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Thioridazine alters the cell envelope permeability of *Mycobacterium tuberculosis*

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Abstract

The increasing occurrence of multidrug resistant tuberculosis exerts a major burden on treatment of this infectious disease. Thioridazine, previously used as a neuroleptic, is active against extensively drug resistant tuberculosis when added to other second and third line antibiotics. By quantitatively studying the proteome of thioridazine treated *Mycobacterium tuberculosis*, we discovered the differential abundance of several proteins that are involved in the maintenance of the cell envelope permeability barrier. By assessing the accumulation of fluorescent dyes in mycobacterial cells over time, we demonstrate that long term drug exposure of *M. tuberculosis* indeed increased the cell envelope permeability. The results of the current study demonstrate that thioridzone induced an increase in cell envelope permeability, and thereby the enhanced uptake of compounds. These results serve as a novel explanation to the previously reported synergistic effects between thioridazine and other anti-tuberculosis drugs. This new insight in the working mechanism of this anti-tuberculosis compound could open novel perspectives of future drug administration regimens in combinational therapy.
Introduction

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is one of the most successful pathogens worldwide. Currently, 8-9 million TB cases and close to 1.5 million deaths due to *M. tuberculosis* are recorded annually.\(^1\)

A major obstacle in the control of TB is the emergence of multidrug resistant TB (MDR-TB), i.e. *M. tuberculosis* strains that are resistant to at least rifampicin and isoniazid, two of the most potent drugs used in anti-TB regimens. In 2013, 3.7% of all new TB cases were reported as MDR-TB, but this is most likely an under estimation.\(^2\) Alarmingly, the reported levels of MDR-TB are around 20% for patients previously treated for TB.\(^2\) Moreover, there is a progression of MDR-TB towards extensively drug resistant TB (XDR-TB), which, in addition to resistance for isoniazid and rifampicin, also involves resistance to any of the fluoroquinolones and at least one aminoglycoside.\(^3\) Finally, the term totally drug resistant TB (TDR-TB) has been introduced to describe resistance against all known TB drugs available at a particular setting. The existence of MDR-TB, XDR-TB and TDR-TB underlines that there is an urgent need for new effective anti-TB compounds.

Thioridazine (THZ), a compound belonging to the class of phenothiazines, is a drug that has previously been used to treat psychotic disorders such as schizophrenia.\(^4\) Over fifteen years ago the *in vitro* effect of THZ on the respiration of *M. tuberculosis* has been reported.\(^5\) The current increase in the frequency of MDR-TB infections and increased level of resistance against anti-TB drugs has renewed the interest in phenothiazines and their anti-mycobacterial properties.\(^6(\text{6})\) In mouse models, a clear positive effect on the killing of *M. tuberculosis* was noticed when THZ was used alone\(^7\) or added to other drugs in treatment regimen.\(^8\) Despite the promising reports of anti-TB treatment with THZ, it should be noted that the anti-mycobacterial effects of THZ, also in combination with other antibiotics, vary between published animal model based studies.\(^9(\text{9})\),\(^10(\text{10})\),\(^11(\text{11})\),\(^12(\text{12})\) Nevertheless, in a clinical setting, THZ has been used for therapy of 17 XDR-TB patients in Buenos Aires, Argentina with remarkable success.\(^13\) As shown by this latter study and others, THZ does not produce significant negative side effects when patients are monitored for possible cardiac effects during treatment.\(^14\)

Three mechanisms have been proposed to explain the mode of action of phenothiazines: 1) accumulation of phenothiazines in macrophages and lung homogenate to concentrations that are equal to those which are bactericidal *in vitro* together with the promotion of intracellular killing by macrophages,\(^15(\text{15})\),\(^16(\text{16})\),\(^17(\text{17})\),\(^10(\text{10})\) 2) potential inhibition of antibiotic extruding efflux pumps that might be over-expressed in MDR-TB infections\(^18(\text{18})\),\(^19(\text{19})\),\(^20(\text{20})\),\(^21(\text{21})\) and which can be experimentally upregulated by exposure to increasing concentrations of anti-TB drugs such as isoniazid\(^20\),\(^22\)
and 3) direct in vitro killing of \textit{M. tuberculosis}.\cite{23,24}

Efflux pumps that possess the ability to transport antibiotics across the cell envelope are thought to play a major role in the development of drug tolerance next to genomic mutations.\cite{25} Moreover, an increased abundance of efflux pumps can stimulate the accumulation of resistance mutations, by enhancing drug tolerance for prolonged time periods.\cite{26} The genome of \textit{M. tuberculosis} encodes for several efflux pumps that may contribute to the development of drug resistance\cite{27} and these have been suggested to be inhibited by phenothiazines.\cite{20} Thus, the use of an efflux pump inhibitor in combination with other antibiotics can prevent the development of efflux pump induced antibiotic resistance and even result in an enhanced activity of other antibiotics. Indeed, synergistic effects between THZ and the first-line antibiotics rifampicin and isoniazid have been reported, as well as synergism between THZ and the anti-mycobacterial drug streptomycin.\cite{10,20,28}

Notably, rifampicin and isoniazid best target replicating, metabolically active cells.\cite{29,31,32,33,34} To the contrary, THZ also displays bactericidal activity against starved cells.\cite{29} Furthermore, THZ monotherapy could cure both drug susceptible and MDR-TB in a mouse model and \textit{in vitro}.\cite{8,24} Both observations demonstrate that THZ is not only able to inhibit efflux pumps, but also show that THZ can be bactericidal.

To get an understanding of the molecular mechanisms used by \textit{M. tuberculosis} to manage THZ induced cell stress, we quantitatively studied the proteome of \textit{M. tuberculosis} \textit{in vitro} with and without the continuous presence of THZ. We report the differential abundance of several proteins and protein clusters upon THZ treatment, which are involved in the maintenance of the cells permeability barrier. In addition, we demonstrate that long-term treatment of \textit{M. tuberculosis} with THZ alters the mycobacterial plasma membrane composition and increases the cell envelope permeability, which influences the uptake of anti-mycobacterial compounds. In addition to the suggested function of THZ as efflux pump inhibitor, we herewith present data that offers a novel explanation to the previously reported synergistic effects between THZ and other anti-tuberculosis drugs.
Material and Methods

Mycobacterial culture conditions

*M. tuberculosis* H37Rv was re-cultured from frozen stocks in 5 ml Tween-Albumin liquid culture broth (Tritium Microbiologie, the Netherlands) at 36°C without shaking until an O.D. at 600 nm of 0.4 AU was reached. Of the mycobacterial pre-culture, 1 ml was transferred to a 250 ml Erlenmeyer flask containing 100 ml Tween-Albumin broth, with 0, 4, 6 and 8 mg/l THZ, and incubated under shaking conditions at 36°C with constant aeration; see Figure S1a. A >100-fold dilution of the pre-culture to prevent the inclusion of death cells for proteomic analysis. The mycobacteria used for proteome analyses were continuously treated with 6 mg/l THZ. Once these cultures reached an O.D. at 600 nm of 0.6 AU, representing the mid-log phase, the cells were washed three times with ice cold PBS, dissolved in 5 ml Lysis-buffer (4% SDS, 100 mM Tris-HCl, pH 7.6) and heat-killed at 95°C for 10 min. Lysates were stored at -20°C until further usage.

Thermostability of thioridazine

The thermostability of THZ, over the culture period of approximately two weeks, was assessed by pre-incubating culture flasks containing 6 mg/l THZ for 21 days at 37°C prior to inoculation with *M. tuberculosis*. We compared the growth curves of this pre-incubated culture broth with that of freshly prepared culture broth containing 6 mg/l THZ; see Figure S1b. The resemblance between the obtained growth curves indicates that the growth rate of *M. tuberculosis* is similar in media containing THZ that is freshly prepared to media containing THZ that was pre-incubated at 37°C for 21 days. This demonstrates that THZ is thermostable on the time-scale of our experiments, and maintained the majority of its activity over the examined culture period.

Proteome analysis

Cells were processed as described previously. In brief, heat inactivated cells for proteomic experiments were mechanically lysed by bead-beating in a mini bead-beater 16 (BioSpec, USA) for 5 min using glass beads. Thereafter, the cells were cooled down on ice for 5 min after which the procedure was repeated twice. The cell lysates were cleared from cell debris by centrifugation for 1 min at 14,000 g and the supernatant was transferred to a fresh tube. Proteins were digested using the filter aided sample preparation (FASP) method. In brief, 100 μg of DTT reduced proteins was loaded on a 30 kDa filter. SDS was removed in three washes with 8 M urea. The proteins were carbamidomethylated, and the excess reagent was removed by three additional washes with 8 M urea. Proteins were then digested overnight using endoproteinase Lys-C (endoLysC), followed by a four hr digestion using trypsin at RT. The tryptic protein digest was desalted on C18 SepPak columns and labelled by reductive amination. A label swap was performed between biological replicates to prevent any experimental bias. A total 100 μg of labeled peptides were fractionated by strong cation exchange (SCX) on an Agilent 1100 system.
equipped with an in-house packed SCX-column (320 μm ID, 15 cm, polysulfoethyl A 3 μm, Poly LC), run at 4 μl/min. The gradient started with a 10 min run at 100% solvent A 70/30/0.1 (water/acetonitrile/formic acid), after which a linear gradient reached 100% solvent B (250 mM KCl, 30% acetonitrile. 0.1% formic acid) in 15 min, followed by 100% solvent C (500 mM KCl, 30% acetonitrile 0.1% formic acid) in the following 15 min. The gradient was held at 100% solvent C for 5 min, then switched back to 100% solvent A. Fifteen fractions were collected in 1 min intervals, lyophilized and reconstituted in 30 μl 95/3/0.1 (water/acetonitrile/formic acid). Dissolved fractions were analyzed by on-line nano-HPLC MS with a system consisting of an Agilent 1100 gradient HPLC system (Agilent, Waldbronn, Germany) as described previously,\textsuperscript{(35)} coupled to a LTQ-FT Ultra mass spectrometer (Thermo, Bremen, Germany). Five μl of each fraction was injected onto a home-made pre-column (100 μm×15 mm; Reprosil-Pur C18-AQ 3 μm, Dr Maisch, Ammerbuch, Germany) and eluted via a home-made analytical nano-HPLC column (50 μm×15 cm; Reprosil-Pur C18-AQ 3 μm). The gradient was run from 0 to 30% solvent B (10/90/0.1 water/acetonitrile/formic acid) in 10-155 min. A tip of approximately 5 μm was drawn at the tip of the nano-HPLC column to act as electrospray needle. Full scan mass spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of $5\times10^6$. The five most intense ions were selected and fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. Peptide and protein identification and quantitation was accomplished using MaxQuant 1.4.0.3, as described previously.\textsuperscript{(35)} In brief, the false discovery rate (FDR) was set to 0.01. Minimal peptide length was set to 6 amino acids. The first search was performed using 20 ppm, while the main search was conducted with 10 ppm. Search of MS/MS spectra was performed with 0.6 Da using the Andromeda search engine. Both the first search and the main search were carried out against a database of \textit{M. tuberculosis} H37Rv (3,996 entries). In total, 262 common contaminants were included in the searches by Andromeda. Enzyme specificity was set as C-terminal to arginine and lysine without proline restriction. A maximum of two missed cleavages was allowed. Variable modifications included N-terminal protein acetylation, methionine oxidation and corresponding dimethyl labels. Carbamidomethylation of cysteine was selected as a fixed modification. Proteins considered for quantification required a cumulative peptide count of two, including both unique and razor peptides. A maximum heavy/light variability of 150% was allowed. Proteins identified by site, which matched against the reverse database or were identified as a contamination, were excluded for further analysis. Statistical analysis of the outcomes was performed by Perseus using the significance B test with a Benjamini-Hochberg FDR 5%. The raw data files and the MaxQuant output files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD001208.

**Cell envelope permeability assay**

The accumulation of Sytox Orange (Life Technologies, USA), Nile Red (Sigma-Aldrich, the
Netherlands) and ethidium bromide (Sigma-Aldrich, the Netherlands) was determined essentially as described previously. In brief, *M. tuberculosis* H37Rv was grown to an O.D. 600 nm of 0.6 AU, washed three times with PBS, and resuspended in Tween-Albumine culture broth (Tritium Microbiologie, the Netherlands) to an O.D. 600 nm of 0.3 AU. Of this cell suspension 500 μl was inserted into a sealed cuvette. Sytox Orange and ethidium bromide were added to final concentration of 100 nM and 6 μM respectively. In addition, the efflux pump inhibitor reserpine (Sigma-Aldrich, the Netherlands) was added to a final concentration of 100 µg/ml in selected samples. The cell suspensions were incubated at 36°C with occasional mixing for 60 min. The accumulation of dyes was determined by fluorescence using a Glomax-Multi Jr Single tube MultiMode Reader (Promega, USA) with an excitation of 525 nm and emission of 580-640 nm. The outcomes of the assay were evaluated using a paired t-test.

**Analysis of phospholipid derived fatty acids**

*M. tuberculosis* H37Rv was cultured as described above, while constantly being exposed to 6 mg/l THZ. Preparation and analysis of fatty acid derived methyl ester (FAME) according to the manufacturers instruction (Sherlock 6.1 Microbial Identification System, MIDI, USA). In brief, cells were washed with ice-cold PBS and resuspended in 1 ml of 15% (w/v) NaOH in 50% (v/v) aqueous methanol. This suspension was then incubated for 30 min in a water bath set at 100°C. The saponified samples were allowed to cool to RT in cold water, acidified and methylated by the addition of 2 ml reagents consisting out of 54% 6 M HCl and 46% aqueous methanol, followed by a 10 min incubation in a water bath set at 80 °C. The samples were cooled by handshaking the sample tubes in ice-cold water. The methylated fatty acids were extracted with 1.25 ml of 50% methyl-tert butyl ether in hexane. The suspensions were allowed to mix for 10 min using end-over-end rotation. The upper phase was washed with 3 ml of 0.3 M NaOH plus 2.2 M NaCl after which the FAMEs were transferred into a gas chromatography sample vial for analysis. Separation of FAMEs was performed using a HP6890 gas chromatograph (Hewlett Packard, USA) with a fused-silica capillary column (25 m x 0.2 mm) cross-linked with 5% phenylmethyl silicone. The operating parameters were set and controlled automatically by the Sherlock 6.1 computer program (MIDI Inc., USA). Identification of peaks and assessment of column performance was achieved using a calibration standard mix (Microbial ID1200-A) containing nC9 – nC20 saturated and 2- and 3-hydroxy fatty acids. Peak areas were determined after careful excision of the selected peaks and weighing it on an analytical balance. The outcomes were evaluated using a paired t-test.

**Lipid analysis**

50 ml of mycobacteria were grown in the presence or absence of 6 mg/l of THZ until the cultures reached mid-logarithmic phase. Cells were washed three times with ice-cold PBS and resuspended in methanol–0.3% NaCl (aqueous) (10:1). Apolar lipids were extracted twice with
petroleum ether (bp. 60-80°C) as described previously. Lipid extracts were dried under a stream of nitrogen and weighted. 60 µg of apolar lipid extract, and a PDIM standard (obtained through BEI Resources, NIAID, NIH: Mycobacterium tuberculosis, Strain H37Rv, Purified Phthiocerol Dimycocerosate (PDIM), NR-20328 were spotted onto silica gel 60 thin-layer chromatography (TLC) plates (Merck Millipore, Germany), which was then developed three times in petroleum ether-ethyl acetate (98:2). PDIM was visualized using 5% phosphomolybdic acid in ethanol and gentle heating using a heat gun.
Results

Culture considerations and proteome analysis
To obtain a reliable model of protein abundance within THZ exposed *M. tuberculosis* cells, a strongly diluted pre-culture was started, which was treated continuously with THZ until the growth reached mid-logarithmic phase. To determine the maximum dosage of THZ that allows the growth of *M. tuberculosis* under the described culture conditions, we inoculated *M. tuberculosis* in the presence of 0, 4, 6 and 8 mg/l THZ; see Figure S1a. THZ partially inhibited the growth of *M. tuberculosis* up to a concentration of 6 mg/l THZ. Exposure of *M. tuberculosis* to 8 mg/l of THZ did not allow for any growth of *M. tuberculosis* within the examined culture period of 24 days. It should be noted that concentrations of 6 mg/l THZ are not clinically achievable within serum. However, compared to the serum concentration THZ is >30-fold concentrated within lungs and alveolar macrophages. As a consequence, it had been demonstrated that clinically achievable serum concentrations of 0.5-1 mg/l and even 0.1 mg/l of THZ can kill intracellular *M. tuberculosis*. A concentration of 6 mg/l THZ, as used in our model, fits well within this clinically relevant range. All cells were cultured, with or without 6 mg/l THZ, to mid-logarithmic phase, harvested and processed according to the protocol outlined in Figure 1a. Proteins were isolated, digested, and the obtained peptides were dimethylated, fractionated using SCX chromatography and analyzed by nanoLC-MS/MS. A total of 15 SCX fractions were analyzed twice. Two biological replicates were analyzed using this approach.

We recently reported how this method yields an unbiased view of the *M. tuberculosis* proteome. The cumulative number of unique proteins that was identified and quantified, based on at least two peptides was 2,479; see Figure 1b. A total of 2,241 proteins were identified in both experiments, corresponding to an overlap of approximately 95% between the biological replicates, which is typically achieved in shotgun proteomics when nearly full proteome coverage is reached. The obtained quantification values of the 2,241 proteins that have been identified in both biological replicates showed a high correlation; see Figure 1c.

Overview of *M. tuberculosis* protein abundance after long-term in vitro thioridazine exposure
To get an overview of proteins that are differentially abundant due to the influence of THZ, we applied strict quantification criteria to our dataset of 2,241 proteins that were identified in both biological replicates, as described in the method section. A total of 59 proteins was identified to be at least two-fold more abundant and 30 proteins to be at least two-fold less abundant upon continuous THZ exposure both with a significance B value ≤0.05; see Table S1 and Figure S2. We classified the differentially abundant proteins according to functional categories as given by Tuberculist; see Figure 2. Thirty-seven proteins that were categorized to be involved in ‘intermediary metabolism and respiration’ were shown to be differentially abundant. Previous
studies already described that phenothiazines inhibit aerobic respiration of *M. tuberculosis* in a NADH-dependent manner.\(^{(39)}\) Using InterPro we identified a total of seven proteins with an NAD(P)-binding domain to be two-fold more abundant upon treatment with THZ; see Table S1.

**FIGURE 1. Proteomic approach to identify differentially regulated proteins in *M. tuberculosis* with and without continuous thioridazine treatment**

A: Schematic design of the experimental approach used in this study. Cells were cultured and processed in parallel until dimethylation of the protein digest. In short, *M. tuberculosis* H37Rv was cultured in the presence or absence of THZ until mid-logarithmic phase was reached. Harvesting of the cells was performed by centrifugation following three washes with ice-cold PBS. The cells were mechanically lysed using bead-beating followed by protein digestion using the FASP procedure. Protein digests were provided with a dimethyl mass label, combined and fractionated using SCX. Fifteen fractions were taken, lyophilized and analyzed by LC-MS/MS.

B: Venn diagram representing the proteins that have been quantified in Biological Replicate 1 and 2. A cumulative number of 2,479 proteins were quantified, of which 2,241 were quantified in both analyses.

C: Comparison of 2,241 normalized protein ratios that were determined in both biological replicates. Proteins were colored using a density gradient. The correlation between biological replicates is presented by the Pearson r (-0.835). Note: A label swap was performed between both biological replicates.
Proteins that were either less (Black filled bars) or more (grey bars) abundant in THZ treated cells compared to the control cells were categorized based on their function as given by Tuberculist. None of the functional categories showed to be significantly enriched after Chi-square analyses.

The potential inhibition of antibiotic extruding efflux pumps by THZ has been studied repeatedly. In this study we have identified and quantified 15 of the 31 previously listed antibiotic efflux pumps, but none of these efflux pumps was observed to be more abundant due to long-term THZ exposure; see Table S2.

Here we examined the proteome of *M. tuberculosis* after continuous exposure to THZ, whereas as a previous transcriptomic study focused on the short, initial response after one, two, four and six hours of exposure THZ. A direct comparison of our proteomic dataset with the previously reported transcriptomic dataset is difficult due the fact that there is not necessarily a correlation between the expression of mRNA and the abundance of a protein in *M. tuberculosis*. Nevertheless, we were able to confirm the differential expression of seven out of ten gene transcripts that were previously reported to be induced at one, two, four and six hrs after THZ treatment, including Rv2710/sigB and the *mce4*/*Rv3492c-Rv3501c* locus; see Table S3. Moreover, 21 out of 59 proteins that we have observed to be two-fold more abundant were

![Functional categories](image_url)
also identified by transcriptomics to be upregulated on at least a single time point. Similarly, 12 out of 30 proteins that we have observed to be two-fold less abundant were also identified by transcriptomics to be downregulated on at least a single time point. Taken together, more than 35% of the proteins that we have identified to be differentially abundant using proteomics were previously also identified by transcriptomics, despite the variability’s between both study designs.

**Differential abundance of proteins involved in maintaining the cell envelope permeability barrier**

Mycobacteria are known for their unique cell wall, typified by mycolic acids which are unusually long-chain fatty acids. (42) This unique mycolic acid bilayer forms an efficient impermeable barrier that protects the bacteria from hostile environments, see Abdallah et al. for a comprehensive overview of the mycobacterial cell envelope. (43) Using our proteomic approach, we witnessed the differential abundance of two protein clusters that are involved in the maintenance of the cell envelope permeability barrier upon long-term THZ treatment of *M. tuberculosis*. A lipid family that retained special attention over the years is phthiocerol dimycocerosates (PDIM). These C83-103 long lipids alter the plasma membrane of host cells, (44) protect the pathogen from nitric oxide, (45),(46) modulate the early innate immune response (45),(46),(47) and lower the cell envelope permeability. (48),(36)

Fourteen proteins are located in the biosynthetic locus of PDIM/Rv2928-Rv2942 and Rv2940c; see Figure 3a. Seven of these proteins displayed an upward trend in our dataset upon continuous THZ exposure of *M. tuberculosis*. The increased abundance of multiple proteins in the PDIM synthesis locus suggests that THZ treated cells need to put in extra effort to maintain normal levels of PDIM in the cell envelope, which prevents the cell envelope from becoming too permeable; see Figure S3. Another protein, Rv2190c, which is involved in cell envelope maintenance and alteration of PDIM levels, was less abundant in THZ treated *M. tuberculosis* cells. (49)

A second cluster of proteins that we observed to be differentially abundant under THZ pressure was the *mce4* operon, as also described on the mRNA level for short-term treated *M. tuberculosis*; see Figure 3b. (23) The *mce4* operon is involved in the uptake of cholesterol, an essential nutrient for *M. tuberculosis* during chronic infection, and thereby an essential virulence factor for *M. tuberculosis*. (50) Similar to PDIM, the accumulation of cholesterol is known to play a role in the mycobacterial cell envelope permeability as the build-up of cholesterol increases the cell envelope permeability for rifampin in *M. tuberculosis*. (51) The lower abundance of *mce4* will lower the uptake of cholesterol by *M. tuberculosis*, which could lead to a decrease of the cell envelope permeability.

In line with the differential abundance of the PDIM and *mce4* locus, which leads to a decrease
in cell envelope permeability, Rv0516c was identified to be threefold less abundant upon continuous THZ treatment. Genetic disruption of Rv0516c increases the osmotic resistance in \textit{M. tuberculosis} and enhances the bacterial tolerance to vancomycin, which affects cell envelope synthesis.\textsuperscript{(52)}

\textbf{FIGURE 3. Differential regulation of the PDIM biosynthetic/mce4 cholesterol uptake locus in thioridazine exposed \textit{M. tuberculosis}}

A: Open reading frame of the genes involved in the synthesis PDIM are represented by the arrows. The proteins that we identified to be approximately two-fold more abundant under THZ pressure in the PDIM biosynthetic locus are highlighted in green.

B: Arrows represent the open reading frames of the genes involved in the uptake of cholesterol. The proteins that were less abundant under the continuous pressure of THZ are highlighted in red.

Finally, Rv0129c/Antigen 85c was found to be threefold more abundant in \textit{M. tuberculosis} strains that were continuously treated with THZ. Inactivation of Antigen 85c results in a 40\% decrease of cell wall bound mycolate.\textsuperscript{(53)} As a consequence of the decreased mycolate content in the mycobacterial cell envelope, the permeability towards both the hydrophobic molecule chenodeoxycholate and the hydrophilic compound glycerol increases.\textsuperscript{(53)} The increased abundance of Antigen 85c might therefore lead to a decrease in cell envelope permeability. Although mycobacteria possess an efficient permeability barrier, when properly maintained, the intrusion of hydrophilic antibiotics may take place via porins.\textsuperscript{(54)} Porins are proteins in bacterial outer membranes that enable the non-specific influx of hydrophilic solutes.\textsuperscript{(55)} OmpATb/ Rv0899 is the most well-known pore forming protein of \textit{M. tuberculosis}. However, THZ treatment did not influence the abundance of this protein; see Table S1. In contrast, the outer membrane channel protein, Rv1698, was observed to be approximately two-fold more abundant in our THZ treated mycobacterial cells. It was reported that a \textit{M. tuberculosis} strain harboring a mutated
Rv1698 gene, accumulated 100-fold more copper than wild-type *M. tuberculosis*.\(^{56}\) The increased abundance of Rv1698 upon THZ treatment might therefore be a necessity to prevent the accumulation of copper when the cell envelope of *M. tuberculosis* becomes compromised. In summary; we have identified a total of 16 proteins to be differentially abundant upon THZ treatment that contribute to a decrease in cell envelope permeability. We hypothesize that the proteins in the PDIM synthesis locus, *mce4* locus, Rv0516c, Rv1698 and Antigen 85c are differentially regulated upon long term exposure of *M. tuberculosis* to THZ to counteract THZ induced cell envelope damage. This hypothesis is supported by electron microscopy images published earlier, which clearly show that the cell envelope of *M. tuberculosis* becomes damaged after four hours of THZ treatment.\(^{23}\)

The effect of thioridazine treatment on the plasma membrane of *M. tuberculosis*

A recently proposed mechanism of THZ, that was supported by a computer-simulation study, is that THZ interacts with lipid-bilayers in bacteria, which causes significant membrane thinning.\(^{57}\) This novel insight is in line with previous studies that also showed how THZ and other phenothiazines interact with negatively charged phospholipids in erythrocytes,\(^{58}\) partitions in lipid-bilayers and the outer and inner membranes of mitochondria.\(^{59}\) The induced damage to the mycobacterial cell envelope upon THZ treatment as suggested by the proteomic data in this study, in light of the latter cited studies, supports the model in which THZ interacts with the bacterial plasma membrane. This interaction of THZ with the plasma membrane of *M. tuberculosis* could lead to a more permeable cell envelope which results in a faster accumulation of antibiotics. To determine whether THZ alters the composition of the mycobacterial phospholipid bilayer, we examined the phospholipid-derived fatty acids (PLFA) of both continuously THZ treated and untreated *M. tuberculosis*. Although a direct effect of THZ on proteins involved in fatty acid metabolism has been suggested, this was not reflected in our proteomic dataset.\(^{60}\) Examination of the PLFA profiles derived from continuously THZ treated *M. tuberculosis* and untreated *M. tuberculosis* cells, we revealed a significant increase in the proportion of tuberculostearic acid (10Me-C18:0) in THZ treated cells; see Figure 4. An increase of tuberculostearic acid has previously been observed in an ethambutol tolerant strain, when compared to an ethambutol susceptible strain.\(^{61}\) Ethambutol is known to increase the cell envelope permeability towards rifampicin in multiple mycobacterial species\(^{62}\), it therefore might be that the increased proportion of tuberculostearic acid is a response of the mycobacteria towards drug-induced cell envelope permeability stress.

Tuberculostearic acid is produced by mycobacteria through the methylation of phospholipid esterified oleic acid, with S-adenosyl methionine as methyl donor followed by a reduction with NADPH as a cofactor.\(^{63}\) Interestingly, an increase of NAD(P)H has been described for THZ treated *M. tuberculosis*.\(^{64}\) Furthermore, as mentioned above, we observed a two-fold increased level
for seven proteins with an NAD(P)-binding domain; see Table S1.

**FIGURE 4.** A larger proportion of tuberculostearic acid is present in thioridazine exposed *M. tuberculosis*. Phospholipid-derived fatty acids of untreated *M. tuberculosis* cells (black bars) and THZ treated cells (grey bars) were analyzed. The composition is given as the average percentage of total integrated chromatographic areas. Error bars represent the mean ± SEM of the average peak area for each of the independent biological triplicates. * P ≤ 0.05

Alteration of the mycobacterial cell envelope permeability upon long-term thioridazine treatment

To determine whether the cell envelope permeability of *M. tuberculosis* is increased upon continuous THZ treatment as hypothesized in our study, we used fluorescence spectrophotometry to measure the accumulation of several fluorescent compounds over a one hour time period. Similar experimental approaches were previously performed using ethidium bromide to determine the efflux pump activity of bacteria.\(^{21,65,66,67,68}\)

However, it is not possible to determine the impact of THZ on the efflux pump inhibition solely by measuring the accumulation of ethidium bromide, as the total accumulation of ethidium bromide is dependent on both efflux pump activity and cell envelope permeability/influx. Therefore, we needed to analyze the accumulation of ethidium bromide alongside another membrane permeable fluorescent dye that does not act as a substrate for efflux pumps. Sytox
Orange, is known as a membrane permeable fluorophore, that, similarly to ethidium bromide, emits fluorescence once it intercalates with mycobacterial DNA. In contrast to ethidium bromide, Sytox Orange has, to our knowledge, not been used as an efflux pump substrate in *M. tuberculosis*. To assure that Sytox Orange is not susceptible to efflux we monitored the accumulation of Sytox Orange and ethidium bromide in the presence and absence of the efflux pump inhibitor reserpine. As presented in Figure 5, ethidium bromide is accumulated to higher levels in the presence of the efflux pump inhibitor reserpine\(^{[37]}\) and THZ, whereas increased accumulation of Sytox Orange was solely observed in the presence of THZ. Thus the data indicates that Sytox Orange does not act as an efflux pump substrate.

The combination of ethidium bromide and Sytox Orange provides a better understanding of cell envelope permeability than the sole usage of ethidium bromide. Since both fluorescent compounds were accumulated to higher levels in continuously THZ treated mycobacterial cells, compared to untreated cells, the data supports our hypothesis that THZ is able to increase the permeability of the mycobacterial cell envelope.

**FIGURE 5.** Ethidium bromide and Sytox Orange accumulate faster in thioridazine treated *M. tuberculosis*. Untreated *M. tuberculosis* cells (white filled bars), THZ treated cells (light grey bars), reserpine treated cells (dark grey bars) and THZ+reserpine treated cells (black bars) were assessed for the accumulation of ethidium bromide and Sytox Orange by fluorescent spectrophotometry. Independent biological duplicates were taken and samples were analyzed in duplicate. Error bars represent the mean ± SD of the average normalized fluorescence units for each biological replicate. Significant differences in accumulation compared to the untreated control group were calculated using two-tailed paired Student’s t-tests. P-values<0.05 were considered to be significant and are indicated with (*).
Discussion

The accumulation of antibiotics inside a bacterial cell is crucial for successful treatment. Efflux pumps provide an intrinsic mechanism in several bacteria that allows the active secretion of drugs.(69) As a result, less of the anti-bacterial compound will be present within the bacteria. The inhibition of efflux pumps is a promising development, as inhibition of efflux pumps has been shown to increase the drug susceptibility of *M. tuberculosis*.(40)

Treatment of *M. tuberculosis* with THZ results in an increased susceptibility of the pathogen towards rifampicin, isoniazid and streptomycin.[9, 10, 20, 28, 70] Furthermore, ethidium bromide, a common substrate for efflux pumps, accumulates faster in THZ treated mycobacteria.[21] Therefore, it has been suggested that THZ inhibited efflux pumps, which leads to an increase of intracellular drug accumulation that could explain the synergistic effects between THZ and other antibiotics. Based on the data obtained in the current study we propose an alternative molecular mechanism that connects the outcomes of several other studies.

Using a proteomic approach, we demonstrated the differential abundance of 16 proteins that are known to fulfill a function in the maintenance of the mycobacterial cell envelope. These observations support a previous study that demonstrated THZ induced cell envelope damage.[23] In fact, we confirmed the differential of SigB on the protein level, that was previously reported to be induced on the mRNA level in the response to THZ.[23] More importantly, our proteomic data points to several molecular processes that might are utilized by *M. tuberculosis* to compensate for THZ induced cell envelope damage. Further studies are warranted to elucidate the role of these proteins in response to THZ stress that might be caused by a direct interaction of THZ with the plasma membrane of *M. tuberculosis*.[57] There is no experimental evidence that THZ binds to the plasma membrane of *M. tuberculosis*. However, in our study we demonstrate that the composition of the mycobacterial plasma membrane is altered upon long-term exposure to THZ; see Figure 4.

Based on the proteomic response of *M. tuberculosis* to THZ we hypothesized that THZ compromises the mycobacterial cell envelope, which allows for rapid accumulation of several antibiotics. To support this hypothesis we performed a fluorescent spectrophotometry based accumulation assay. To this end, we have selected two fluorophores with unique properties; ethidium bromide and Sytox Orange. Ethidium bromide was used to monitor both cell envelope permeability and efflux pump activity, whereas we demonstrated that the accumulation of Sytox Orange is solely dependent on cell permeability; see Figure 5. Using this experimental setup we demonstrated that THZ treated cells accumulate more Sytox Orange and ethidium bromide than untreated cells, which supports our hypothesis that the cell envelope of *M. tuberculosis*
becomes more permeable after THZ treatment; see Figure 5.

Synergistic effects between antibiotics, due to alteration of the cell envelope permeability, have previously been observed for ethambutol, which increases the accumulation of rifampicin in multiple mycobacteria: *M. aurum, M. smegmatis* and *M. tuberculosis*.\(^{(62)}\) In addition, it has also been shown in *M. marinum* that the susceptibility towards multiple antibiotics increases once the cell envelope permeability was significantly increased.\(^{(36, 71)}\) The THZ induced cell envelope permeability could explain the synergistic effects between THZ and other drugs in *M. tuberculosis* as described previously.\(^{(9, 10, 20, 28, 70)}\)

The exceptional cell envelope of *M. tuberculosis* provides the pathogen with an intrinsic form of antibiotic tolerance.\(^{(43)}\) By permeabilizing the cell envelope antibiotics can more readily enter the cell and accumulate to a bactericidal concentration. In fact, due to the increased permeability of the cell envelope to old drugs and new anti-tuberculosis antibiotics, not only would the old drug(s) be restored to an effective therapeutic level, the dose levels of new but toxic anti-tubercular compounds may be significantly reduced.

Regarding the accumulation of ethidium bromide, we did not observe a synergistic effect when we combined THZ with the efflux pump inhibitor reserpine, at the concentrations and timespan examined; see Figure 5. However, the observation that Sytox Orange accumulates to higher levels in THZ treated cells than reserpine treated cells demonstrates that there is a role for cell envelope permeabilizing agents next to efflux pump inhibitors. As demonstrated, some compounds only accumulate to high intracellular levels through the induction of cell permeability.

In the here described model we demonstrate that THZ induces cell envelope permeability. The corresponding proteomic data revealed that proteins involved in the synthesis of PDIM and uptake of cholesterol are differentially abundant during THZ treatment. Similarly, we demonstrated that the relative content of tuberculostearic acid increases in the plasma membrane upon exposure to THZ. Both the differential abundance of these proteins and the alteration of the plasma membrane composition can play a vital role to counteract the THZ induced cell envelope permeability. The extent to which these changes contribute to THZ tolerance in *M. tuberculosis* requires further assessment.

The increase in cell envelope permeability upon THZ treatment is an alternative explanation for the synergistic effects of THZ with other antibiotics that has so far only been attributed to the inhibition of efflux pumps. Nevertheless, there remain two conceivable hypotheses in which THZ inhibits mycobacterial efflux pumps. First, the THZ induced cell envelope permeability might
reduce the proton motive force, which indirectly inhibits efflux pumps by denying its source of energy. Secondly, the THZ induced alteration of the phospholipid bilayer might also result in a membrane-mediated inhibition of efflux pumps by THZ as suggested previously. It is important to note that both the indirect inhibition of efflux pumps and an increase of cell envelope permeability can contribute to a more rapid accumulation of antibiotic compounds in the cell. Cell envelope influencing agents like THZ may therefore increase the efficacy of other drugs significantly.

Finally, permeabilization of the cell envelope could lead to the loss of metabolites, ions and thereby the loss of, for example, the proton motive force which will ultimately lead to cell death. Thereby, the permeabilization of the mycobacterial cell envelope by THZ could also explain why THZ is active against metabolically inactive cells.

Conclusion
By analysing the proteome of THZ treated *M. tuberculosis* cells we observed the differential abundance of 16 proteins that are involved in the maintenance of the mycobacterial permeability barrier. We further demonstrated that long-term THZ treatment yields a relatively permeable *M. tuberculosis* cell envelope. Finally, we also showed how the plasma membrane composition of *M. tuberculosis* is altered under the influence of THZ. This renewed insight in the working mechanism of THZ offers a better understanding on the molecular basis of this promising new antibiotic, which will hopefully result in a more efficient use of this drug, possibly in combination with other efflux pump inhibitors.
References


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Supporting information

Supporting Information Table S1. Table showing the identified and quantified proteins in both biological replicates with additional information. This table can be downloaded on the website of the journal: https://pubs.acs.org/doi/suppl/10.1021/acs.jproteome.5b01037

Supporting Information Table S2. Table showing the identified drug efflux pumps with their corresponding drug targets. This table can be downloaded on the website of the journal: https://pubs.acs.org/doi/suppl/10.1021/acs.jproteome.5b01037

Supporting Information Table S3. Table showing a comparison between gene transcript expression data derived from short-term THZ-treated *M. tuberculosis* reported previously (23) and the identified protein abundance ratios in the reported proteomic data set. This table can be downloaded on the website of the journal: https://pubs.acs.org/doi/suppl/10.1021/acs.jproteome.5b01037
FIGURE S1. Growth patterns of *M. tuberculosis* H37Rv treated with thioridazine.

(A) Exposure of *M. tuberculosis* to 0 (control), 4, 6 & 8 mg/L thioridazine.

(B) Growth curves of media containing 6 mg/L thioridazine media that has been pre-incubated for 21 days at 37°C (stability) and media containing 6 mg/L that has freshly been prepared.
FIGURE S2. Protein ratio distribution comparison of thioridazine treated and untreated *M. tuberculosis* H37Rv. Normalized Log2 transformed protein ratio’s plotted against the summed Log2 peptide intensity for both biological replicates. Data points are colored based on their significance B values. Turquois is >0.5, blue >0.25, red >0.1, yellow >0.05 and green <0.05. Note: a label swap was performed between both biological replicates.
FIGURE S3. Thin Layer Chromatography analysis of phthiocerol dimycocerosate (PDIM) abundance in *M. tuberculosis* H37Rv. Apolar lipids were extracted, analysed on silica gel TLC plates and three times developed in petroleum ether:ethyl acetate (98:2). I: a spot of PDIM standard, II/III: spots of untreated *M. tuberculosis* H37Rv, IV/V: spots of thioridazine treated *M. tuberculosis*. 

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