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Elucidating the antimycobacterial mechanism of action of ciprofloxacin using metabolomics

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Abstract: In the interest of developing more effective and safer anti-Tuberculosis treatment, we aimed for a better understanding of the antimycobacterial action of ciprofloxacin against Mycobacterium tuberculosis (Mtb). We used GCxGC-TOF-MS and well described metabolomics statistical approaches, to investigate and compare the metabolic profiles of Mtb in the presence and absence of the drug. The metabolites that best describe the differences between the compared groups were identified as markers characterizing the changes induced by ciprofloxacin. Malic acid was ranked as the most significantly altered metabolite marker induced by ciprofloxacin, indicative of an inhibition of the tricarboxylic acid (TCA) and glyoxylate cycle of Mtb. The altered fatty acid, myo-inositol and triacylglycerol metabolism seen in this group, supports the previous observations of ciprofloxacin action on the Mtb cell wall. Furthermore, the altered pentose phosphate intermediates, glycerol metabolism markers, glucose accumulation, and the reduction in the glucogenic amino acids specifically, indicates a flux towards DNA (as well as cell wall) repair, also supporting previous findings of DNA damage caused by ciprofloxacin. This study further provides insights useful for designing network whole-system strategies for the identification of possible modes of actions of various drugs and possibly adaptations by Mtb resulting in resistance.

Keywords: Fluoroquinolones; Ciprofloxacin; Untargeted Metabolomics; Mycobacterium tuberculosis; Tuberculosis; GCxGC-TOFMS

1. Introduction

Tuberculosis (TB), caused by the Mycobacterium tuberculosis (Mtb), is the leading cause of death globally from a single infectious agent [1], resulting in a mortality rate of 1.5 million and an infection rate of about 10 million annually [2]. Furthermore, the prevalence of drug resistant TB is also on the rise, which is mainly attributed to poor patient adherence to the drug regimen, [3] as a result of the many side-effects experienced by patients being treated with first-line anti TB medication, accompanied by the long treatment duration required [4,5]. Further contributing factors to developing drug resistant TB include; inaccurate diagnosis, unsupervised treatment, poor economic status [6], and in 2020, was further exacerbated by the COVID-19 pandemic [2,7]. Currently, the WHO’s approved first-line therapy for patients with active TB, is a 6-month “directly observed treatment short-course” (DOTS) regimen, and consists of isoniazid, ethambutol, pyrazinamide and rifampicin [8,9]. Infection with multi-drug resistant (MDR)-TB and extensively drug resistant (XDR)-TB requires treatment using various second-line antibiotics, which are expensive, have far more side effects due to their higher toxicity and need to be consumed for even a longer duration [2,10]. The only newly approved drugs for TB over the last 50 years, are the second-line drugs for treating MDR-TB; linezolid, bedaquiline and delamanid, and, not long after, resistance followed [9,11,12]. Consider-
ing this, there is an urgent need for well-tolerated and effective treatment for TB, using drugs with novel mode of action against the infectious organism.

A suggested approach for avoiding the long drug trial-phases usually required for approving new drug candidates, is further investigation of already existing drugs, repurposed for use in treating TB/MDR-TB, [13,14]. In order for a drug to be selected for possible repurposing applications, it should preferably be affordable, easily available and show good pharmacokinetic/pharmacodynamic properties. For these reasons and of late, several fluoroquinolones (FQs) are intensively being investigated for use as anti-TB therapy [15], FQs, originally used to treat urinary tract infection [16], were first shown to be effective against \textit{Mtb} in 1984, and have since gained continuous interest for such applications [17-19]. Currently, they are among the most frequently prescribed drugs [20] and are considered the backbone of MDR-TB treatment [10,21]. FQs target two \textit{Mtb} topoisomerase deoxyribonucleic acid (DNA) enzymes, DNA gyrase and topoisomerase IV [22]. The first introduces negative super helical twists in the bacterial DNA-double helix and catalyses the separation of daughter chromosomes [23], whereas the latter is responsible for the segregation into two daughter cells at the end of DNA replication [24]. Earlier generations, ciprofloxacin and levofloxacin, exhibit greater activity against gram-negative bacteria (and some gram-positive bacteria), and target mainly DNA gyrase [25-27]. Newer generation FQs, gatifloxacin, ol Roxacin and moxifloxacin, show greater activity against gram-positive bacteria and anaerobes, and targets both, DNA gyrase and topoisomerase IV [28]. In \textit{Mtb}, however, only DNA gyrase is present, which is capable of carrying out the action of both topoisomerases [4]. FQs inhibit DNA gyrase by binding to the enzyme and DNA, which leads to double-stranded DNA breaks [29]. When stabilized, replication and transcription cannot happen, leading to slow \textit{Mtb} cell death. If the topoisomerase is removed, the double stranded breaks are processed into single-strand DNA, which, if left unrepaired, leads to lethal chromosomal fragmentation [30]. These are recognized by the DNA damage response regulon, recA/lexA, which in turn induce the bacterial stress (SOS) response. During the SOS response, DNA repair is activated, reactive oxygen species (ROS) are released, and cell growth arrest is initiated [31]. The underlying molecular mechanisms are, however, still largely unclear [32].

The innate resistance mechanism of \textit{Mtb} to many anti-TB drugs can be attributed to its complex cell wall [33], however, the specific, highly lipophilic characteristics of FQs [34], provide great permeability over this [35]. These antibiotics chelate with Mg2+ cations and electrostatically interact with membrane phosphodiester, subsequently traversing the \textit{Mtb} cell wall [36]. FQ’s are not exempt from resistance however, of which the best described are mutations in genes \textit{gyrA} and \textit{gyrB}, encoding subunit Gyra and GyrB of DNA gyrase [37-39]. Furthermore, several resistance-forming proteins have also been identified; the efflux pumps, \textit{LfrA} [40,41] and \textit{MmpL} (mycobacterium membrane protein large) [42], the target protection proteins, \textit{MfpA} and \textit{MfpB} (\textit{Mycobacterium} fluoroquinolone resistance proteins) [43,44], and the cell-survival promoter, \textit{HtrA2} (high temperature requirement A) [45]. DNA repair and mutations by the SOS regulon have also been described [18].

Interactions between DNA gyrase and FQs have been thoroughly investigated [22,38,46]. Still, little is known about the overall biochemical mechanisms of action against \textit{Mtb} specifically, and drug resistance to these [10,19]. The results published thus far are somewhat contradictory. In a study by Verma, \textit{et al.} [47], the macromolecular composition of the \textit{M. smegmatis} cell wall after sub-MIC cipro treatment, indicated a significant decrease in the total lipids, phospholipids and sugars, suggesting ciprofloxacin-induced alterations of the cell wall. In contrast, Halouska, \textit{et al.} [48] indicated ciprofloxacin-induced inhibition of transcription, translation and DNA supercoiling, without effects on the cell wall. While most data suggest cell death due to the inhibition of DNA replication [49], altered DNA biosynthesis could set in motion secondary events contributing to its bacteriostatic or bacteriocidic effects. Furthermore, it is important to remember that stronger target activity does not predict better antimycobacterial activity. This is perfectly demonstrated by ciprofloxacin-dimers, which show enhanced DNA gyrase in-
hilation, while less effectively killing \textit{Mtb} [50]. Proposely, this is a result of stronger cleavage of \textit{FQ} to the DNA-enzyme complex, which results in less single strand DNA fragments, and subsequently prevents RecA from recognizing damaged DNA and inducing the SOS regulon [32]. The SOS response assists in killing by releasing ROS [51,52], yet simultaneously activates DNA repair and creates a dormancy-state, ultimately leading to resistance. Before the SOS response can be used as an advantage, this phenomenon, and how it connects to the mechanism of ciprofloxacin, still needs to be elaborated on.

The selection of ciprofloxacin as our investigational compound is predominantly based on its safety profile. Although less potent than moxifloxacin (MIC 0.12–0.5 \(\mu\)g/mL), and levofloxacin (MIC 1 \(\mu\)g/mL), ciprofloxacin (MIC 0.5–4.0 \(\mu\)g/mL) [53] has the lowest risk for causing serious ventricular arrhythmia, cardiovascular mortality, and hepatotoxicity [54,55]. Furthermore, ciprofloxacin demonstrates the highest clearance rate of all \textit{FQ}'s [56] and is thus the preferred option for treatment of renally-impaired patients [17]. The levels of ciprofloxacin in cerebrospinal fluid can be as high as 40–90% compared to that of plasma [53], which offers further advantages for its use in the treatment of tuberculous meningitis. Adverse drug reactions (ADRs) are usually minimal (5\% or less) and the most common ADRs are usually gastrointestinal in nature (nausea, vomiting, diarrhea, and abdominal pain [57,58]). Previous studies demonstrated that mitochondrial topoisomerases bear less than 30\% homology to their prokaryotic counterparts and are not inhibited [23,59], and it has been reported that ciprofloxacin does affect mitochondrial DNA synthesis [60]. These and many other advantages of ciprofloxacin have not gone unnoticed, as stated by the World Health Organization [61], who has included ciprofloxacin as a critically important antibiotic.

Most of the evidence brought to light thus far has been discovered using genomics, transcriptomics or proteomics [62]. Metabolomics, the latest addition to “omics” technologies, identifies the down-stream metabolites of altered pathways and therefor presents a more sensitive level of organization, of which up-stream deductions can be made [8,63]. We identified the metabolite markers best differentiating \textit{Mtb} with and without ciprofloxacin, using a two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-TOF-MS) metabolomics approach, combined with universally connected metabolic libraries and advanced statistical analysis, in order to better elucidate its mechanism of action.

2. Materials and Methods

2.1. Cell culture

Antimycobacterial minimum inhibitory concentration (MIC) and sub-MIC (50\% inhibitory concentrations (MICs)) of ciprofloxacin was determined via the Alamar Blue Essay [64]. The cell cultures (5 individually cultured samples per group) were prepared as previously described [65], in the presence and absence of ciprofloxacin. All reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise stated. Briefly, \textit{Mtb} H37Rv ATCC 27294 (kindly obtained from the Medical Research Council, Pretoria, Gauteng, South Africa) was cultured and maintained for 4 weeks on LJ slants. The bacterial inoculum was prepared to a McFarland standard of 1 (approximately 3 \(\times\) 10\(^8\) colony-forming units/mL) in Middlebrook 7H9 broth supplemented with 10\% OADC (oleic acid, albumin, dextrose, catalase) (Becton, Dickinson, UK) and 2\% PANTA (polymyxin B, amphotericin B, nalidixic acid and trimethoprim, azlocillin) (Becton, Dickinson, UK). PANTA was added for the prevention of contamination with negligible impact on \textit{Mtb}'s growth [66]. Ciprofloxacin was dissolved in DMSO (150 \(\mu\)M) and diluted into Middlebrook 7H9 broth to a final concentration of 0.3 \(\mu\)M (0.12 \(\mu\)g/mL) (0.2 \% DMSO). One milliliter of the prepared inoculum was added to yield a final assay volume of 5 mL, with a bacterial test concentration of 6 \(\times\) 10\(^7\) CFU/mL ciprofloxacin. For the untreated \textit{Mtb} control samples, 4 mL of Middlebrook 7H9 broth (0.2 \% DMSO) was added to each replicate culture, followed by the addition of the bacterial inoculum as described.
above. The DMSO solvent was kept constant throughout the assay. After 5 days of incubation at 37 °C, the samples were centrifuged to pellet the bacteria at 4500 rpm for 15 min. The pellets were washed with 1 mL of PBS and pelleted again under the same conditions. Finally, the PBS was aspirated from the samples and the pellets were stored immediately at -80 °C until further testing.

2.2. Whole metabolome extraction procedure and derivatization

The metabolites were extracted from the samples and derivatized as previously described by Beukes, et al. [67], with slight modifications. Briefly, 8 mg of each of the individually cultured samples were weighed out into an Eppendorf tube, followed by the addition of 50 µL 3-Phenylbutyric acid (0.13 mg/ml H2O) (Sigma-Aldrich, Lot#536478V) as internal standard. One milliliter of a chloroform: methanol: water (1:3:1 ratio) solution was added, after which the Eppendorf tubes were shaken in a vibration mill at 30 Hz for 5 min, with a 3 mm carbide tungsten bead in each. The samples were centrifuged at 12 000 rpm for 5 min and the supernatant was transferred to a GC glass vial. The extracts were dried under a nitrogen stream, followed by the addition of 50 µL methoxamine hydrochloride (Sigma-Aldrich, Lot#BCBP2843V) in pyridine (Lot#52BC3355V) at a concentration of 15 mg/mL. The glass vials were heated at 50°C for 90 min. Following, methoximation, 40 µL N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylsilyl chloride (Lot#BCBW2670) was added, and vials were heated again for 60 min at 50°C. Each extract was then transferred to a 0.1 mL vial insert in a GC sample vial and injected into GCxGC-TOF-MS.

2.3. GCxGC-TOFMS analysis

A 4D Pegasus GCxGC-TOF-MS (LECO Africa (Pty) Ltd, Johannesburg, South Africa), equipped with a Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany) and an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA) coupled to TOF-MS (LECO Africa) was used for the analysis. The samples were analyzed in random sequence, in a split-less ratio. To monitor the analytical performance throughout the entire analysis, a quality control (QC) sample was analyzed at regular intervals. The processed samples were injected into Rxi-5Sil MSprimary capillary column (28.8 m x 0.25 mm internal diameter, 0.25 µm film thickness, Restec), and a Rxi-17 secondary capillary column (1.2 m x 0.25 mm internal diameter, 0.25 µm film thickness), for GC compound separation. The primary GC oven temperature was set at 70°C for 2 min, and then increased at a rate of 4°C/min to a final temperature of 300°C, at which it was maintained for an additional 2 min. The secondary oven was set at 85°C for 2 min, increased at 4.5°C/min, to a final temperature of 300°C, at which it was maintained for 4.5 min. Helium, set to a column flow rate of 1 mL/min, was used as a carrier gas, and held at a constant temperature of 270°C. Mass spectrometric data acquisition was carried out at -70 eV, with a solution delay of 350 sec, and a mass range of 50-800 m/z was scanned with a rate of 200 spectra/sec.

2.4. Data processing, clean-up and statistics

ChromaTof software (version 4.32) was used for mass spectral deconvolution (at a signal to noise ratio of 20), peak alignment and peak identification on the obtained mass spectra. Metabolites were identified by comparing their mass fragment patterns to that of compounds in commercially available databases containing previously injected standards. For normalization and assessment of data quality, the data were pretreated using a standardized metabolomics data clean-up procedure [67]. Each detected compound was normalized using MS total useful signal (TUS), which is based on a factor calculated from the sum of all metabolites identified in all samples, and by calculating the relative concentration of each by using the internal standard. All missing/zero values were replaced by a value calculated as 20% of the minimum detection limit of the entire dataset, as these are most likely present in sub-minimum concentrations rather than being completely
An 80% data filter was then applied to eliminate compounds with more than 80% zero values within both groups. To provide a balanced representation of all metabolites, log transformation and auto-scaling (mean-centered and divided by the standard deviation of each variable) was applied. This prevents compounds with minor concentrations to be overlooked, due to domination of compounds with higher concentrations. Making use of MetaboAnalyst (Version 5.0), multivariate statistical methods in the form of unsupervised principal component analysis (PCA) and supervised partial least squares-discriminant analysis (PLS-DA) were applied. Subsequently, uni-variate analysis was done by calculating t-test and effect size values.

Relationships between the selected metabolites were mapped using the KEGG, MetaCyc and BioCyc databases, in addition to intensive research of the previously published literature on the topic.

3. Results

3.1. Data overview

When visualising the analytical technique’s repeatability graphically (Figure 1), approximately 86% of all the compounds identified (n=260) had a coefficient of variation (CV) value under 50%, of which 70% had CV values under the 20%. The analytical technique used during this analysis thus proves to be highly repeatable and can be trusted to provide reliable results. PCA was initially used to get an overview of the natural grouping of metabolic data (Figure 2). The total variance between the groups, described by the first two principal components (PCs), was 57.1%, of which PC1 and PC2 accounted for 32.1% and 25%, respectively. The PCA scores plot of the metabolite data analyzed by GCxGC-TOF-MS shows clear clustering between Mtb in the presence and absence of ciprofloxacin, as represented in Figure 2.
Figure 2. PCA scores plot obtained from GCxGC-TOFMS whole metabolome analysis of Mtb samples in the presence and absence of ciprofloxacin. The variances accounted for are indicated in parenthesis.

3.2. Marker selection

The met metabolite markers (n=26) best describing the differences between the ciprofloxacin and control samples were selected based on compliance with the following criteria: a PLS-DA VIP value > 1 [74], a t-test P-value < 0.05 [75] or an effect size > 0.8 [76] (Figure 3).

The selected metabolites markers are listed according to their PLS-DA VIP values in Table 1, along with their respective average concentrations and univariate test outcomes. Of the total, 61.5 % (16/26) markers were elevated, of which most include fatty acids. The most differentiating marker was malic acid, with an exceptionally high d-value of 6.621 and low p-value of >0.0001.

![Venn diagram](image)

Figure 3. Venn diagram illustrating the multi-statistical approach for selecting the metabolites that best describe the variation detected in the metabolome of Mtb cultured with and without ciprofloxacin.

| Metabolite marker | Average concentration (mg/g cell mass, standard deviation) | t-test (P-value) | Effect size (d-value) | PLS-DA (VIP) | Fold Change (log2) |
|-------------------|----------------------------------------------------------|-----------------|----------------------|--------------|------------------|
| Mtb with ciprofloxacin |                                                     |                 |                      |              |                  |
| Mtb controls      |                                                     |                 |                      |              |                  |
| Metabolite                                   | Control Mean (SE) | Ciprofloxacin Mean (SE) | p-Value |
|---------------------------------------------|-------------------|-------------------------|---------|
| Glutamic acid (16015)                      | 0.10 (0.001)      | 0.15 (0.006)            | 0.124   |
| Hexadecanoic acid (15756)                  | 0.575 (0.051)     | 1.0 (0.077)             | 0.128   |
| 9-Octadecenoic acid (36021)                | 0.679 (0.060)     | 0.6 (0.041)             | 0.132   |
| Tetradecanoic acid (28875)                 | 0.089 (0.010)     | 0.2 (0.017)             | 0.141   |
| Eicosanoic acid (28822)                    | 0.005 (0.001)     | 0.004 (0.000)           | 0.146   |
| Erythritol (17113)                         | 0.021 (0.002)     | 0.023 (0.001)           | 0.202   |

4. Discussion

In this study, we identified a number of significantly altered metabolites induced by the administration of ciprofloxacin to Mtb culture, which when interpreted, in the light of known metabolism and previous ciprofloxacin findings, better elucidate its mechanisms of action against Mtb, as shown in Figure 4. The most prominently altered pathways included gluconeogenesis, fatty acid metabolism, amino acid metabolism, the pentose phosphate pathway (PPP) and the urea cycle.

Of note, was the elevation of many of the even and odd chain saturated fatty acids of between 14 to 20 carbons (C14:0-C20:0) in length, in Mtb treated with ciprofloxacin. This was also true for two Δ^2-unsaturated fatty acids; 9-hexadecenoic (Δ^2C16:1) and 9-octadecenoic (Δ^2C18:1) acids. These indicate a strongly upregulated synthesis towards cell wall repair, supporting previous evidence associating ciprofloxacin with cell wall damage [47]. Simplified, lying outside of the cytoplasmic membrane, a peptidoglycan (PG) layer is covalently attached to arabinogalactan (AG), which itself attaches to mycolic acids (MA), to form the MA-AG-PG complex (MAPc) [77,78]. Interspersed within the
MAPc, are the glycerolipids, phosphatidyl myo-inositol mannosides (PIM) and lipoarabinomannans (LAM) [79]. PIM is a crucial part of the membrane structure and serves as a precursor of LAM [80]. The saturated fatty acid markers in this study are produced by fatty acid synthase type I (FAS I). FAS I generates 16 to 26 carbon length fatty acyl-coenzyme A’s (CoA) [81], which are fed into FAS II for elongation. FAS I and FAS II provide acyl-groups for the synthesis of all cell envelope components, except for AG [82,83], Δ⁹C16:1 and Δ⁹C18:1, and their precursors, hexadecanoic (C16:0) and octadecanoic (C18:0) acid, respectively, are considered major fatty acids of glycerolipids and mycolic acids [84-86], Δ⁹C16:1 and Δ⁶C18:1 are reduced from C16:0 and C18:0, in the presence of Fe²⁺, a flavin, NADPH and O₂ [87,88]. Interestingly, some mmpL genes, encoding fatty acid transporter protein MmpL, have been shown to be repressed when their transcriptional regulator proteins bind to C:16 fatty acids and monoacylglycerols (MAG) [89,90]. Even so, further research is needed to establish possible activity of different fatty acids on different MmpL regulator proteins. It is, however, important to note, that the damage that ciprofloxacin administration induces to the cell wall, may be direct, but is most likely indirect, by inhibition of other energy producing mechanisms/or simply by inducing the SOS response in Mtb, shifting energy production away from glucose, toward using preferably fatty acids [91], and hence less of these fatty acids are now available to cell wall synthesis.

The dramatically elevated synthesis of intracellular fatty acids would be expected to consume a considerable amount of carbon, which can be supplied from various sources [91]. However, as will be explained, glucose and glyceral seem to be the major suppliers for such, and the various components required for DNA repair. This is supported by previous findings indicating ROS, produced during the FQ-induced SOS-response, causes oxidative stress, which in turn activates utilization of triacylglycerol (TAG) and cell wall lipids for energy [92-94], as is generally the case during the non-replicative phase of Mtb [95,96]. These results are also supported by previous findings showing reduced concentrations of phospholipids and mycolic acids in the cell wall macromolecules of sub-MIC ciprofloxacin-treated M. smegmatis [47], since these are now being preferentially used for energy production, with glucose supplying the necessary carbon substrates for the continued synthesis of these much-needed fatty acids which are now preferentially used for energy production.
A shift in energy supply to β-oxidation of fatty acids, as opposed to the TCA cycle, reserves NAD⁺, in addition to CO₂, for de novo synthesis of nucleotides (for DNA repair and fatty acids). NADH and NADPH released during fatty acid metabolism, fuels the upregulated non-oxidative PPP and glycero lipid metabolic pathway. In this study, the downregulated TCA cycle is indicated by a reduction in malic acid and aspartic acid, which according to the uni-and multivariate statistics, were ranked as the two most important metabolite markers (Table 1). Aspartic acid is considered a validated reporter of oxaloacetate (OAA) [97], and together with malic acid, support TCA cycle inhibition. Although oxidative stress is normally associated with an increase in glyoxylate shunt activity, in this study, the glyoxylate shunt is clearly downregulated. This indicates a greater need of carbon flux through gluconeogenesis towards the PPP and glucose, for subsequent fatty acid and nucleotide synthesis [98,99]. This supports the SOS response-induced decrease in oxidative phosphorylation and increase in energy reserves for DNA repair, in response to FQs [32,97]. It is noteworthy to mention that the ATPase activity of DNA gyrase (29), shows reduced adenosine triphosphate (ATP) conversion in the presence of ciprofloxacin [100], which most likely is for the purpose of reserving ATP for other energy consuming pathways, such as gluconeogenesis and DNA repair [101].

Various PPP intermediates were detected to be altered in the ciprofloxacin treated Mtb, which contribute to DNA and cell wall repair [94,102,103]. Under normal circumstances, erythrose-4-phosphate and glyceraldehyde-3-phosphate produce xylulose-5-phosphate (xylulose-5P) and fructose-6-phosphate (fructose-6P) [91]. The latter is converted to glucose-6-phosphate, for subsequent PIM synthesis, via the myo-inositol pathway [104], and to glucose-N-acyl 6-phosphate (GlcN6P), for subsequent PG synthesis [105]. Xylose-5P is the precursor of ribose-5-phosphate (ribose-5P), which, in the presence of ATP, is converted to 5-phosphoribosyl-1-pyrophosphate (pRpp) [98,106]. PRpp is the branch point intermediate of decaprenyl-phospho-arabinofuranose (DPA) [107], the only donor of arabinose to AG and LAM [99,108] and nucleotide synthesis. In this investigation, the upregulated non-oxidative PPP in the ciprofloxacin treated Mtb, is supported by the elevated concentrations of xylofuranose and reduced erythritol (Figure 4).

Furthermore, it is well known, that PPP metabolism is fueled by glucose and the glucogenic amino acids; valine, aspartic acid and glutamic acid [109], all of which were specifically and significantly reduced in the ciprofloxacin-treated Mtb, in addition to their degradation products; β-aminoisobutanoic acid [110], 5-oxoproline [111] and N-acetyl-lysine [112], respectively (Figure 4). Furthermore, the reduced levels of glutamic acid and aspartic acid in our investigation, support an oxidative state in the ciprofloxacin treated group [113]. The above-mentioned amino acids also serve as precursors of cell wall related intermediates. Alanine, derived from aspartic acid, and valine combined, produce CoA, which is used for the use of fatty acids during the synthesis of cell wall lipids [114-116]. Valine also serves as a precusor of propionyl-CoA [110,117] for the elongation of odd chain fatty acids [118]. Aspartic acid is the precursor of NAD⁺, 2,6-diaminopimelate (DAP) [119,120] and S-adenosyl methionine (SAM) [121]. Here, NAD⁺ serves as a cofactor for FAS I [122], and SAM is required for the methylation of cell-wall fatty acids [123]. DAP, along with alanine and glutamic acid, serve as substrates for PG [124]. The flux through aspartic acid can be supported by the elevated levels of urea in Mtb treated with ciprofloxacin [125]. Considering that aspartic acid is one of the top 3 metabolite markers identified (Table 1), it is possibly utilized for many, if not all, of these cell-envelope associated intermediates.

Furthermore, the FQ induced SOS response is known to cause cell growth arrest [126]. In this study, inhibited cell growth is suggested by increased TAG metabolism and reduced TCA activity, in addition to the inhibited protein synthesis. Protein synthesis requires ATP-dependent activation of glutamate and aspartate, followed by amination by...
ammonia, to form aminoacyl-tRNA [109]. However, in this study, urea was found elevated in \( Mtb \) treated with ciprofloxacin, indicating accumulation of ammonia and reduced recycling of nitrogen via glutamine and proline metabolism. This, in addition to a decreased degradation of urea by urease, also linked to the \( Mtb \) stress-response [127], retains ammonia from protein synthesis [128]. Inhibited protein formation has also been suggested in a proteomics study of \( Mtb \) treated with ciprofloxacin [129], as well as a metabolomics study of \( M. smegmatis \) treated with ciprofloxacin [48]. Inhibited protein synthesis would in turn disrupt the functionality of membrane proteins, subsequently inhibiting nutrient uptake as well as fatty acid transport to the cell wall [42]. Additionally, urea acts as an osmolyte preventing dehydration or water loss as a result of the seemingly damaged cell wall [130]. Increased urea could also be indicative of polyamine synthesis (Figure 4) [131]. Polyamines, such as putrescine, have been reported to reduce accumulation of trans-membrane proteins [132] and contribute to the phenotypic drug resistance to FQs [133].

Changes to the monoacylglycerols (MAG); 1-monomyrystin, 2-monopalmitin and 1-monohexadecanoyl, are indicative of cell envelope changes via the glycerolipid and triacylglycerol (TAG) pathways. TAG in the cell wall [134] can be metabolized during the stress-induced transition to the non-replicated phase [135] and during infection [136]. \( Mtb \)'s adaption during these circumstances involves the use of lipids as main energy reserves as previously mentioned [137]. During the deacylation of TAG to diacylglycerol (DAG), and from DAG to MAG, acyl-CoA’s are released, which are either directed towards of glycerolipid synthesis (Figure 4) or towards energy production [138,139]. Glycerolipid and TAG metabolism share an important intermediate at the branch point, 1,2-diacyl-sn-glycerol 3-phosphate, commonly known as phosphatidate (PA) (Figure 4) [140]. PA is synthesized via 2 pathways: 1) the phosphorylation of DAG, by DAG kinase [141] or 2) the acylation of glycerol-3-phosphate (glycerol-3P) by glycerol phosphate- and acyl glycerol phosphate acyltransferase [142]. In this study, the concentrations of glycerol-3-phosphate, and its precursor, glycerol, were decreased, supporting a MAG metabolic flux towards TAG and PIM in the ciprofloxacin treated \( Mtb \) group. Glycerol-3P appears to recycle the transport of membrane lipids [143] and can thus be expected to be the rate limiting step, which would explain the accumulation of the fatty acids and the accompanied reduction in glycerol levels. The synthesis of glycerolipids also requires myo-inositol, which is produced via glucose-6-phosphate (glucose-6P) [144]. Firstly, myo-inositol 3-phosphate synthase converts glucose-6P into myo-inositol 3-phosphate, which in turn is dephosphorylated by several myo-inositol monophosphates to produce myo-inositol [84]. The elevated levels of myo-inositol monophosphates in the ciprofloxacin-treated \( Mtb \), hence further supports the aforementioned flux towards glycerolipids [145]. Furthermore, myo-inositol-1-phosphate is converted by a glycosyltransferase, Msh, mycothiol. Mycothiol is also considered an important antioxidant required for balancing the cytosolic NAD\(^+\)/NADH ratio [146]. In summary, the mycobacterial cell wall metabolism is visibly linked to the SOS response, which has frequently been proposed to cause resistance towards ciprofloxacin and challenge the otherwise impressive bactericidal activity of this drug [18,147-149].

5. Conclusions

In this study, we investigated the metabolic changes to \( Mtb \) induced by sub-MIC of ciprofloxacin, in order to better understand its mechanism of action and perhaps also the adaptations of \( Mtb \) to this, which may lead to possible drug resistance. Previous studies have identified alterations in transcription, translation, and cell wall synthesis, as part of the mechanism of action of ciprofloxacin against \( Mtb \) (51, 52, 135). Our metabolomics study identified metabolite markers which support previous results, as indicated by the drastic accumulation of cell wall related fatty acids, metabolites associated with an elevated gluconeogenesis and PPP flux towards DNA repair, further supported by a reduc-
tion in specifically the glycogenic amino acids, and increased urea indicative of protein catabolism, as opposed to protein synthesis.

Moreover, many of these markers indicate that ciprofloxacin induces an SOS-induced non-replicative phase in Mtb. The DNA damage-induced SOS response is a key mechanism determining Mtb persistence and tolerance to the drug. Differentiating the pathways resulting in ROS from those leading to drug resistance, and subsequently combining FQ with medication inhibiting the mutation-related pathways, could lead to eradication of resistance and more effective killing. This study not only gives a better understanding of ciprofloxacin’s mode of action but provides helpful insight for further investigation of antibiotic-induced resistance by Mtb, and also perhaps the use of ciprofloxacin, in combination with existing anti-TB drugs.

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