Effects of lipid-lowering drugs on vancomycin susceptibility of mycobacteria

Running title: Mtb inhibited by vancomycin and THL

Céline Rens[a], Françoise Laval[b], Mamadou Daffé[b], Olivier Denis[c], Rosangela Frita[d], Alain Baulard[d], Ruddy Wattiez[e], Philippe Lefèvre[a] and Véronique Fontaine[a#]

[a]Université Libre de Bruxelles (ULB), Unit of Pharmaceutical Microbiology and Hygiene, CP205/2, Boulevard du Triomphe, 1050 Brussels, Belgium

[b]Institute of Pharmacology and Structural Biology, University of Toulouse, CNRS, University Paul Sabatier (UMR 5089), Department of "Tuberculosis and Infection Biology" 205 route de Narbonne, BP64182, 31077 Toulouse cedex 04, France.

[c]Laboratory of Bacteriology, Department of Microbiology, Hôpital Erasme, Université Libre de Bruxelles (ULB), 808, route de Lennik, 1070 Brussels, Belgium

[d]Institut Pasteur de Lille, Université de Lille, CNRS, Inserm, CHU Lille, U1019 - UMR 8204 - CIIL - Center for Infection and Immunity of Lille, F-59000 Lille, France

[e]Department of Proteomics and Microbiology, University of Mons, 20, place du Parc, B-7000 Mons, Belgium

Address correspondence to Véronique Fontaine, vfontain@ulb.ac.be

ABSTRACT

Tuberculosis is still a cause of major concern, partly due to the emergence of multi-drug resistant strains. New drugs are therefore needed. Vancomycin can target mycobacteria with cell envelop deficiency. In this study, we used a vancomycin susceptibility assay to detect drugs hampering the lipids synthesis in *M. bovis* BCG and in *M. tuberculosis*. We tested three drugs already used to treat human obesity: tetrahydrolipstatin (THL), simvastatin and fenofibrate. Only vancomycin and THL were able to synergize on *M. bovis* BCG and on *M. tuberculosis* although mycobacteria could also be inhibited by simvastatin alone. Lipid analysis allowed to identify several lipid modifications in *M. tuberculosis* H37Rv treated with those drugs. THL treatment mainly reduced the phthiocerol dimycocerosate (PDIM) content in the mycobacterial cell wall, providing an explanation for the synergy, as PDIM deficiency has been related to vancomycin susceptibility. Proteomic analysis suggested that bacteria treated with THL, on the opposite to simvastatin, tried to recover, inducing, among others, lipid synthesis. The combination of THL with vancomycin should be considered as a promising solution in new strategies to treat MDR-TB.

INTRODUCTION

Tuberculosis (TB), mainly caused by *Mycobacterium tuberculosis*, is still killing 1.5 million people annually in the world (1). Although TB death rate has decreased by 47% since 1990 according to the Millennium Development Goals targets, reaching a 86% treatment success in 2013, the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) infections (9.7% of MDR-TB) is highly worrisome. In some regions of Eastern Europe, the percentage of XDR-TB even reached 25% (2). MDR-TB refers to TB with both resistance to isoniazid and rifampicin and XDR-TB to a subgroup of MDR with additional resistance to any fluoroquinolone and at least one second line injectable drug. In 2015, the overall cure rate of the MDR infections, using restricted, expensive, prolonged, second-line treatment regimens is falling to 50 % (1). There is a growing concern to discover new drugs to fight MDR and XDR-TB. As such, Bedaquiline and Delamanid have been recently approved for treating MDR-TB and others are currently in clinical trials (3,4).

It is well established that the *M. tuberculosis* antibiotic resistance is highly dependent on its unique waxy cell wall, consisting in a covalently linked skeleton, composed from inside to outside, of peptidoglycan, arabinogalactan and mycolic acids (5). Unique, extractable lipids, such as acyltrehaloses, including sulfolipids (SL), trehalose dimycolate (TDM), diacyltrehalose (DAT) and penta- or poly-acyltrehalose (PAT), but also phthiocerol and phthiodolone dimycocerosates (PDIM), particularly involved in virulence and antibiotic resistance, are embedded in an outer membrane. In addition, phosphatidyl-myo-inositol mannosides (PIM), and hypermannosylated PIM, lipomannan and lipoarabinomannan are anchored though their phosphatidyl-myo-inositol moieties into the inner and outer membrane of the cell wall and have been shown to be involved in *M. smegmatis* antibiotics resistance (6).

Recently, we described an innovative approach to identify compounds targeting mycobacterial lipids biosynthesis that can synergize with glycopeptides to inhibit the growth of mycobacteria. Interestingly, we showed that the combination of vancomycin with a drug targeting long-chain lipid synthesis, such as cerulenin, act synergistically against MDR and XDR clinical strains (7).

In this study, we used our vancomycin susceptibility assay to test commercially available drugs focusing on one's that could potentially lower lipid biosynthesis, in order to identify potential synergistic activity with vancomycin (7). This choice is justified by the importance of complex lipids composing the mycobacterial envelope, their roles in the cell wall impermeability, the susceptibility to antibiotics and the virulence of *M. tuberculosis* (5, 7, 8). Three drugs commonly used to treat obesity and cholesterol disorders were tested: tetrahydrolipstatin (THL), simvastatin and fenofibrate (7). THL is an esterase inhibitor well known for inhibiting pancreatic lipases (9) but also several bacterial enzymes (10-17). Parket et al. reported that, in *M. smegmatis*, THL can target Rv3802 acting both as a thioesterase and a phospholipase on phosphatidylinositol mannoside 2 (18) but also the ES-31 serine protease (19). Interestingly, Ravindran et al. pointed out 9 lipases and one thioesterase, TesA, as potential THL targets (20). This last enzyme, TesA, is involved in the synthesis of phthiocerol dimycocerosate (PDIM) (21). PDIM are lipid compounds in the outer membrane of defined slow-growing mycobacterial species, including *M. tuberculosis* and *M. bovis* BCG, essential among others for the cell envelope impermeability and antibiotic resistance, in particular towards glycopeptides (5, 7). Simvastatin is a cholesterol lowering drug that has been recently reported to enhance phagosomal maturation and autophagy, increasing *M. tuberculosis* burden in macrophage and mouse (22, 23). This drug has been reported to improve the efficiency of first line antituberculous drugs in vivo, probably by lowering lipids in foamy macrophages (23). Finaly, fenofibrate belongs to the fibrate lipid lowering drugs family. Grabes et al.

reported some toxicity of fibrates on *M. tuberculosis* (24). Gemfibrozil has been indeed shown to inhibit *M. tuberculosis* growth with a potential action on a mycobacterial enoyl Coenzyme A (24, 25). More recently, Kim et al. reported that fibrates could lower bacterial burden and inflammation in a *M. smegmatis* macrophage invasion model by a PPAR-α independent pathway (26).

Here, using three different antibacterial assays, we show the antimycobacterial activity of the simvastatin and THL and demonstrate that THL synergizes with vancomycin. Lipid and protein analysis indicated distinct mode of action, with THL inhibiting the cell wall synthesis, as expected.

MATERIALS AND METHODS

Bacteria and cultures. *M. bovis* BCG GL2 and *M. tuberculosis* H37Rv were used. Mycobacteria were grown in 7H9 medium containing 0.05% Tween 80 supplemented with 10% albumin-dextrose complex (Difco Laboratories) or on 7H11 Middlebrook agar supplemented with oleic acid-albumin-dextrose complex (Difco Laboratories).

Drug susceptibility tests. Drug susceptibility was investigated using three methods: the macro-dilution method, the standardized agar proportion method, and the BacT/Alert MP method (Mycobacteria Process) according to the bioMérieux protocol and previous reports (27, 28). For M. bovis BCG, in the context of a first assay, we used the macrodilutions method to determine more rapidly and in a smaller volume the minimal inhibitory concentration (MIC) and the fractional inhibitory concentration index (FICI). For *M. tuberculosis*, we additionally used the standardized agar proportion method. Bact/alert MP method allowed to perform a fast preliminary test on *M. bovis* BCG and to evaluate the

119 potential synergy of drug combination on *M. tuberculosis*. For all methods, cultures less than
120 4 weeks old were used to prepare homogeneous suspension inoculum.

121 The macrodilution method, used for *M. bovis BCG*, was done in 7H9 medium without Tween,
122 supplemented with albumin-dextrose complex in polycarbonate tube to reach a final volume
123 of 1.2 ml, thus mimicking the BacT/Alert method at a 1:10 scale. A 600 µl inoculum, at an
124 $O.D_{600}$ of 0.2, diluted 1 in 24, was added to 600 µl serial drug dilutions (one in two). The FICI
125 of the drug in combination with vancomycin was calculated as follow: FICI= FICa + FICb=
126 MICab/ MICa + MICba/ MICb. In agreement with the Checkerboard method, synergy is
127 reached when FICI $\leq$ 0.5, indifference could be observed when 0.5 < FICI < 2, and an
128 antagonistic effect is noted when FICI > 2 (29).

129 For the standard agar proportion method (27), we used mycobacterial suspensions (1
130 McFarland turbidity) to perform serial dilutions from 10^{-1} to 10^{-4}, in the presence or absence
131 of various concentrations of tetrahydrolipstatin (THL), simvastatin, fenofibrate and
132 vancomycin alone or in combination. The MIC was defined as the lowest drug concentration
133 that inhibited more than 99% of the bacterial population. Vancomycin, THL, fenofibrate and
134 simvastatin were purchased from Sigma Aldricht. THL was also commercially obtained from
135 Sandoz and fenofibrate from Eurogenerics.

136 The BacT/Alert MP system (Mycobacteria Process), using BacT/ALERT MP bottles (11 ml)
137 supplemented with restoring fluid was inoculated with drugs and mycobacterial suspensions
138 as previously described (7). The growth index (GI) values were recorded every 10 min. A
139 1/100 proportional growth control condition consisted in a 100-fold diluted bacterial
140 inoculum, injected in a drug-free control vial. The concentration of the drug(s) simultaneously
141 giving a flagged positive bottle as the 1/100 control vial was considered as the MIC.
142 Combined drug effect was investigated using concentrations at sub-MICs and previously

published 'x/y' methodology (30-33). Briefly, when the 1:100 control vial was flagged and the daily ΔGI of the 1:100 control vial reached at least 30, the GI was read for at least 1 additional day to calculate the ΔGI from the previous day. In the case of a two-drug combination, a 'Δx/Δy' quotient of < 0.5 indicates synergy with Δx being the ΔGI value obtained for the vial with the combination of drugs and Δy being the lowest ΔGI value obtained with any of the single drug used within the combination tested (7).

Lipid analysis. Lipids from *M. tuberculosis* H37Rv (350 ml 7H9 culture without Tween 80 at OD_{600} 0.5) treated with THL (50 µg/ml) or treated with same volume of DMSO (as control) were extracted first with $CHCl_3/CH_3OH$ (1:2, v/v) for 24h at room temperature, and twice with $CHCl_3/CH_3OH$ (2:1, v/v) for 2 days. Crude extracts were washed twice with distilled water and evaporated to dryness, according to standard protocols as described previously (34).

The lipid extracts were comparatively analyzed by HPTLC (High Performance Thin-Layer Chromatography) on HPTLC Silica Gel 60 (Merck), using various solvent systems, mainly petroleum ether/diethyl ether (9:1, v/v) for phthiocerol dimycocerosates (PDIM) and $CHCl_3/CH_3OH/H_2O$ (30:8:1, 65:25:4 and 60:35:8, v/v/v) for more polar lipids (trehalose mycolates and phospholipids). Visualizations were performed by immersion of the plates in primuline solution or by spraying the plates with 10% phosphomolybdic acid and charring for PDIM or 0.2% anthrone solution (w/v) in concentrated H_2SO_4 and charring for glycolipids. The various lipid spots were quantified by either absorption measurement at the specific wavelength with TLC Scanner 3 using wincats software and/or colorimetric measure using ChemiDoc XRS+ with Image Lab Sofware (BioRad).

SWATH acquisition LC-MS/MS Analysis. Quantitative proteomic analyses were performed on proteome extracts of the different *M. tuberculosis* samples. Cells from 3 biological replicates were harvested by centrifugation (16,000×g, 10 min, 4°C) when OD_{600} reached 0.5.

Proteins were extracted by sonication (3×10 sec, amplitude 40%, IKA U50 sonicator) in 6 M guanidinium chloride solution. Extracted proteins were reduced, alkylated and precipitated with acetone. Proteolytic peptides were obtained by overnight enzymatic digestion using trypsin at a ratio of 1:50 (w/w).

MS experiments were conducted following a label-free strategy on UHPLC-HRMS/MS plateform (Eksigent 2D ultra & AB Sciex TripleTOF™ 5600) in SWATH data independent acquisition (DIA) mode. Two micrograms of peptides were separated on a C18 column (Acclaim PepMap100, 3 µm, 150 µm × 25 cm, Dionex) with a linear acetonitrile gradient (5 to 35% (v/v), 450 nl.min-1, 30 min) in water containing 0.1% (v/v) formic acid. Each MS survey scan (400-1500 m/z, 50 ms accumulation time) was followed by 34 SWATH acquisition overlapping windows of 25 Da with covering the precursor m/z range. For each window, ions were fragmented using rolling collision energy, and fragment ion spectra were accumulated for 95 ms in high sensitivity mode.

SWATH spectra were identified by comparison to a reference spectral library obtained with traditional data-dependent acquisition (DDA) experiments on proteins extracted from the different *M. tuberculosis* samples. Sample preparation and separation procedures were identical to the one previously mentioned. DDA spectra were acquired with the following parameters: MS scan (400-1500 m/z, 500 ms accumulation time) in high resolution mode (>35000) followed by 50 MS/MS scans (100-1800 m/z, 50 ms accumulation time, intensity threshold at 200 c.p.s). The DDA mass spectrometry data were processed with AB Sciex ProteinPilot™ 4.5 software. Spectra identification was performed by searching against the *M. tuberculosis* H37Rv UniProt entries with parameters including carbamidomethyl cystein, oxidized methionine, all biological modifications, amino acid substitutions and missed cleavage site. Proteins identified at a false discovery rate below 1% were used as the SWATH reference spectral library.

SWATH wiff files were processed by AB Sciex PeakView 2.1 software and his SWATH™ Acquisition MicroApp. Up to 6 peptides with at least 99% confidence were selected with 6 transitions per peptide. XIC extraction windows was set to 25 min, and XIC width to 75 ppm. XIC peak area was extracted and exported in AB Sciex MarkerView™ 1.2 software for normalization and statistical analysis. Protein functional classification of tuberculist was used (http://genolist.pasteur.fr/TubercuList/). Only differentially expressed proteins with a p value < 0.05 were selected and analyzed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) version 9.1 (http://string.embl.de/). STRING uses a score to define interaction confidence; all interactions with a confidence score >0.9 (highest confidence) were collected (35).

RESULTS

Preliminary test on *M. bovis* BCG. We tested tetrahydrolipstatin (THL), simvastatin and fenofibrate for their ability to weaken the mycobacterial cell wall and consequently to potentiate the vancomycin inhibitory effect (7). The first experiment was done on *M. bovis* BCG using the BacT/ALERT MP method and a fixed 5 µg/ml vancomycin concentration, far below the vancomycin MIC (> 500 µg/ml), as previously reported (7). We observed that only THL remarkably increased the susceptibility of the bacteria to vancomycin (Fig. 1). In order to investigate if this growth inhibition resulted from a synergy or from the effect of THL alone, we determined the MIC of THL and its FICI in combination with vancomycin. As shown in Table 1, the MIC of THL alone, determined by the macrodilution method, was 5 µg/ml. The MIC dropped to 0,625-1,25 µg/ml in the presence of vancomycin (10 µg/ml), revealing that this combination acts synergically (FICI = 0,185-0,31). In contrast, simvastatin alone showed an MIC of 100 µg/ml and could act with vancomycin but not in synergy

(FICI=1). Interestingly no synergistic effect of THL was observed in the presence of amoxicilline/clavulanic acid, suggesting a specific mechanism responsible for the combined effect of THL with vancomycin (data not show).

Synergistic inhibition confirmation on *M. tuberculosis*. The BacT/ALERT assay on *M. tuberculosis* H37Rv showed an effect of simvastatin 100 µg/ml and THL 50 µg/ml when combined with vancomycin 5 µg/ml, in contrast to fenofibrate showing no effect to concentrations up to 100 µg/ml (Fig. 2). We further investigated with the BacT/ALERT system the combination of THL (25 µg/ml) with vancomycin (5 µg/ml) and obtained a $\Delta x/\Delta y$ = 0,03 showing a synergy for those drugs. To calculate the FICI by the checkerboard method we performed macrodilution experiments for both THL and simvastatin with vancomycin (Table 2). THL MIC was 50 µg/ml when tested alone and fell to 3,1 µg/ml when combined with vancomycin (10 µg/ml). Simvastatin alone showed an MIC of 100 µg/ml decreasing (50 µg/ml) in combination with vancomycin (10 µg/ml). Based on this data the FICI calculation showed synergism for THL with vancomycin (FICI= 0,31) and a non-synergistic effect for simvastatin with vancomycin (FICI= 1,5). Finally, the NCCLS agar proportion method confirmed these data (Table 2). Using this method, THL MIC was 5 µg/ml and fell to 0,625 µg/ml in combination with 2 µg/ml vancomycin. Confirming the BactAlert results, simvastatin showed an MIC of 100 µg/ml which decreased to 50 µg/ml in the presence of 2 µg/ml vancomycin. The checkerboard allowed us to calculate a FICI of 0,375 for THL with vancomycin and a FICI of 1 for simvastatin with vancomycin. Fenofibrate was also retested with the agar proportion method, which confirmed its inefficacy to inhibit the growth of H37Rv (up to 200 µg/ml) including in combination with 2 µg/ml of vancomycin.

Membrane lipid alteration by THL and simvastatin on *M. tuberculosis*. Total lipid extract from treated *M. tuberculosis* H37Rv were compared to those of untreated to assess the effect of these drugs on cell wall lipids. As shown in Fig. 3, 24 h after the addition of 50 µg/ml

241 THL, the amount of phthiocerol dimycocerosates (PDIM A) and phthiodiolone
242 dimycocerosate (PDIM B) outer membrane lipids clearly decreased, as well as the amount of
243 triacyl glycerol (TAG) TDM and SL-1. Some compounds, exhibiting a mobility close to those
244 of trehalose dimycolate (TDM) and the major sulfated compounds (SL-1), were observed only
245 in THL-treated extracts. Mycolic acids were unchanged (data not shown).

246 In contrast to THL, treatment with 100 µg/ml simvastatin did not affect the amount of PDIM
247 A and PDIM B in the cell wall. We also observed increased amounts of phosphatidyl-myo-
248 inositol dimannosides (Ac_2PIM_2 and Ac_2PIM_6) and reduced amounts of phosphatidyl glycerol
249 (PG), phosphatidyl ethanolamine (PE), $AcPIM_2$, DAT/TMM (trehalose monomycolate) and
250 TAG. Mycolic acids were equally unchanged (data not shown)

251 **Proteomic response to THL and simvastatin in *M. tuberculosis*.** To improve our
252 comprehension of the drugs action, we compared the proteome of untreated *M. tuberculosis*
253 with the proteome of *M. tuberculosis* treated 24h with two fold reduced sub-MIC
254 concentration of either THL (25 µg/ml) or simvastatin (50 µg/ml) and no vancomycin, in
255 order to study bacteria reaction in condition avoiding cell killing. Differential proteomic data
256 were obtained using the sequential window acquisition of all theoretical spectra (SWATH)
257 approach. SWATH-MS is a recent label free, quantitative proteomics based to mass
258 spectrometric method that combines data-independent acquisition (DIA) and targeted data
259 analysis. In this way, robust quantitative data of thousands of proteins in a single
260 measurement was obtained in MS2 mode (36). As shown in Table 3, the THL treatment
261 induced more protein changes (around 7.5 % of the protein content) in *M. tuberculosis* than
262 the simvastatin treatment (around 1.8 % change). THL treatment mainly involved protein
263 upregulation (97 %), in contrast to simvastatin inducing mainly protein downregulation
264 (78%). Based on this simple analysis, it is clear that the bacterial response is completely
265 different after a THL or simvastatin treatment. However, few proteins were commonly

regulated (0.3 %), such as four proteins downregulated from the intermediary metabolism (e.g. AldC and ProB) and two upregulated ribosomal proteins (RplW and RpmJ). THL treatment induced many protein up-regulations, mainly involved in intermediary metabolism and respiration (34 %), information pathways (16 %) and lipid metabolism (14 %) (Table 3, Fig. S1 and Fig. S2). Proteins involved in transcription (RpoA, RpoC, Rho, NusG, Mfd, …), traduction (RpmJ, RpsM, RplT, RpsR1, RpmD, …), the metabolic tricarboxylic acid cycle (MDH, GltA2, CitA, Can, Icd2, Kgd), glutamate metabolism (GltB, GlnA1, Gdh) and numerous enzymes of FAS I (Fas) and II (AcpM, KasA, FabG1, Pks13, …) lipid synthesis were upregulated in THL treated *M. tuberculosis* (Fig. S2 and Table S3). Simvastatine downregulated proteins mainly involved in intermediary metabolism and respiration (33 %) and lipid metabolism (12 %) (Table 3, Fig. S1, Fig. S2 and Table S3).

DISCUSSION

Tetrahydrolipstatin (THL), simvastatin and fenofibrate were tested for their ability to potentiate the antimycobacterial activity of glycopeptides by weakening the mycobacterial cell wall (7). Our first approach, using the BacT/ALERT system on *M. bovis* BCG, identified an interesting inhibitory effect of THL with vancomycin. A synergy could be identified by the Checkerboard method from the macrodilution assay. This synergy was also observed on *M. tuberculosis* by both the BacT/ALERT method and the Checkerboard method using the macrodilution and the NCCLS agar proportion results. This synergy suggested that THL destabilized the outer membrane of the cell envelope and facilitated the action of vancomycin. The impact of THL on the outer membrane integrity was verified by lipid analysis. As expected for a drug synergizing with vancomycin against *M. tuberculosis* and *M. bovis* BCG growth, we observed a reduction of PDIM A and PDIM B in THL treated *M. tuberculosis*.

290 The interaction of THL with TesA might explain, at least partly, this result (20). In agreement
291 with Belardinelli et al., we also observed that the outer membrane SL-1 lipids were strongly
292 decreased (37). Belardinelli et al. recently described that THL inhibits PAT biosynthesis and
293 even more SL-1 biosynthesis suggesting a potential action on Chp 1 (37). The amount of
294 mycolic acids was unchanged by THL treatment. The effect of THL on *M. tuberculosis* is
295 therefore different than on *M. kansasii*, as Kremer et al. describing for the first time the
296 antimycobacterial activity of this drug in *M. kansasii*, underlined its partial inhibitory effect
297 on mycolic acids and meromycolyl-diacylglycerol synthesis (38). It is worth noting that we
298 also observed 48h and 72h after treatment an accumulation of degradation products of the cell
299 wall mycolyl-arabinogalactan. These products consisted mainly in di-mycolyl-di-
300 arabinoglycerol, mono-mycolyl-arabinoglycerol and mono-mycolyl-di-arabinoglycerol (data
301 not shown). We also observed a reduction of triacylglycerol (TAG). Altogether, our results
302 suggest that THL induced a loss of cell wall integrity by targeting several cell envelope lipid
303 pathways, potentially among them, Chp 1, involved SL-1 synthesis as previously reported
304 (30) and TesA, involved in PDIM synthesis as suggested by Ravindran et al. (20).

305 Simvastatin, although not able to synergize with vancomycin, inhibited already alone (100
306 µg/ml) the growth of both *M. bovis* BCG and *M. tuberculosis*. This result, observed in three
307 antimycobacterial drug susceptibility assays, is in contrast with a previous publication in
308 which no inhibitory effect for the simvastatin alone was detected (23). Our hypothesis is that
309 it could be due either to the fact that they were using a different macrodilutions method or that
310 the quality of the simvastatin was not identical (23). Lipid analysis of simvastatin treated *M.*
311 *tuberculosis* confirmed that on the opposite to THL treated cells, PDIM were not reduced, in
312 agreement with the absence of synergy with vancomycin. The amount of PG, PE and TAG
313 was reduced, as the amount $AcPIM_2$ but Ac_2PIM_2 and Ac_2PIM_6 were increased. The action of
314 THL and simvastatin on the mycobacterial cell wall was thus clearly different.

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315 For the fenofibrate, we could not detect any effect, either alone or in combination with
316 vancomycin, on *M. bovis* BCG or *M. tuberculosis*.

317 It is interesting to understand how the bacteria respond to an antibiotic/drug stress, as it could
318 give us a clue to the drug tolerance or resistance developed by bacteria. In this study, we
319 observed that *M. tuberculosis* treated either by THL or simvastatin have a smaller intracellular
320 TAG contents than untreated cells. The TAG reservoir could serve as a fatty acyl/carbon
321 source for recovery, metabolism but could also have been decreased due to the action of these
322 lipid lowering drugs. Proteomic analyses suggested that *M. tuberculosis* under THL stress are
323 trying to recover, expressing more proteins, especially those involved in intermediary
324 metabolism, respiration and information pathways, probably in order to gain energy. The
325 protein upregulation observed in FAS I and FAS II lipid synthesis pathway in THL treated *M.*
326 *tuberculosis* suggests that the bacteria is remodeling his cell wall. These modifications in lipid
327 metabolism were consistent with 3 gene cluster signature profiles (GC82, GC89 and GC120)
328 out of 6 proposed by Boshoff et al. for inhibitors of cell wall biosynthesis (isoniazid,
329 ethionamide, cerulenin), in agreement with our lipid results and previous reports as described
330 above (39). Our proteomic data with THL were compared to the microarray analysis of
331 Waddell et al. providing gene expression profile for THL treated *M. tuberculosis* (40). In
332 agreement with our results, Waddell et al. mostly observed gene upregulation with THL
333 treatment.

334 On the opposite, *M. tuberculosis* under simvastatin, did not seem to try recovering, as very
335 few change in proteins expression were detected. Interestingly, some downregulated proteins
336 are listed in three gene clusters, GC29, GC34 and GC76, described by Boshoff et al.
337 downregulated by the 5-chloropyrazinamide, rifampicin, H_2O_2 (GC29), rifampicin,
338 pyrazinamide, nicotinamide and benzamide (GC34) and rifampicin, pyrazinamide,

nicotinamide, H_2O_2 and UV (GC76), respectively (39). It would be therefore interesting to carry out further studies to investigate whether simvastatin could damage DNA.

In conclusion, we identified a synergy between THL and vancomycin to inhibit *M. bovis* BCG and *M. tuberculosis* growth. Lipids analysis confirmed that THL destabilized the outer membrane of the cell envelope, among other by reducing the amount of PDIM, facilitating the vancomycin action. The concordance of the results obtained by the macrodilution method, the NCCLS agar proportion experiments and the BacT/ALERT MP assays, suggests that the BacT/ALERT MP system is suitable for antituberculous drugs screening. Simvastatin, by itself, also seems a promising antimycobacterial drug, as mycobacteria seemed unable to act in order to recover.

Further studies, especially in vivo assays, should be performed with these drugs, in order to assess their potential use against TB. New formulations should be developed to allow their delivery into the lungs.

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REFERENCES

1. **World Health Organization. 2015.** WHO report 2015. Global tuberculosis control.
2. **Acosta CD, Dadu A, Ramsay A, Dara M.** 2014. Drug-resistant tuberculosis in Eastern Europe: challenges and ways forward. Public Health Action **4**:S3-S12.

3. **Olaru ID, von Groote-Bidlingmaier F, Heyckendorf J, Yew WW, Lange C, Chang KC.** 2015. Novel drugs against tuberculosis: a clinician's perspective. Eur Respir J **45:**1119-1131.
4. **Mdluli K, Kaneko T, Upton A.** 2015. The tuberculosis drug discovery and development pipeline and emerging drug targets. Cold Spring Harb Perspect Med **5:**a021154.
5. **Neyrolles O, Guilhot C.** 2011. Recent advances in deciphering the contribution of *Mycobacterium tuberculosis* lipids to pathogenesis.Tuberculosis (Edinb) **91:**187-195.
6. **Parish T, Liu J, Nikaido H, Stoker NG.** 1997. A *Mycobacterium smegmatis* mutant with a defective inositol monophosphate phosphatase gene homolog has altered cell envelope permeability. J Bacteriol **179:**7827-33.
7. **Soetaert K, Rens C, Wang XM, De Bruyn J, Lanéelle MA, Laval F, Lemassu A, Daffé M, Bifani P, Fontaine V, Lefèvre P.**2015. Increased Vancomycin Susceptibility in Mycobacteria: a New Approach To Identify Synergistic Activity against Multidrug-Resistant Mycobacteria. Antimicrob Agents Chemother **59:**5057-5060.
8. **Daffé, M., Crick, D. and Jackson, M.** 2014. Genetics of capsular polysaccharides and cell envelope (glyco)lipids. Microbiol Spectrum **2:**MGM2-0021-2013.
9. **Hadváry P, Sidler W, Meister W, Vetter W, Wolfer H.** 1991. The lipase inhibitor tetrahydrolipstatin binds covalently to the putative active site serine of pancreatic lipase. J Biol Chem **266:**2021-2027.
10. **Haalck L, Spener F.** 1997. On the inhibition of microbial lipases by Tetrahydrolipstatin. Methods in Enzymology **286:**252–263.
11. **Saxena AK, Roy KK, Singh S, Vishnoi SP, Kumar A, Kashyap VK, Kremer L, Srivastava R, Srivastava BS.** 2013. Identification and characterisation of small-

molecule inhibitors of Rv3097c-encoded lipase (LipY) of *Mycobacterium tuberculosis* that selectively inhibit growth of bacilli in hypoxia. Int J Antimicrob Agents **42:**27-35.

12. **Dhouib R, Ducret A, Hubert P, Carrière F, Dukan S, Canaan S.** 2011. Watching intracellular lipolysis in mycobacteria using time lapse fluorescence microscopy. Biochim Biophys Acta **1811:**234-241.
13. **Singh G, Arya S, Narang D, Jadeja D, Singh G, Gupta UD, Singh K, Kaur J.** 2014. Characterization of an acid inducible lipase Rv3203 from *Mycobacterium tuberculosis* H37Rv. Mol Biol Rep **41:**285-296.
14. **Côtes K, Dhouib R, Douchet I, Chahinian H, de Caro A, Carrière F, Canaan S.** 2007. Characterization of an exported monoglyceride lipase from *Mycobacterium tuberculosis* possibly involved in the metabolism of host cell membrane lipids. Biochem J **408:**417-427.
15. **Low KL, Rao PS, Shui G, Bendt AK, Pethe K, Dick T, Wenk MR.** 2009. Triacylglycerol utilization is required for regrowth of in vitro hypoxic nonreplicating *Mycobacterium bovis* bacillus Calmette-Guerin. J Bacteriol **191:**5037-5043.
16. **Crellin PK, Vivian JP, Scoble J, Chow FM, West NP, Brammananth R, Proellocks NI, Shahine A, Le Nours J, Wilce MC, Britton WJ, Coppel RL, Rossjohn J, Beddoe T.** 2010. Tetrahydrolipstatin inhibition, functional analyses, and three-dimensional structure of a lipase essential for mycobacterial viability. J Biol Chem **285:**30050-30060.
17. **Seeliger JC, Holsclaw CM, Schelle MW, Botyanszki Z, Gilmore SA, Tully SE, Niederweis M, Cravatt BF, Leary JA, Bertozzi CR.** 2012. Elucidation and chemical modulation of sulfolipid-1 biosynthesis in *Mycobacterium tuberculosis*. J Biol Chem **287:**7990-8000.

18. **Parker SK, Barkley RM, Rino JG, Vasil ML.** 2009. *Mycobacterium tuberculosis* Rv3802c encodes a phospholipase/thioesterase and is inhibited by the antimycobacterial agent tetrahydrolipstatin. PLoS One **4:**e4281.

19. **Majumdar A, Wankhade G, Kamble PD, Harinath BC.** 2011. Effect of HIV protease inhibitors and Orlistat on mycobacterial ES-31 serine protease, a potential drug target in *Mycobacterium tuberculosis*. Indian J Tuberc **58:**4-10.

20. **Ravindran MS, Rao SP, Cheng X, Shukla A, Cazenave-Gassiot A, Yao SQ, Wenk MR.** 2014. Targeting lipid esterases in mycobacteria grown under different physiological conditions using activity-based profiling with tetrahydrolipstatin (THL). Mol Cell Proteomics **13:**435-448.

21. **Rao A, Ranganathan A.** 2004. Interaction studies on proteins encoded by the phthiocerol dimycocerosate locus of *Mycobacterium tuberculosis*. Mol Genet Genomics **272:**571-579.

22. **Parihar SP, Guler R, Khutlang R, Lang DM, Hurdayal R, Mhlanga MM, Suzuki H, Marais AD, Brombacher F.** 2014. Statin therapy reduces the *Mycobacterium tuberculosis* burden in human macrophages and in mice by enhancing autophagy and phagosome maturation. J Infect Dis **209:**754-763.

23. **Skerry C, Pinn ML, Bruiners N, Pine R, Gennaro ML, Karakousis PC.** 2014. Simvastatin increases the in vivo activity of the first-line tuberculosis regimen. J Antimicrob Chemother **69:**2453-2457.

24. **Garbe TR.** 2004. Co-induction of methyltransferase Rv0560c by naphthoquinones and fibric acids suggests attenuation of isoprenoid quinone action in Mycobacterium tuberculosis. Can J Microbiol **50:**771-778.

25. **Reich-Slotky R, Kabbash C, Della-Latta P, Blanchard J, Feinmark S, Freeman S, Kaplan G, Shuman H, and Silverstein S.** 2009. Gemfibrozil Inhibits Legionella

pneumophila and *Mycobacterium tuberculosis* Enoyl Coenzyme A Reductases and Blocks Intracellular Growth of These Bacteria in Macrophages. J Bacteriol **191:**5262–5271.

26. **Kim SJ, Hong M, Song KD, Lee HK, Ryoo S, Heo TH.** 2014. Normalization of the levels of inflammatory molecules in *Mycobacterium smegmatis*-infected U937 cells by fibrate pretreatment. Biol Res **47:**42.
27. **National Committee for Clinical Laboratory Standards.** 2003. Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes. Approved standard M24-A. National Committee for Clinical Laboratory Standards, Wayne, PA.
28. **Lorian V, M.D.** 2005. Antibiotics in Laboratory. Medicine.Editor, 5th ed. Lippincott Williams&Wilkins, Philadelphia.
29. **Hsieh MH, Yu CM, Yu VL, Chow JW.** 1993. Synergy assessed by checkerboard. A critical analysis. Diagn Microbiol Infect Dis **16:**343-349.
30. **Mathys V, Wintjens R, Lefevre P, Bertout J, Singhal A, Kiass M, Kurepina N, Wang XM, Mathema B, Baulard A, Kreiswirth BN, Bifani P.** 2009. Molecular genetics of para-aminosalicylic acid resistance in clinical isolates and spontaneous mutants of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 53:2100-2109.
31. **Werngren J, Klintz L, Hoffner SE.** 2006. Evaluation of a novel kit for use with the BacT/ALERT 3D system for drug susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol **44:**2130-2132.
32. **David S.** 2001. Synergic activity of D-cycloserine and beta-chloro-D-alanine against *Mycobacterium tuberculosis*.J Antimicrob Chemother **47:**203-206.
33. **Singh P, Wesley C, Jadaun GP, Malonia SK, Das R, Upadhyay P, Faujdar J, Sharma P, Gupta P, Mishra AK, Singh K, Chauhan DS, Sharma VD, Gupta UD,**

459 **Venkatesan K, Katoch VM.** 2007. Comparative evaluation of Löwenstein-Jensen
460 proportion method, BacT/ALERT 3D system, and enzymatic pyrazinamidase assay for
461 pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol
462 **45:**76-80.

463 34. **Roxane Simeone, Gaelle Huet, Patricia Constant, Wladimir Malaga, Anne**
464 **Lemassu,Francoise Laval, Mamadou Daffe, Christophe Guilhot, Christian**
465 **Chalut.** 2013. Functional Characterization of Three O-methyltransferases Involved in
466 the Biosynthesis of Phenolglycolipids in *Mycobacterium tuberculosis*. PLoS One
467 **8:**e58954.

468 35. **Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J,**
469 **Simonovic M, Roth A, Santos A, Tsafou KP, Kuhn M, Bork P,Jensen LJ, von**
470 **Mering C.** 2015. STRING v10: protein-protein interaction networks, integrated over
471 the tree of life. Nucleic Acids Res **43:**D447-52.

472 36. **Gillet LC, Navarro P, Tate S, Röst H, Selevsek N, Reiter L, Bonner R, Aebersold**
473 **R.** Targeted data extraction of the MS/MS spectra generated by data-independent
474 acquisition: a new concept for consistent and accurate proteome analysis. 2012. Mol
475 Cell Proteomics **11:**O111.016717-1-17

476 37. **Belardinelli JM, Larrouy-Maumus G, Jones V, Sorio de Carvalho LP, McNeil**
477 **MR, Jackson M.** 2014. Biosynthesis and translocation of unsulfated acyltrehaloses in
478 *Mycobacterium tuberculosis*. J Biol Chem **289:**27952-27965.

479 38. **Kremer L, de Chastellier C, Dobson G, Gibson KJ, Bifani P, Balor S, Gorvel JP,**
480 **Locht C, Minnikin DE, Besra GS.** 2005. Identification and structural
481 characterization of an unusual mycobacterial monomeromycolyl-diacylglycerol. Mol
482 Microbiol **57:**1113-1126.

39. **Boshoff HI, Myers TG, Copp BR, McNeil MR, Wilson MA, Barry CE.** 2004. The transcriptional responses of *Mycobacterium tuberculosis* to inhibitors of metabolism: novel insights into drug mechanisms of action. J Biol Chem **279:**40174-40184.

40. **Waddell SJ, Stabler RA, Laing K, Kremer L, Reynolds RC, Besra GS.** 2004. The use of microarray analysis to determine the gene expression profiles of *Mycobacterium tuberculosis* in response to anti-bacterial compounds. Tuberculosis **84:**263-274.

Figure Legends

FIG 1 Drug susceptibility assay on *M. bovis* BCG. Fluorometric reflectance experiment record the normal and delayed growth of *Mycobacterium bovis* BCG with or without drugs. Growth curves were compared to a 1/100 inoculum dilution to evaluate the MIC. Representative growth curves of the initial inoculum (BCG 1/1), of the dilution (BCG 1/100) and of the initial inoculum with a fixed 5 µg/ml vancomycin (V5) combined with 10 µg/ml THL (THL10) or with 10 µg/ml simvastatin (S10) or with 10 µg/ml of fenofibrate (F10).

FIG 2 THL synergizes with vancomycin. Representative growth curves of the initial inoculum (H37Rv 1/1), the dilution (H37Rv 1/100), and the initial inoculum with a fixed 5 µg/ml vancomycin (V5) added to 50 µg/ml THL (THL50) or to 100 µg/ml fenofibrate (F100) or to 100 µg/ml simvastatin (S100).

FIG 3 Lipid modifications detected in *M. tuberculosis* treated with THL (T) or simvastatin (S) when compared to the DMSO control (C). A. HPTLC of PDIMs A, PDIMs B and TAG separated with petroleum ether/diethylether (9:1, v/v) and visualized by primuline. B. HPTLC of lipids separated with $CHCl_3/CH_3OH/H_2O$ (60:35:8, v/v/v), visualized by spraying with a 0.2% anthrone solution (w/v) in concentrated H_2SO_4, followed by heating. C. Lipid

quantification of PDIM A, PDIM B and TAG based on colorimetric and fluorometric analysis of TLC. D. Lipid quantification of representative lipids based on colorimetric and fluorometric analysis of TLC. In each graph, means ± error bars of three independent experiments data converted to a percentage, with untreated samples taken as 100%, are shown. For THL treated or simvastatin treated samples versus untreated samples, three independent experiments were analyzed by TLC and quantified.

527 TABLE 1 Drug susceptibility results (MIC) obtained in the macrodilution series and analyzed by the
528 checkboard method for *M. bovis* BCG

	vancomycin	THL	simvastatin
	MIC (µg/ml) / FIC / FICI	MIC (µg/ml) / FIC / FICI	MIC (µg/ml) / FIC / FICI
Alone	250 / - / -	5 / - / -	100 / - / -
+ 1µg/ml THL	15 / 0,06 / 0,185-0,31	-/ - / -	-/ - / -
+ 25µg/ml simvastatin	125 / 0,5 / 1	-/ - / -	-/ - / -
+ 10µg/ml vancomycin	-/ - / -	0,625-1,25 /0,125-0,25/0,185-0,31	50 / 0,5 / 1

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TABLE 2 Drug susceptibility results (MIC) obtained with the macrodilution series and with the NCCLS agar proportion methods, both analyzed by the Checkboard method on *M. tuberculosis*

		vancomycin	THL	simvastatin	fenofibrate
		MIC (µg/ml) / FIC / FICI	MIC (µg/ml) / FIC / FICI	MIC (µg/ml) / FIC / FICI	MIC (µg/ml) / FIC / FICI
Alone	MS	100 / - / -	50 / - / -	100 / - / -	-/ - / -
	NCCLS	40 / - / -	5 / - / -	100 / - / -	> 200 / - / -
+ 1µg/ml THL	MS	25 / 0,25 / 0,31	-/ - / -	-/ - / -	-/ - / -
	NCCLS	10/ 0,25 / 0,375	-/ - / -	-/ - / -	-/ - / -
+ 25µg/ml simvastatin	MS	100 / 1 / 1,5	-/ - / -	-/ - / -	-/ - / -
	NCCLS	20 / 0,5 / 1	-/ - / -	-/ - / -	-/ - / -
+ 50µg/ml fenofibrate	MS	-/ - / -	-/ - / -	-/ - / -	-/ - / -
	NCCLS	40 / 1 / 2	-/ - / -	-/ - / -	-/ - / -
+ 10µg/ml vancomycin	MS	-/ - / -	3,1 / 0,06 / 0,31	50 / 0,5 / 1,5	-/ - / -
	NCCLS	-/ - / -	-/ - / -	-/ - / -	-/ - / -
+ 2µg/ml vancomycin	MS	-/ - / -	-/ - / -	-/ - / -	-/ - / -
	NCCLS	-/ - / -	0,625 / 0,125 / 0,375	50 / 0,5 / 1	>200 /1 /2

546 TABLE 3 Summary of up- and down-expressed proteins after 24 h THL or simvastatin

547 treatment[a]

Functional classification	THL		Simvastatin	
	Up	Down	Up	Down
Intermediary metabolism and respiration	99	6	4	19
Lipid metabolism	40	0	2	7
Information pathways	48	1	4	3
Virulence, detoxification, adaptation	12	0	1	2
Cell wall and processes	26	0	0	4
Regulatory proteins	13	0	0	4
Conserved hypotheticals	55	2	5	19

548 [a]*Proteins with a p-value < 0,05*

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