702) [51, 52], or reduction of antibiotic resistance systems of the bacterium are expected to be fruitful. For this purpose, recombinant M. tuberculosis strains deficient in or overexpressing specific efflux pumps may be used to investigate the efficacy of THZ as a putative efflux inhibitor. With respect to studying the effect of THZ on the M. tuberculosis membrane, recombinant strains deficient in or overexpressing porins could be used. Such studies could validate THZ as an anti-TB drug with novel mechanisms of action, which may be used to combat MDR- and XDR-TB.

ACKNOWLEDGEMENTS

We gratefully acknowledge the contribution of the researchers for their published data, Gene Expression Omnibus database and patents available on the PubMed, Medline, SciFinder, Cochrane library, Delphilon integrated view and Espacenet database that helped to make this compilation without any language restriction.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
REFERENCES


New Patentable Use of an Old Neutrophic Compound Thoridiazene


