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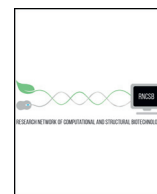
Mechanistic Insights From Global Metabolomics Studies into Synergistic Bactericidal Effect of a Polymyxin B Combination With Tamoxifen Against Cystic Fibrosis MDR *Pseudomonas aeruginosa*

Maytham Hussein [a,b], Mei-Ling Han [c], Yan Zhu [c], Elena K. Schneider-Futschik [a], Xiaohan Hu [a], Qi Tony Zhou [d], Yu-Wei Lin [c], Dovile Anderson [b], Darren J. Creek [b], Daniel Hoyer [a,e,f], Jian Li [c,*], Tony Velkov [a,*]

[a] Department of Pharmacology & Therapeutics, School of Biomedical Sciences, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Parkville 3010, VIC, Australia
[b] Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia
[c] Monash Biomedicine Discovery Institute, Department of Microbiology, School of Biomedical Sciences, Faculty of Medicine, Nursing and Health Sciences, Monash University, Melbourne, Australia
[d] Department of Industrial and Physical Pharmacy, College of Pharmacy, Purdue University, 575 Stadium Mall Drive, West Lafayette, IN 47907, USA
[e] The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville 3052, VIC, Australia
[f] Department of Molecular Medicine, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla 92037, CA, USA

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A B S T R A C T

Polymyxins are amongst the most important antibiotics in modern medicine, in recent times their clinical utility has been overshadowed by nosocomial outbreaks of polymyxin resistant MDR Gram-negative 'superbugs'. An effective strategy to surmount polymyxin resistance is combination therapy with FDA-approved non-antibiotic drugs. Herein we used untargeted metabolomics to investigate the mechanism(s) of synergy between polymyxin B and the selective estrogen receptor modulator (SERM) tamoxifen against a polymyxin-resistant MDR cystic fibrosis (CF) *Pseudomonas aeruginosa* FADDI-PA006 isolate (polymyxin B MIC=8 mg/L , it is an MDR polymyxin resistant *P. aeruginosa* isolated from the lungs of a CF patient). The metabolome of FADDI-PA006 was profiled at 15 min, 1 and 4 h following treatment with polymyxin B (2 mg/L), tamoxifen (8 mg/L) either as monotherapy or in combination. At 15 min, the combination treatment induced a marked decrease in lipids, primarily fatty acid and glycerophospholipid metabolites that are involved in the biosynthesis of bacterial membranes. In line with the polymyxin-resistant status of this strain, at 1 h, both polymyxin B and tamoxifen monotherapies produced little effect on bacterial metabolism. In contrast to the combination which induced extensive reduction (≥ 1.0-log2-fold, $p \leq 0.05$; FDR ≤ 0.05) in the levels of essential intermediates involved in cell envelope biosynthesis. Overall, these novel findings demonstrate that the primary mechanisms underlying the synergistic bactericidal effect of the combination against the polymyxin-resistant *P. aeruginosa* CF isolate FADDI-PA006 involves a disruption of the cell envelope biogenesis and an inhibition of aminoarabinose LPS modifications that confer polymyxin resistance.

1. Introduction

Multi-drug resistant (MDR) *P. aeruginosa* is associated with life-threatening infections, commonly observed in cystic fibrosis (CF) lung, pneumonia, and surgical-site infections [1,2]. *P. aeruginosa* infections are notorious for developing high-level resistance towards a wide array of antibiotics, often leaving clinicians with few treatment options [3–5]. In 2015, the European Antimicrobial Resistance Surveillance Network (EARS-Net) reported that >13.7% of the *P. aeruginosa* isolates were MDR, showing resistance to most of the major antibiotic classes. The World Health Organization (WHO) has recently classified MDR *P. aeruginosa* as a top-priority critical pathogen, urgently requiring the development of new treatment options [6]. In patients suffering from CF, colonization of lungs with *P. aeruginosa* is associated with poorer quality of life and higher mortality rates [7,8].

Polymyxins (colistin and polymyxin B) are currently used as last-line treatment against MDR *P. aeruginosa* infections [9,10]. Although polymyxins exert their antibacterial activity by disorganizing the bacterial outer membrane (OM) via electrostatic interactions with the lipid A component of lipopolysaccharide (LPS), the exact antibacterial killing mechanism is still enigmatic [11]. The most common mechanism of polymyxin resistance seen in *P. aeruginosa* involves LPS modifications, including the addition of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) to its lipid A structure, or by deacylation, hydroxylation and palmitoylation of the lipid A fatty acyls

[12–14]. These structural modifications reduce the overall net negative charge and hydrophobicity of the OM, thereby ablating the binding of the amphipathic polymyxin [2]. Treatment failure due to sub-optimal plasma drug concentrations or resistance to polymyxins in MDR *P. aeruginosa* can emerge after polymyxin monotherapy, which highlights the need for novel combination therapies e.g. with FDA-approved non-antibiotics [15–18]. The use of synergistic combinations of antibiotics with FDA approved non-antibiotic drugs is a highly valuable and cost-effective strategy to enhance the clinical efficacy of currently used antibiotics against MDR bacterial pathogens [19–22]. It has been proposed that polymyxins can increase the OM permeability and enable the non-antibiotic compounds to access their intracellular target(s) [19,21]. We recently showed that combining polymyxin B with selective estrogen receptor modulators (SERMs), namely tamoxifen, raloxifene and toremifene, resulted in synergistic bactericidal activity against a wide range MDR Gram-negative pathogens, particularly MDR *P. aeruginosa* CF isolates [21]. Polymyxin B in combination with toremifene synergistically caused a significant release in cytosolic green fluorescence protein, depolarizing the cytoplasmic membrane and permeabilizing activity in a nitrocefin assay. The combination also induced an increase of cellular reactive oxygen species and a distinctive damage to the outer membrane of *P. aeruginosa* cells. Given the importance of repurposing "off-the-shelf" non-antibiotics drugs to treat MDR *P. aeruginosa*, it is crucial to elucidate the mechanism(s) by which the polymyxin B-SERMs combinations achieve this powerful bacterial killing activity as well as their ability to suppress the emergence of polymyxin resistance.

Antibiotic combination therapies focus on phenotypic killing without systematic or mechanistic investigations [23]. Metabolomics represent a cutting-edge platform that is suitable for investigating the mode of action of and bacterial resistance to antibiotics through analysis of the metabolic response to drug treatment [24,25]. The profiling of significantly perturbed metabolites and reconstruction of their metabolic pathways provides a rapid and effective means for understanding how bacteria respond to the antimicrobials at the molecular level, the mechanism by which antibiotics kill bacterial cells and ultimately the identification of novel antibiotic targets [26–31].

The present study employs a global metabolomics approach to decipher the complex interplay of multiple cellular pathways in a clinical MDR *P. aeruginosa* CF isolate in response to polymyxin B combination treatment with the SERM tamoxifen. The novel findings reveal for the first time that the synergistic bactericidal effect and suppression of polymyxin resistance of the combination involves the disruption of pathways that mediate bacterial cell envelope biogenesis and via inhibition of the OM remodeling processes that confer polymyxin resistance, such as modification of the lipid A phosphates with cationic aminoarabinose moieties.

2. Results

2.1. Multivariate and Univariate Analyses of the Metabolites Affected by Polymyxin B and Tamoxifen in P. aeruginosa.

Multivariate data analysis using partial least squares discriminant analysis (PLS-DA), and univariate data analysis using one-way analysis of variance (ANOVA) followed by Fishers least square difference (LDS), were performed to determine the metabolites in *P. aeruginosa* FADDI-PA006 that were significantly affected by the polymyxin B and tamoxifen treatments (≥ 0.59 -log$_2$-fold; $p \leq .05$; FDR ≤ 0.05). The analytical reproducibility of the LC-MS analysis was determined by periodic analysis of a pooled biological quality control (PBQC) sample (Table S2), which demonstrated a median relative standard deviation (RSD) of 13%, which is well below the acceptable limits (< 20%) in standard metabolomics practice [32]. The reproducibility of all sample groups was acceptable across all time points (15 min, 1 and 4 h), where the median RSD across all time points was (20–29%) for

untreated (control) groups and (20–28%) for the treated groups; consistent with some baseline variability in the dynamics of ordinary bacterial metabolism commonly seen with or without antibiotic treatments (Table S2) [33,34]. The multivariate analysis with PLS-DA revealed that the polymyxin B and tamoxifen monotherapy treatment groups did slightly overlap with untreated (control) groups at 15 min in the first two components, whereas a greater overall difference was observed between the combination treatment group and the untreated (control) group (Fig. S1A(i)). At 1 h, the PLS-DA scores plots showed a marked separation between the combination treatment group and all other treatment groups (i.e. the polymyxin B and tamoxifen monotherapy, and untreated control) in component 1, indicating that polymyxin-tamoxifen therapy at 1 h was more impactful on the bacterial metabolome (Fig. S1A(ii)). At 4 h, nearly all treatment samples clustered with minimal separation from the untreated group control. The effectiveness of all treatments (polymyxin B, tamoxifen monotherapy and their combination) to induce significant changes in bacterial metabolome was similar at 15 min; however, more bacterial metabolome perturbations were observed after polymyxin B-tamoxifen treatment in the subsequent time points (1 and 4 h), which induced perturbations of 115 and 38 metabolites in total, respectively (Fig. S2). Fig. S1B reveals that although most of the significantly affected metabolites (≥ 0.59 -log$_2$-fold; $p \leq .05$; FDR ≤ 0.05) were unique to the polymyxin B-tamoxifen combination treatment particularly at 1 and 4 h; polymyxin B monotherapy had more metabolites in common with the combination than tamoxifen monotherapy. A large proportion of affected metabolites following polymyxin B and tamoxifen monotherapy displayed a significant increase in levels at 15 min, whilst almost all significantly affected metabolites induced by combination therapy exhibited decreased levels (Figs. 1 & 2(i)). Lipids, carbohydrates, and amino acids were the most commonly affected classes. The combination treatment induced more metabolome perturbations compared to polymyxin B and tamoxifen monotherapy at 1 h. Notably, nearly all of the significantly affected metabolites showed a remarkable decline in their levels after combination treatment (Figs. 1 & 2(ii)), and a different distribution of metabolite classes was observed, where carbohydrate intermediates were primarily affected, followed by amino acids, unclassified metabolites and lipid metabolites (Fig. 2(ii)). Only a few metabolites showed were significantly affected following polymyxin B and tamoxifen monotherapy at 1 h (Fig. 2(ii)). At 4 h, different patterns were observed for the combination treatment in which fewer metabolites were impacted and the level ~50% of the metabolites increased dramatically. Similarly to the early time points (15 min and 1 h), most of the significantly affected metabolites following polymyxin B or tamoxifen monotherapy showed an increase (Fig. 2(iii)).

2.2. Impact of the Combination on Lipid Metabolism in P. aeruginosa

Lipids and lipid intermediates underwent markedly more perturbations at 15 min compared to later time points (1 and 4 h) across all treatment groups; notably, all treatments caused greater perturbations in fatty acids compared to glycerophospholipids (GPLs). Both tamoxifen alone and the combination therapy, caused a similar extent of lipid perturbations: all metabolites, primarily fatty acids and glycerophospholipids, were decreased following the combination therapy. Intriguingly, the combination caused a significant reduction in the levels of essential fatty acids involved in synthesis of outer membrane lipids FA (16:0) (hexadecanoic acid) and FA (14:0) (tetradecanoic acid) (≥ 1.0-log$_2$-fold, $p \leq .05$; FDR ≤ 0.05) (Fig. 3A). Furthermore, two fundamental phospholipid metabolites (*sn*-glycero-3-phosphoethanolamine and *sn*-glycerol 3-phosphate) were significantly reduced after combination treatment (≥ 2.0-log$_2$-fold, $p \leq .05$; FDR ≤ 0.05) (Fig. 3A). Importantly, the combination decreased palmitoleyl-CoA, a key intermediate involved in fatty acid elongation, at 15 min (≥ 1.0-log$_2$-fold, $p \leq .05$; FDR ≤ 0.05) (Fig. 3A).
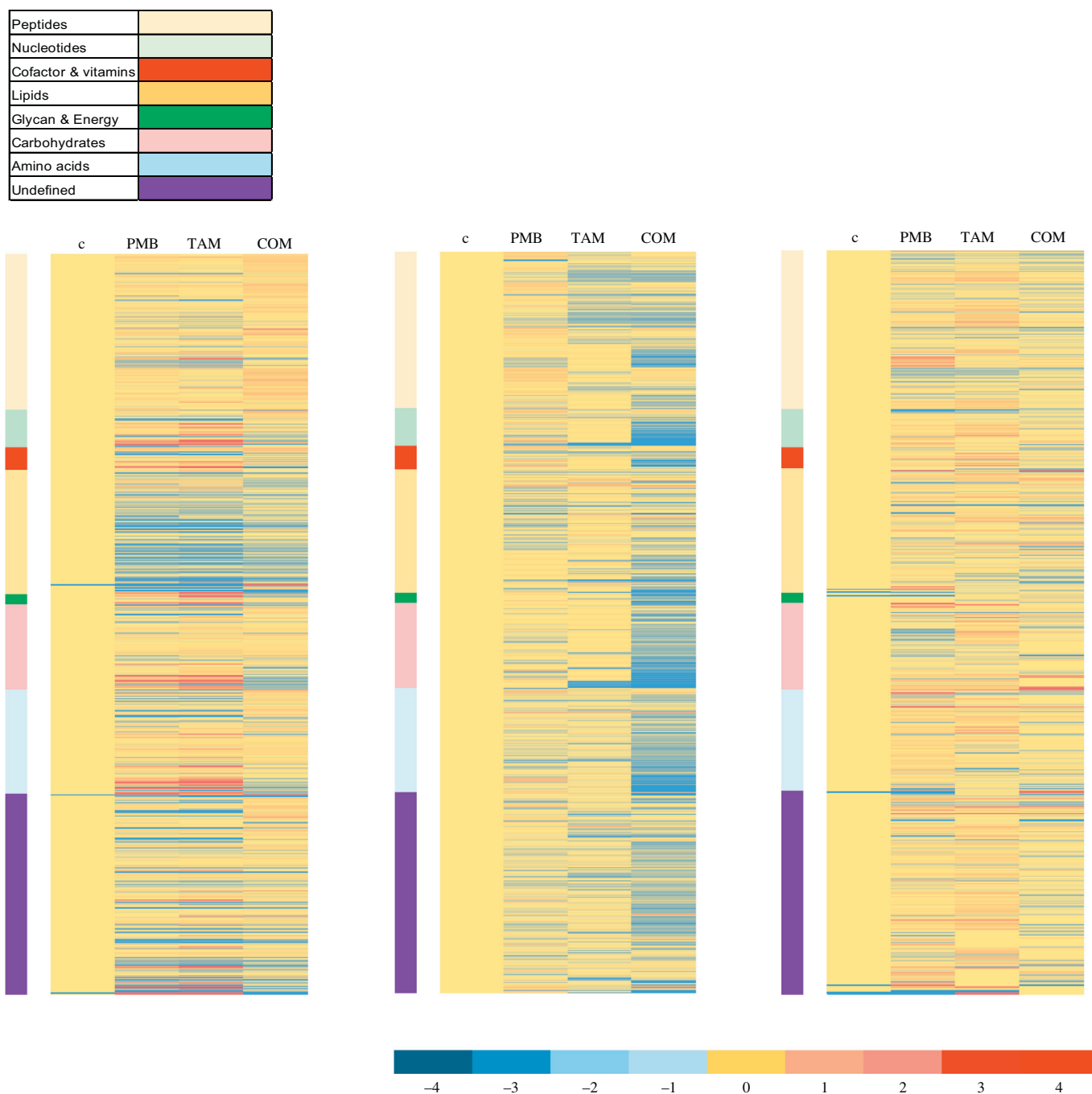
Fig. 1. Monotherapy and combination of polymyxin B and tamoxifen induce global metabolic changes in *P. aeruginosa* FADDI-PA006. Heatmap profiles of all identified metabolites clustered by metabolite class after treatment with single and combination (COM) of polymyxin B (PMB) and tamoxifen (TAM) at 15 min, 1 h, and 4 h.

Tamoxifen monotherapy had a considerable impact on lipid metabolites, in particular fatty acids, at 15 min (Fig. 3A). Although the levels of two important lipid metabolites, namely tetradecanoic acid and *sn*-glycero-3-phosphoethanolamine decreased after tamoxifen monotherapy ($\geq$ 1.0-$\log_2$-fold, $p \leq .05$; FDR ≤ 0.05), the levels of key intermediates in fatty acid synthesis, palmitoleyl-CoA and fatty acid (16:0) increased remarkably ($\geq$ 2.0-$\log_2$-fold, $p \leq .05$; FDR ≤ 0.05) (Fig. 3A). At 15 min, polymyxin B monotherapy only induced significant changes to a smaller number of fatty acids and glycerophospholipids (Fig. 3A).

On the other hand, the influence of all treatments (polymyxin B or tamoxifen monotherapy and their combination) was less pronounced on lipid pathways at the later time points (1 and 4 h) (Figs. 3B). However, combination therapy remained highly effective at reducing the levels of the aforementioned crucial lipid precursors at 1 h, including

palmitoleyl-CoA, *sn*-glycero-3-phosphoethanolamine and *sn*-glycerol 3-phosphate ($\geq$ 1.0-$\log_2$-fold, $p \leq .05$; FDR ≤ 0.05) (Fig. 3B).

2.3. Impact of the Combination on Amino Sugar and Nucleotide Sugar Metabolism and Downstream Peptidoglycan, and Lipopolysaccharide Formation and Modification Pathways in P. aeruginosa.

Several metabolites related to amino sugar and nucleotide sugar metabolism were significantly perturbed at 15 min across the treatment groups (Fig. 4A(i)). Tamoxifen monotherapy lead to a substantial increase in the levels of four essential amino- and nucleotide sugar metabolites that are directly related to interrelated peptidoglycan and LPS biosynthesis, namely UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc), *N*-Acetyl-D-glucosamine 6-phosphate, D-Glucosamine 6-phosphate,
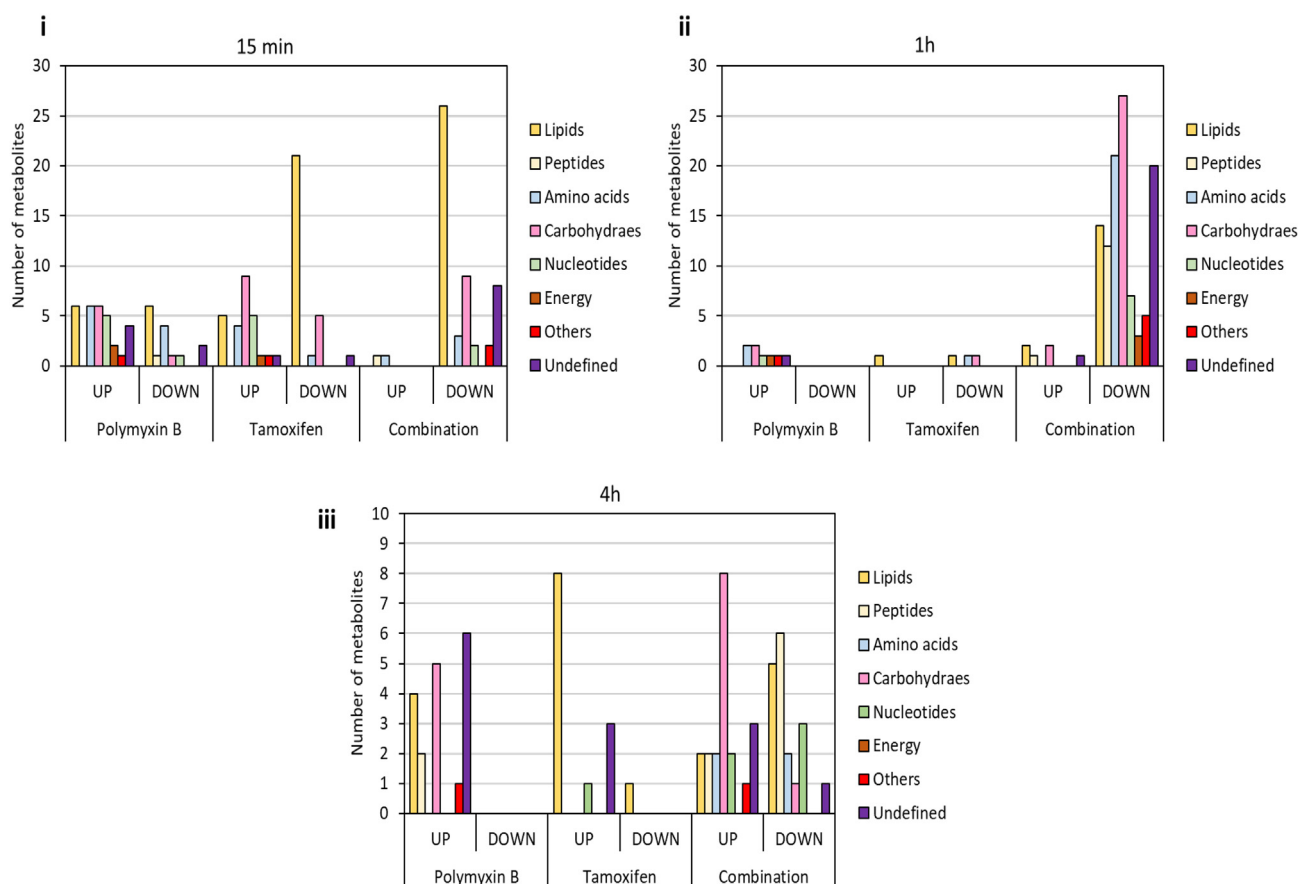
Fig. 2. Total number of significant metabolite changes classified according to metabolite classes after antibiotic treatment of *P. aeruginosa* FADDI-PA006 at (i) 15 min, (ii) 1 h, and (iii) 4 h. (≥ 0.59 -log$_2$-fold; p ≤ .05; FDR ≤ 0.05).

and *N*-acetylmuramate 6-phosphate (≥ 2.5-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Fig. 4B). Only one intermediate, namely D-Glucosamine, was slightly decreased following tamoxifen monotherapy (> 0.59-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Fig. 4A(i)). Likewise, four of these metabolites and one intermediate of peptidoglycan biosynthesis displayed a marked increase in their levels (except for D-Glucosamine) after polymyxin B monotherapy, namely UDP-GlcNAc, D-Glucosamine 6-phosphate, *N*-Acetyl-D-glucosamine 6-phosphate, *N*-acetylmuramate 6-phosphate, and UDP-MurNAC-L-Ala-gamma-D-Glu-m-DAP (≥ 1.5-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Fig. 4A(i)). In contrast, the combination treatment reduced the levels of six of these metabolites at 15 min, including D-Glucosamine, UDP-GlcNAc, D-Glucosamine 6-phosphate, *N*-Acetyl-D-glucosamine 6-phosphate, *N*-acetylmuramate 6-phosphate and UDP-MurNAC-L-Ala-gamma-D-Glu-m-DAP (> 0.59-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Fig. 4A(i)).

At the 1 h time point, both tamoxifen and polymyxin B monotherapy lacked effect on amino sugar and nucleotide sugar metabolites related to peptidoglycan biosynthesis, whilst the combination therapy induced significant changes in key peptidoglycan pathway intermediates (Fig. 4A(ii)). Specifically, three metabolites declined considerably following polymyxin B – tamoxifen combination treatment, namely D-alanyl-D-alanine, UDP-MurNAC-L-Ala-gamma-D-Glu-m-DAP, and UDP-MurNAC-L-Ala-gamma-D-Glu-m-DAP-D-Ala-D-Ala (≥ 1.5-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Fig. 4A(ii)). Moreover, five other essential precursors of amino sugar and nucleotide sugar metabolites were depleted, namely D-Glucosamine, D-Glucosamine 6-phosphate, *N*-Acetyl-D-glucosamine 6-phosphate, *N*-acetylmuramate, and UDP-GlcNAc (≥ 1.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Fig. 4). At 4 h, the impact of all treatments was minimal, with perturbations of only two intermediates for each of polymyxin B mono- and combination therapy, including *N*-Acetylmuramate

and UDP-*N*-acetyl-D-glucosamine for polymyxin B monotherapy, and D-Glucosamine and *N*-acetylmuramate for the combination treatment. Tamoxifen monotherapy had no signifcant impact on amino sugar or nucleotide sugar metabolism at 4 h.

In addition to the aforementioned pathways, the combination treatment had a significant impact on LPS biosynthetic metabolites and the lipid A aminoarabinose modification pathway at both 15 min and 1 h. The concentrations of two crucial components of LPS biosynthesis 3-Deoxy-D-manno-octulosonate (KDO), and UDP-GlcNAc were significantly decreased after combination therapy at 15 min (> 0.59-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Figs. 5A). Furthermore, the combination treatment decreased the levels of two fundamental metabolites from the aminoarabinose lipid A modification pathway including UDP-L-Ara4FN (> 1.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) and UDP-glucuronate (> 0.59-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) at 15 min (Figs. 5A). In contrast, both tamoxifen and polymyxin B monotherapy increased the levels of these metabolites. Tamoxifen monotherapy induced a prominent increase in the concentrations of KDO and UDP-GlcNAc (> 2.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05); and the levels of two lipid A aminoarabinose modification intermediates (UDP-glucuronate and UDP-L-Ara4FN) were also elevated (> 3.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Figs. 5A). Similarly, the UDP-glucuronate and UDP-L-Ara4FN levels were significantly elevated following polymyxin B monotherapy at 15 min (> 1.5-log$_2$-fold, p ≤ .05; FDR ≤ 0.05).

At 1 h, the combination treatment reduced the levels of three main intermediates of LPS biosynthesis including KDO, CMP-KDO, and D-glycero-D-manno-Heptose 7-phosphate (≥ 1.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Fig. 5B). The main precursors that are involved in the aminoarabinose modification of lipid A, were significantly decreased following the combination treatment at 1 h, particularly UDP-glucose,
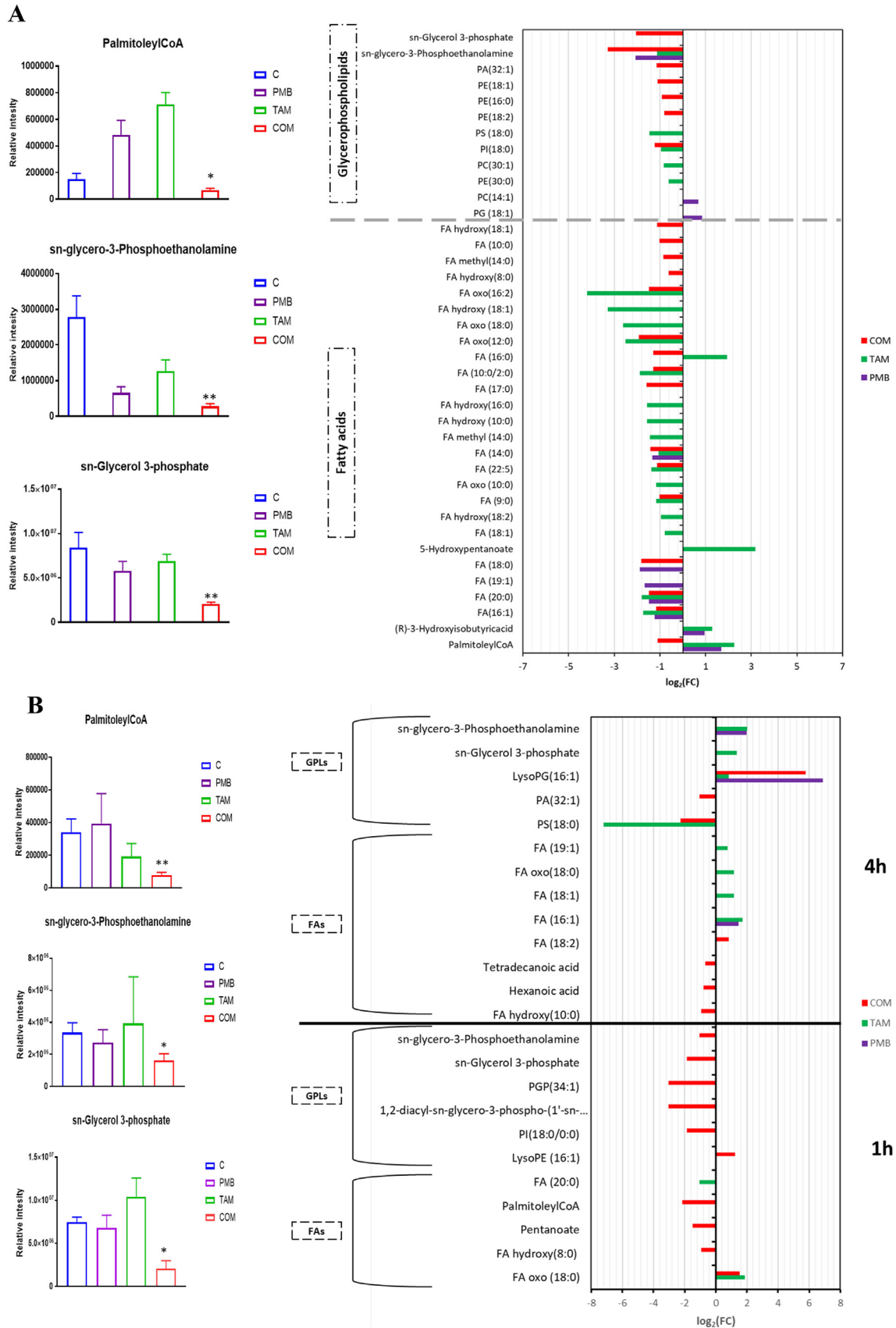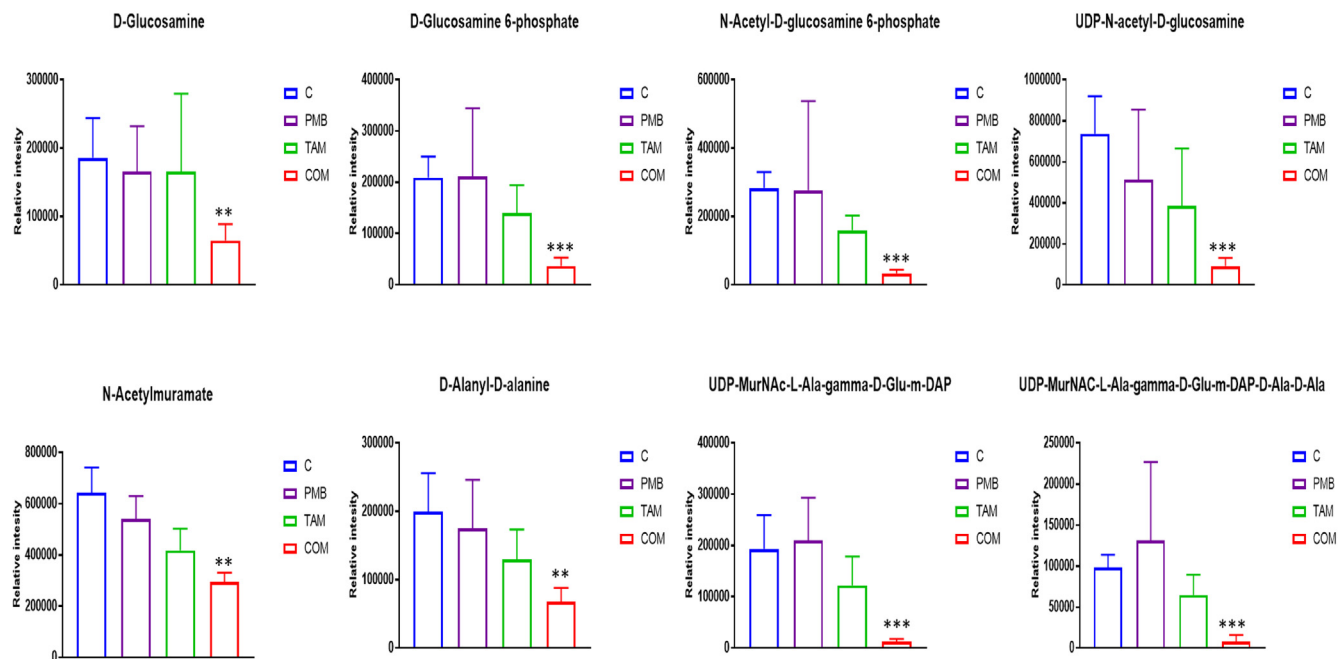
Fig. 3. Significantly impacted lipids in *P. aeruginosa* FADDI-PA006 following treatment with polymyxin B (PMB, purple), tamoxifen (TAM, green) and the combination (COM, red) at (A) 15 min, and (B) 1 h and 4 h. Lipid names are putatively assigned based on accurate mass. (** ≥ 2.0-log$_2$-fold, *p* ≤ .05; FDR ≤ 0.05); (* ≥ 1.0-log$_2$-fold, *p* ≤ .05; FDR ≤ 0.05).

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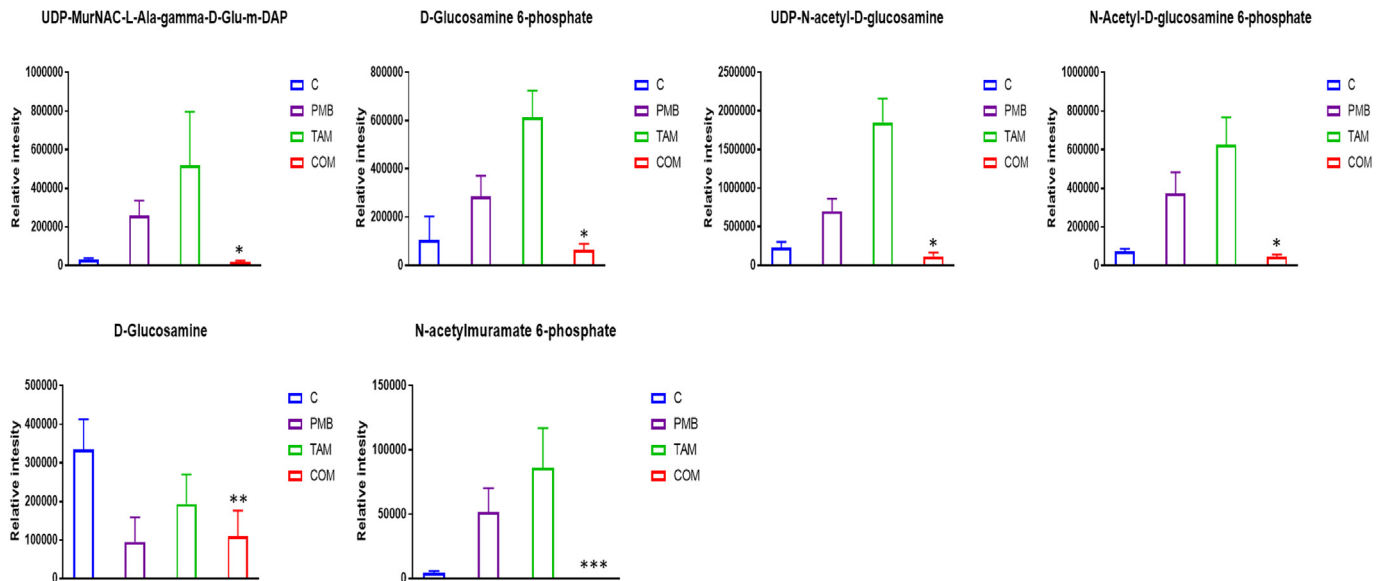
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Fig. 4. (A) Bar charts for the significantly impacted metabolites involved in amino sugar and nucleotide sugar and peptidoglycan biosynthesis of *P. aeruginosa* FADDI-PA006 following treatment with polymyxin B (PMB, purple), tamoxifen (TAM, green) monotherapy and in combination (COM, red) at (i) 15 min and (ii) 1 h (* > 0.59 -log$_2$-fold; p ≤ .05; FDR ≤ 0.05), (** ≥ 1.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) & (*** ≥ 2.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05). (B) Schematic diagram for amino sugar and nucleotide sugar metabolism and direct interrelated peptidoglycan biosynthetic pathway. Blue boxes indicated significantly impacted metabolites.

UDP-glucuronate, and UDP-L-Ara4FN (≥ 2.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Fig. 5B). By contrast, both polymyxin B and tamoxifen monotherapies did not influence LPS biosynthesis, nor lipid A aminoarabinose modification intermediates at 1 h (Fig. 5B). Notably, the levels of UDP-L-Ara4FN were greatly elevated following polymyxin B monotherapy at 4 h (≥ 3.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) while a much smaller increase was observed after the combination treatment at 4 h (> 0.59˜1.0-log$_2$-fold, p ≤ .05; FDR ≤ 0.05) (Table S2). None of the treatments affected the LPS biosynthesis at 4 h (Table S2).

2.4. Impact of the Combination on Central Carbohydrate Metabolism and Energy Production Pathways in P. aeruginosa.

Central carbohydrate metabolism and energy production pathways were only affected at 1 h across all treatments. Both polymyxin B and tamoxifen monotherapies marginally affected only one metabolite, namely fumarate (Fig. 6A). The combination treatment induced marked changes of several intermediates involved in the main pathways of central carbohydrate metabolism, namely glycolysis, pentose phosphate
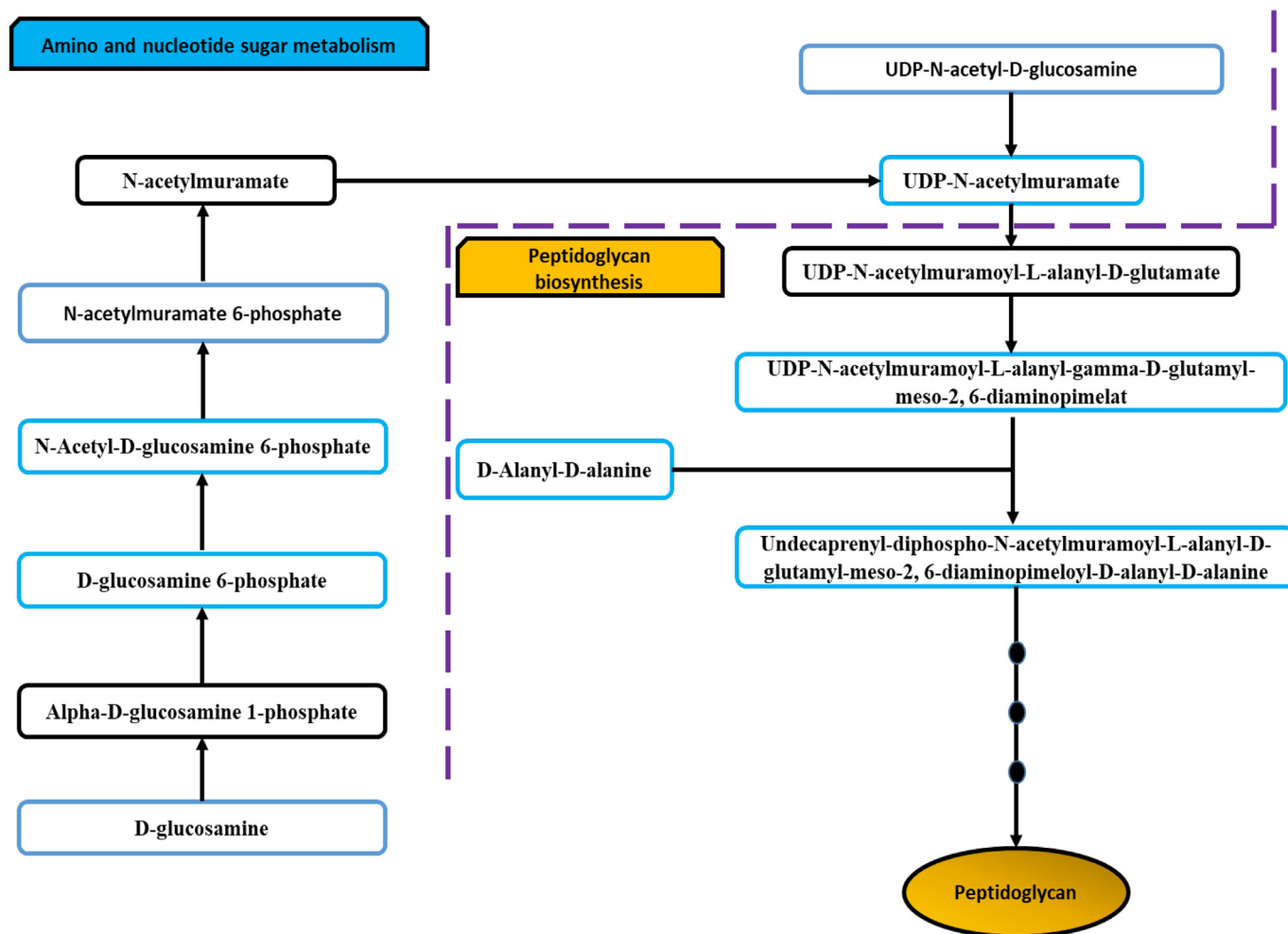
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Fig. 4 (*continued*).

pathway (PPP), tricarboxylic acid cycle (TCA) and the electron transport chain (ETC) (Fig. 6B). Interestingly, five intermediates of PPP decreased after combination treatment at 1 h, namely D-Glucono-1,5-lactone, D-Glycerate, D-Gluconic acid, D-Sedoheptulose 7-phosphate, and D-Ribose 5-phosphate ($\geq$ 1.0-log$_2$-fold, $p \leq$ .05; FDR $\leq$ 0.05) (Fig. 6A). Besides the PPP, another related pathway that was impacted by the combination therapy was glycolysis, of which five intermediates showed a significant reduction, namely D-Glucose 6-phosphate, D-Fructose 6-phosphate, glyceraldehyde-3-phosphate, phosphoenol-pyruvate (PEP), and glycerone phosphate ($\geq$ 1.0-log$_2$-fold, $p \leq$ .05; FDR $\leq$ 0.05) (Figs. 6A & B). The TCA cycle also exhibited substantial changes for four intermediates following combination treatment: *cis*-aconitate, fumarate, CoA, and succinate ($\geq$ 1.0-log$_2$-fold, $p \leq$ .05; FDR $\leq$ 0.05) (Fig. 6B). Concomitantly, the concentration of four essential metabolites of the ETC was significantly decreased following the combination treatment, NADH, NAD$^+$, ATP, and ADP ($\geq$ 1.0-log$_2$-fold, $p \leq$ .05; FDR $\leq$ 0.05) (Figs. 6A & B).

2.5. Effect of the Combination on Nicotinate and Nicotinamide Metabolism in P. aeruginosa.

Nicotinate and nicotinamide metabolism is vital for bacterial cell viability, as it generates the precursors of the coenzymes NAD+ and NADP+, which are crucial for cellular electron transfer reactions [35]. Strikingly, the combination treatment caused significant perturbations

in the levels of six fundamental intermediates of nicotinate and nicotin-amide metabolism, but only at 1 h (Fig. 7). The concentrations of glycerone phosphate, NAD$^+$, nicotinate, nicotinamide, nicotinate D-ribonucleotide (NMN), and quinolinate (pyridine-2,3-dicarboxylate) were reduced following combination therapy ($\geq$ 1.5-log$_2$-fold, $p \leq$ .05; FDR $\leq$ 0.05) (Fig. 7). These pathways were not affected by either poly-myxin B or tamoxifen monotherapy.

3. Discussion

In recent years, combination therapies with FDA approved non-antibiotic drugs has emerged as a novel strategy for maintaining the clinical efficacy of polymyxins and suppressing outbreaks of polymyxin resistance [17,36,37]. We have previously shown that the combination of polymyxin B with a SERM produces a highly synergistic bactericidal effect against MDR CF *P. aeruginosa* isolates and prevents the emergence of polymyxin resistance [21]. SERMs are non-steroidal estrogen receptor modulators approved for chemotherapy and chemoprevention of breast cancers and osteoporosis [38]. Along with their antineoplastic activity, SERMs have been found to exhibit direct antimicrobial activities (i.e. antifungal, antiviral, antibacterial) [39–42]. It has been proposed that SERMs operate via multiple mechanisms of antifungal activity, including membrane damage, inhibition of membrane peroxidation, cell cycle arrest, and interference with calmodulin [41]. However,

A

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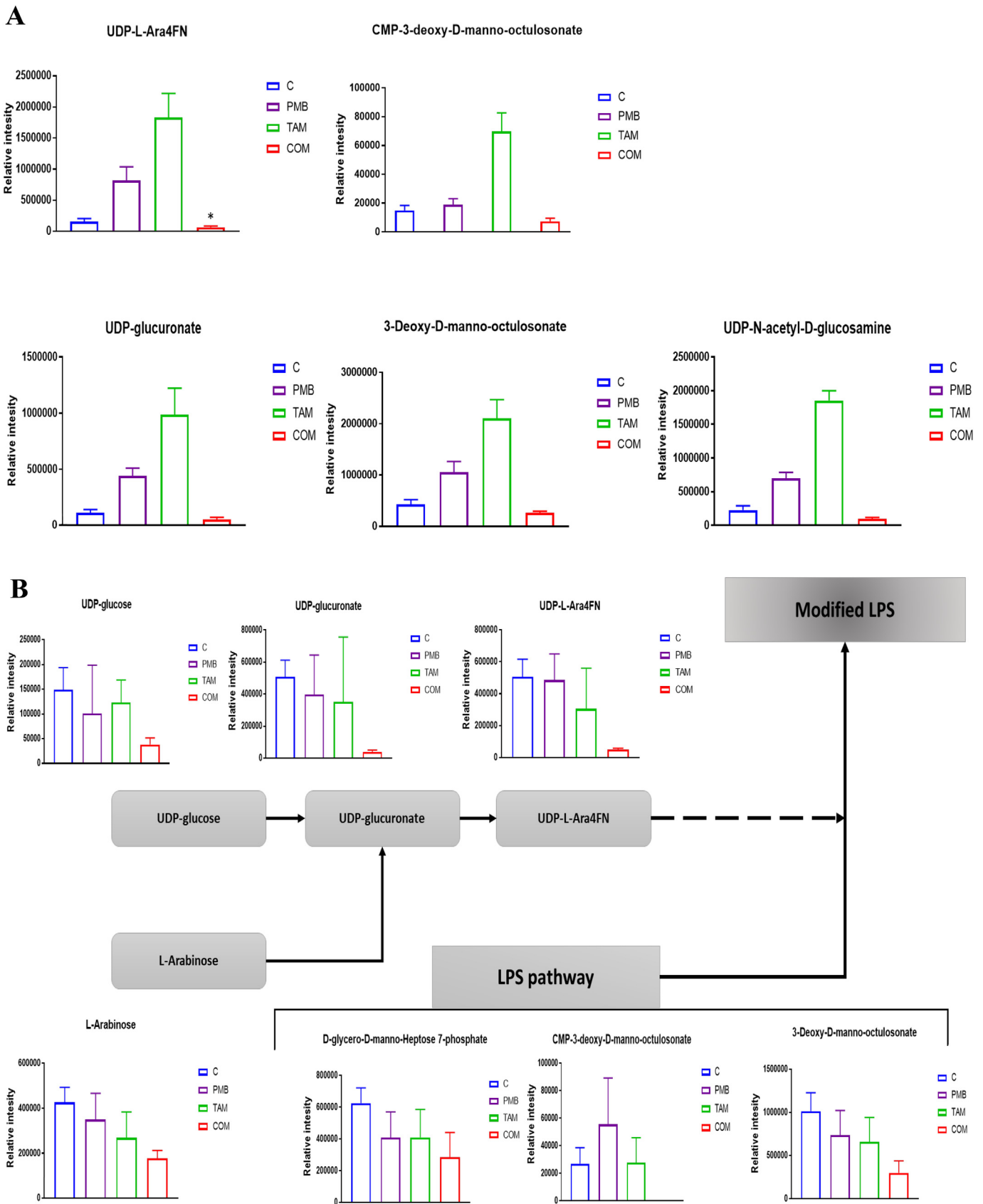
Fig. 5. (A) Bar charts for the significantly affected metabolites involved in LPS biosynthesis and its aminoarabinose modification following polymyxin B (PMB), tamoxifen (TAM), and combination (COM) treatment at 15 min *(≥ 1.0-log$_2$-fold, $p ≤ .05$; FDR ≤ 0.05); (≥ 0.59-log$_2$-fold, $p ≤ .05$; FDR ≤ 0.05). Control samples (blue), PMB (purple), TAM (green), and COM (red). (B) Bar charts and a diagram for the significantly impacted intermediates of LPS formation and its aminoarabinose modification after polymyxin B (PMB), tamoxifen (TAM), and the combination treatment at 1 h (≥ 1.0-log$_2$-fold, $p ≤ .05$; FDR ≤ 0.05).

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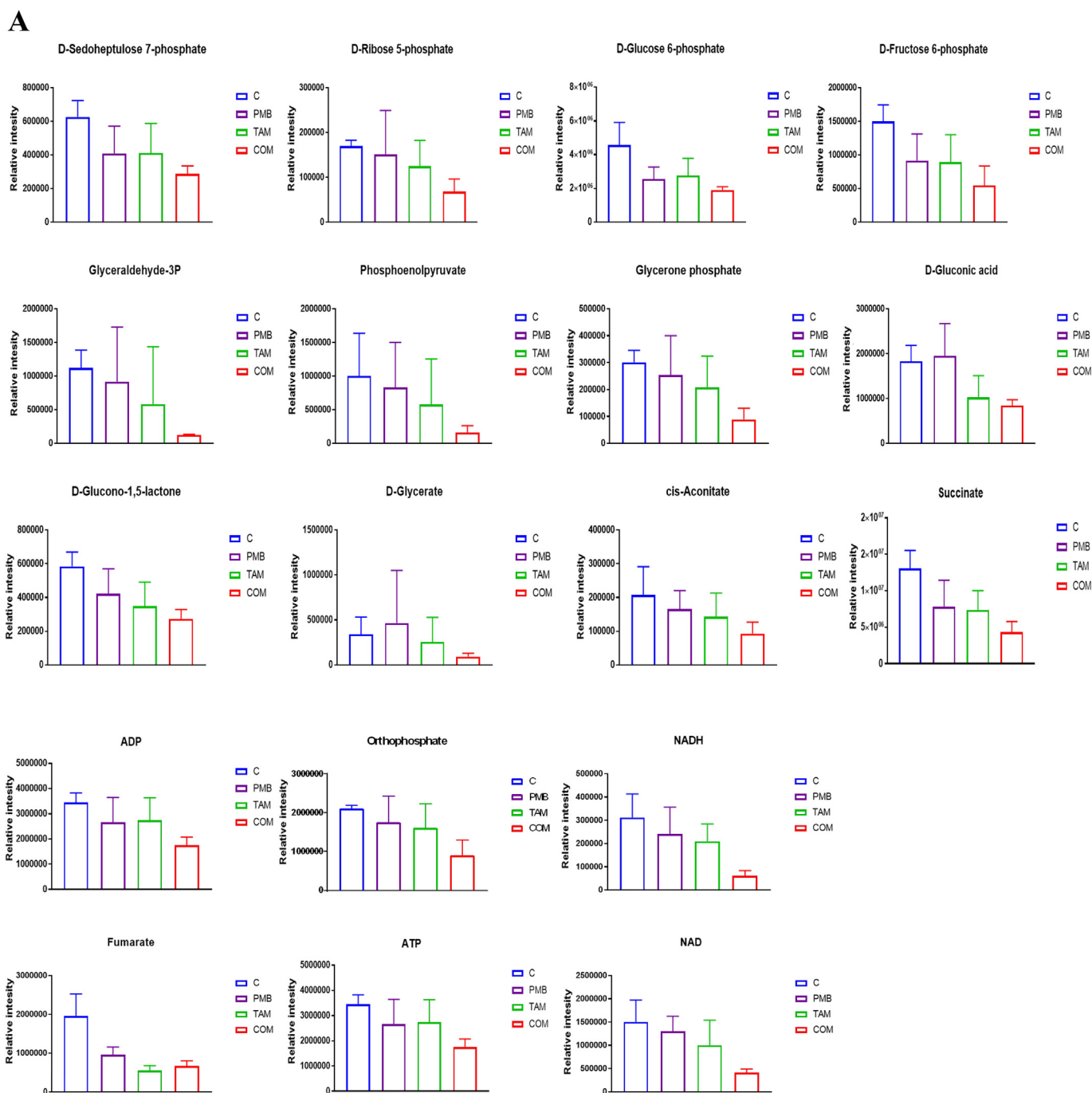
Fig. 6. (A) Bar charts for the significantly affected intermediates of the interrelated pathways of glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA), and electron transport chain of *P. aeruginosa* FADDI-PA006 after polymyxin B (PMB, purple), tamoxifen (TAM, green), and the combination (COM, red) treatment at 1 h (≥ 1.0-log$_2$-fold, $p \leq .05$; FDR ≤ 0.05). (B) Schematic diagram for the impacted pathways (glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA), and electron transport) following treatment with polymyxin B, tamoxifen, and combination treatment at 1 h. Blue boxes indicated significantly impacted metabolites.

their mechanism of antibacterial activity remains largely enigmatic. Understanding the biochemical mechanism(s) by which polymyxin B in combination with SERMs act synergistically against *P. aeruginosa* is vital for their potential future repurposing as antimicrobial agents. This report is the first to employ untargeted metabolomics to decipher the mechanisms of action of this novel combination against a MDR polymyxin resistant *P. aeruginosa* FADDI-PA006 isolated from the lungs of a CF patient. In order to ensure the study is clinically relevant, all experiments were performed using clinically achievable concentrations of polymyxin B

(2 mg/L) and tamoxifen (8 mg/L) [2,38,43,44]. Three different drug exposure times, 15 min, 1 h and 4 h were examined.

It has been proposed that polymyxins exert their bactericidal activity via disorganization and perforation of the bacterial OM [45,46]. However, as the tested strain FADDI-PA006 is resistant to polymyxins, it is not surprising that polymyxin B monotherapy caused minimal perturbations of OM lipids (Fig. 3A & B). Although polymyxin B decreased the levels of essential membrane lipids such as the fatty acid (18:0) octadecanoic acid, a significant increase in the level of palmitoleyl-CoA was also reported. The saturated fatty acid (18:0) octadecanoic acid is
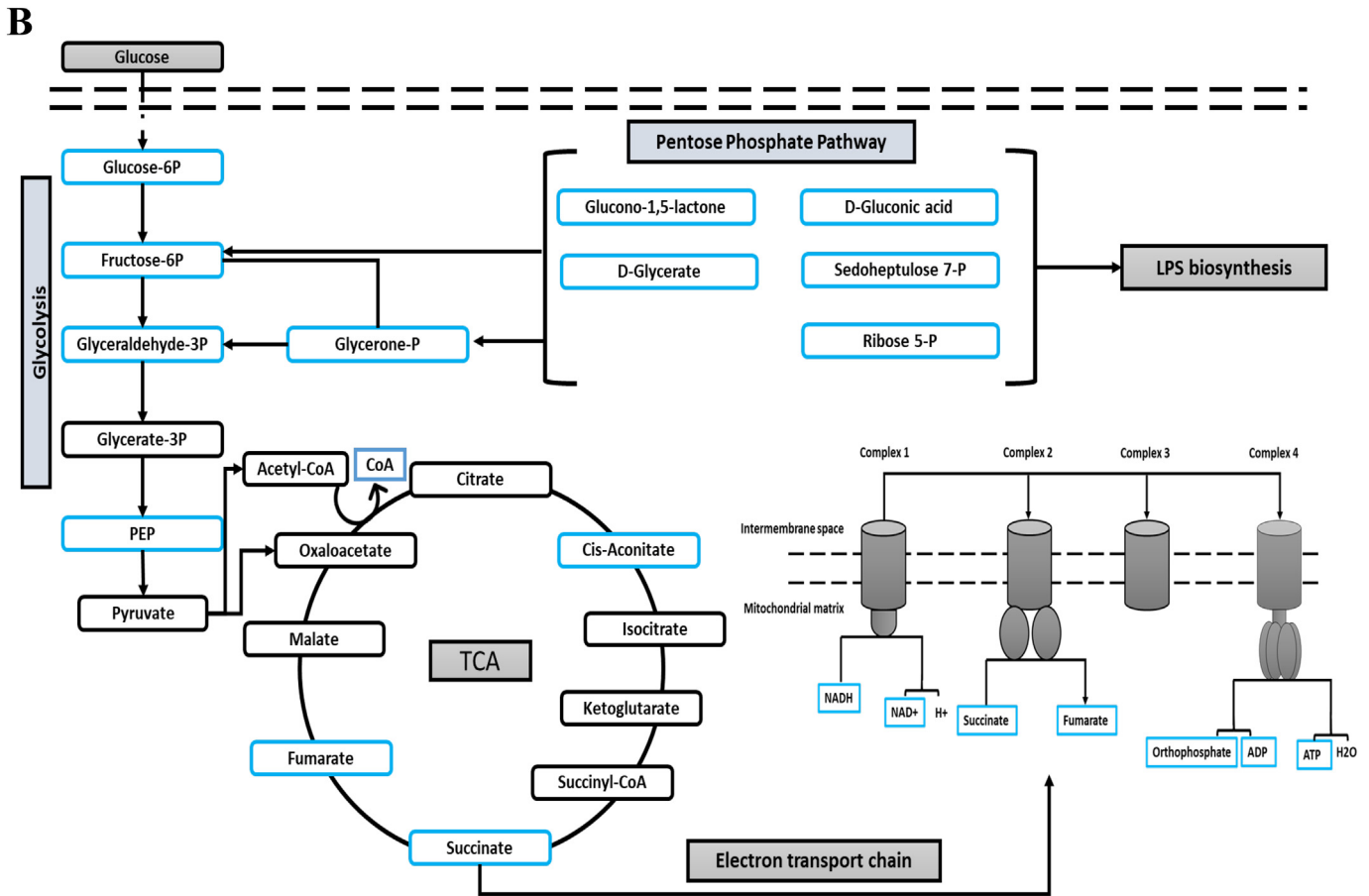
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Fig. 6 (*continued*).

one of the principal fatty acids making up the composition of the *P. aeruginosa* OM [47]; and a reduction in saturated fatty acids is usually associated with increased permeability and fluidity of the bacterial OM [48]. In addition to its estrogen receptor binding activity, tamoxifen is known to have multiple secondary cellular effects related to its direct molecular interactions with the membrane phospholipids and its ability to decrease membrane fluidity [49,50]. As illustrated in Fig. 3A, tamoxifen induced a remarkable increase in the levels of palmitic acid (FA (16:0)), an important membrane fatty acid which is known to decrease membrane fluidity and hence, reduce membrane permeability [51]. However, tamoxifen caused a significant reduction in the levels of key intermediates involved in LPS and phospholipid biosynthesis including tetradecanoic acid and *sn*-glycero-3-phosphoethanolamine [52]. In contrast, the combination treatment produced markedly different changes in lipid pathways, wherein most of the significantly perturbed lipids were decreased across all time points such as a significant decrease in the levels of *sn*-glycerol 3-phosphate and *sn*-glycero-3-phosphoethanolamine at 15 min and 1 h (Fig. 3A & B). *Sn*-glycerol 3-phosphate is a crucial intermediate required for the formation of membrane phospholipids, it undergoes acylation at the 1-position to form lysophosphatidic acid (LPA), and then a second acylation step produces phosphatidic acid (PA), the key intermediate in the synthesis of bacterial membrane glycerolipids [51,53]. Interestingly, in response to combination treatment, the levels of lysophosphatidylethanolamines (PE (18:1), PE (18:2) and LysoPE (16,0)) were significantly reduced, which indicates that the combination therapy potentially inhibits LPS modifications with ethanolamine compounds (Fig. 3A & B) [54]. The combination treatment also reduced the levels of fatty acids that are essential for LPS biosynthesis, tetradecanoic acid and palmitoleyl-CoA at early exposure times [52,55].

In addition to the effect on the lipid metabolism, pathway analysis revealed that polymyxin B monotherapy negatively impacted intermediates from the LPS and peptidoglycan biosynthetic pathways. Notably, our results are in line with a previous metabolomics study which revealed polymyxin B perturbs the main intermediates involved in LPS and peptidoglycan biosynthesis of the Gram-negative bacterium *Acinetobacter baumannii* [33]. Both Polymyxin B and tamoxifen monotherapy induced a significant increase in the concentrations of fundamental amino sugar and nucleotide sugar intermediates of the peptidoglycan biosynthetic pathway including *N*-Acetyl-D-glucosamine 6-phosphate, UDP-*N*-acetyl-D-glucosamine, and UDP-MurNAC-L-Ala-gamma-D-Glu-m-DAP (Fig. 4A). Furthermore, polymyxin B and tamoxifen monotherapy increased the levels of LPS biosynthetic pathway intermediates and the associated L-Ara4N lipid A modification intermediates, but only at 15 min. *P. aeruginosa* can develop polymyxin resistance via intrinsic (PmrB mutations) or extrinsic (*mcr-1*) mechanisms, which manifest the modification of LPS with the cationic moieties aminoarabinose (L-Ara4N) or phosphoethanolamine (PEtn) that act to repel the like-charged polymyxin [56,57].

The combination treatment induced a marked reduction of fundamental precursors (amino sugar and nucleotide sugar metabolism) of the interrelated peptidoglycan and LPS biosynthesis pathways; it is an important to note that this effect was more pronounced at 1 h than 15 min, opposite to the combination effect on lipid metabolism (Figs. 4 & 5). For instance, the levels of UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc), *N*-Acetyl-D-glucosamine 6-phosphate (GlcNAc-6P), and D-Glucosamine 6-phosphate decreased dramatically (Fig. 4A & B). Amino sugars and nucleotide sugars form the essential components of cell surface structures of bacteria (peptidoglycan, LPS and the polysaccharide capsule). UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc) in
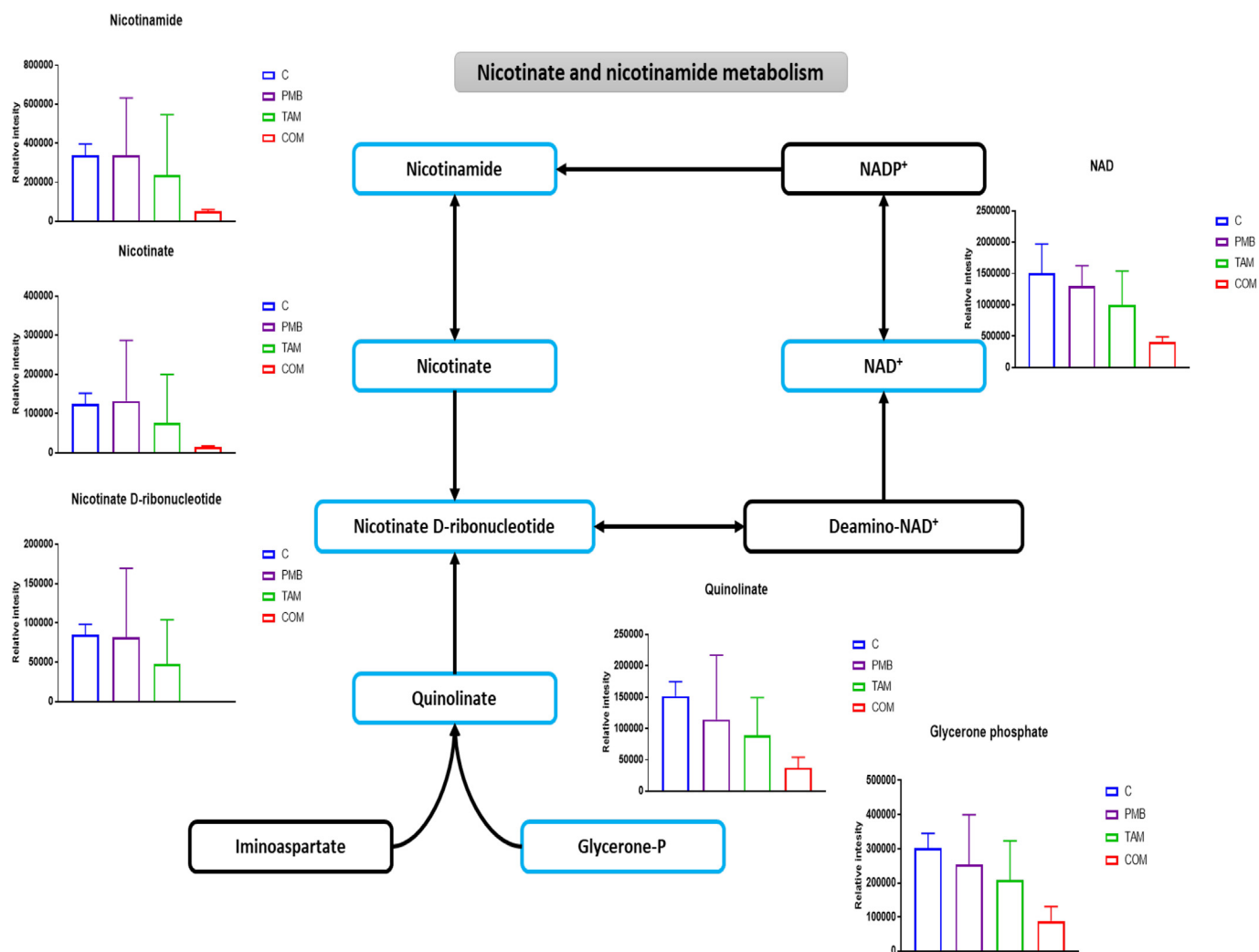
Fig. 7. Schematic diagram and bar graphs for significantly affected metabolites of nicotinate and nicotinamide metabolism of *P. aeruginosa* FADDI-PA006 after polymyxin B (PMB, purple), tamoxifen (TAM, green), and the combination (COM, red) treatment at 1 h (≥ 1.0-log$_2$-fold, $p \leq .05$; FDR ≤ 0.05). Blue boxes indicated significantly affected metabolites.

particular plays an important role as amino sugar donor in many transferase reactions in the biogenesis of peptidoglycan, the core lipid A moieties of the LPS, and certain *O*-antigens of Gram-negative bacteria [58–61]. It has been previously proposed that drugs interfering with bacterial nucleotide sugar biosynthetic pathways might be potential targets for the discovery of new therapeutics [62]. Our findings are in agreement with the previous metabolomics study in which the impact of a set of common antibiotics was tested against the *Staphylococcus aureus* metabolome [63]. The study revealed that vancomycin, a glycopeptide which acts via inhibition of bacterial cell wall synthesis, was greatly effective in reducing the amino sugar and nucleotide sugar intermediates after a 30 min treatment period [63]. Maifiah *et al.,* found that colistin monotherapy and in combination with doripenem caused a significant depletion in the intracellular levels of several important metabolites associated with amino sugar and nucleotide sugar metabolism of *A. baumannii* at 1 and 4 h [33].

The bacterial cell envelope is composed of an inner cell membrane and a cell wall in Gram-positive bacteria, in addition to the outer membrane in Gram-negative bacteria. This structure provides structural integrity to the cell and protect these organisms from their unpredictable and often hostile environment [64]. Owing to its importance as a crucial cell wall structural component of bacteria, peptidoglycan biosynthesis has been extensively investigated as a target for developing of new antibacterial agents [65,66]. It is well known that

several antibiotics exert their action by attacking peptidoglycan assembly, including vancomycin (which interferes with D-Alanyl-D-alanine) and fosfomycin [67,68]. A recent metabolomics study showed that fosfomycin and erythromycin significantly reduced levels of major precursors of cell wall biosynthesis in *S. aureus*[63]. Furthermore, it has been found that the combination of colistin and doripenem affected greatly the main intermediates of peptidoglycan biosynthesis of *A. baumannii* [33]. Our results are in agreement with these previous studies, where marked reductions were observed for key intermediates involved in the biosynthesis of peptidoglycan (Figs. 4A & B).

Importantly, our study is the first to demonstrate that combining tamoxifen with polymyxin B causes a remarkable decrease in the essential precursor metabolites of aminoarabinose (i.e. UDP-L-Ara4FN, UDP-glucuronate, and L-Arabinose), a major mechanism of polymyxin resistance (Figs. 5A & B) [56,57]. Although, the level of LPS components increased with polymyxin B and tamoxifen treatment, they display a considerable reduction after the combination treatment at 15 min and 1 h, in particular KDO, CMP-KDO, and D-glycero-D-manno-heptose 7-phosphate (Figs. 5A & B). The reduction in the concentrations of LPS and its aminoarabinose modification might result from either inhibition of amino sugar and nucleotide sugar metabolism, or from depletion of the main intermediates of PPP e.g., D-Ribose 5-phosphate and D-Sedoheptulose 7-phosphate (Figs. 6A & B). ADP-heptose, a key downstream metabolite of the heptose biosynthesis pathway, is an important

component of the LPS inner core [69]. Mutations in the gene (GmhA) associated with ADP-glyceromannoheptose synthesis in *Haemophilus influenza*, which cause deficiencies in heptose biosynthesis, result in an a virulent phenotype, increased membrane permeability and increased susceptibility to antibiotics [70,71]. Additionally, KDO synthesis is initiated by activation of D-arabinose-5-phosphate isomerase (API), an enzyme that promotes the reversible isomerization of D-ribulose-5-phosphate (Ru5P) to D-arabinose-5-phosphate, a KDO precursor; inactivation of this enzyme causes death of *E. coli* [72]. Thus, the depletion in the level of main components of amino sugar and nucleotide sugar metabolism and its direct downstream pathways, peptidoglycan and LPS biosynthesis, suggest synergistic bactericidal activity of our combination against *P. aeruginosa*.

In addition to its potential impact on membrane structure, our combination treatment was effective in depleting several components of glycolysis and tricarboxylic acid cycle (TCA) and a directly connected pathway, the electron transport chain at 1 h (Figs. 6A & B). The levels of glyceraldehyde-3P, PEP, Fructose-6P, succinate, and fumarate significantly declined after combination therapy (Figs. 6A & B). Central carbohydrate metabolism is a metabolic network that involves glycolysis and the TCA cycle; it has been recently explored for new targets for antibacterial agents [73,74]. The significant decrease of fundamental intermediates of the TCA cycle, in particular succinate, likely results in a reduction of reducing equivalents for the electron transport chain. Succinate dehydrogenase is a vital enzyme that links the TCA cycle and the electron transport chain and is able to oxidize of succinate to fumarate [74]. The deletion of succinate dehydrogenase in *S. enterica* impaired its ability to colonize mice [75]. Succinate dehydrogenase has also been investigated in other bacteria such as *P. aeruginosa* as a promising next-generation drug target [76]. In addition to the TCA cycle defects, the polymyxin B – tamoxifen combination treatment resulted in depletion of several intermediates of the electron transport chain, including ATP, ADP, NAD^+ and orthophosphate (Figs. 6A & B).

Nicotinate and nicotinamide metabolism was another strongly interrelated pathway that was extensively perturbed by the polymyxins B – tamoxifen combination at 1 h (Fig. 7). Six crucial precursors were significantly reduced following combination treatment e.g., quinolinate, NMN, and nicotinamide (Fig. 7). This biochemical pathway is a vital source of NAD^+ (H) and $NADP^+$ (H) cofactors, which are necessary for multiple complex reactions in several metabolic pathways including glycolysis, PPP, TCA cycle and fatty acid biosynthesis [35,77]. An investigation into the importance of bacterial NAD biosynthesis demonstrated that knock-down of the protein levels for the two genes, NadD and NadE, which are necessary for the last two steps of NAD biogenesis resulted in cell death in Mycobacteria [78]. Intriguingly, our combination produced a marked inhibition of nicotinate and nicotinamide biosynthesis (Fig. 7).

To sum up, the current study is the first to decipher the mechanism of synergistic bactericidal activity of a novel combination of a polymyxin with a SERM, using a metabolomics platform. Polymyxin B and tamoxifen monotherapy caused minor perturbations to the FADDI-PA006 metabolome at 15 min; which were mainly comprised of increased levels of selected metabolites. On the other hand, the combination treatment showed greater perturbation to the metabolome at 15 min, 1 and 4 h. The combination inhibited most affected pathways across all time exposures, with the greatest differences observed at 1 h. It is possible that the synergistic killing associated with this novel combination is largely due to inhibition of multiple complex interconnected pathways, in particular those related to bacterial cell envelope biosynthesis, and inhibition of bacterial resistance development via outer membrane modification. Additional synergistic effects were also noticed involving inhibition of key central metabolic pathways including glycolysis, PPP, TCA cycle and electron transport chain. Importantly, this study highlights the potential for repurposing of tamoxifen for an antimicrobial indication in combination with polymyxins. Multi-omics study along with further molecular work should be performed to validate the key pathways

recognized from the metabolomics analysis. To do so, mutant libraries, *in silico* genome scale metabolic modelling and metabolite feeding experiments would be required. Further studies are warranted to examine other non-antibiotic drugs as antimicrobial agents in combination with the OM permeabilizing antimicrobial agents such as the polymyxins and aminoglycosides.

4. Methods

Detailed methods of all experimental procedures are provided in the supporting information

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2018.11.001.

References

[1] Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. Pharm Ther 2015;40:277.
[2] Velkov T, Roberts KD, Nation RL, et al. Pharmacology of polymyxins: new insights into an 'old'class of antibiotics. Future Microbiol 2013;8:711–24.
[3] Mesaros N, Nordmann P, Plésiat P, et al. Pseudomonas aeruginosa: resistance and therapeutic options at the turn of the new millennium. Clin Microbiol Infect 2007; 13:560–78.
[4] Hancock RE, Speert DP. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. Drug Resist Updat 2000;3:247–55.
[5] Livermore DM. Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare? Clin Infect Dis 2002;34:634–40.
[6] Johnson C. The 12 superbugs threatening human health.
[7] Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. Clin Microbiol Rev 2002;15:194–222.
[8] Verkman A, Song Y, Thiagarajah JR. Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease. Am J Physiol-Cell Physiol 2003;284:C2–C15.
[9] Li J, Nation RL, Turnidge JD, et al. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. Lancet Infect Dis 2006;6:589–601.
[10] Nation RL, Li J, Cars O, et al. Framework for optimisation of the clinical use of colistin and polymyxin B: the Prato polymyxin consensus. Lancet Infect Dis 2015;15: 225–34.
[11] Trimble MJ, Mlynárčik P, Kolář M, et al. Polymyxin: alternative mechanisms of action and resistance. Cold Spring Harb Perspect Med 2016;6:a025288.
[12] Ernst RK, Guina T, Miller SI. Salmonella typhimurium outer membrane remodeling: role in resistance to host innate immunity. Microbes Infect 2001;3:1327–34.
[13] Raetz CR, Reynolds CM, Trent MS, et al. Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem 2007;76:295–329.
[14] Llobet E, Campos MA, Giménez P, et al. Analysis of the networks controlling the antimicrobial-peptide-dependent induction of Klebsiella pneumoniae virulence factors. Infect Immun 2011;79:3718–32.
[15] Li J, Rayner CR, Nation RL, et al. Heteroresistance to colistin in multidrug-resistant acinetobacter baumannii. Antimicrob Agents Chemother 2006;50:2946–50.
[16] Cai Y, Chai D, Wang R, et al. Colistin resistance of acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. J Antimicrob Chemother 2012; 67:1607–15.
[17] Bergen PJ, Bulman ZP, Saju S, et al. Polymyxin combinations: pharmacokinetics and pharmacodynamics for rationale use. Pharmacotherapy 2015;35:34–42.
[18] Henry R, Crane B, Powell D, et al. The transcriptomic response of Acinetobacter baumannii to colistin and doripenem alone and in combination in an in vitro pharmacokinetics/pharmacodynamics model. J Antimicrob Chemother 2015;70: 1303–13.
[19] Ejim L, Farha MA, Falconer SB, et al. Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. Nat Chem Biol 2011;7:348.
[20] Schneider EK, Reyes-Ortega F, Velkov T, et al. Antibiotic–non-antibiotic combinations for combating extremely drug-resistant Gram-negative 'superbugs'. Essays Biochem 2017;61:115–25.

[21] Hussein MH, Schneider EK, Elliott AG, et al. From breast cancer to antimicrobial: combating extremely resistant Gram-negative "superbugs" using novel combinations of polymyxin B with selective estrogen receptor modulators. Microb Drug Resist 2017;23:640–50.

[22] Schneider EK, Azad MA, Han M-L, et al. An "unlikely" pair: the antimicrobial synergy of polymyxin B in combination with the cystic fibrosis transmembrane conductance regulator drugs KALYDECO and ORKAMBI. ACS Infect Dis 2016;2:478–88.

[23] Lim T-P, Lee W, Tan T-Y, et al. Effective antibiotics in combination against extreme drug-resistant Pseudomonas aeruginosa with decreased susceptibility to polymyxin B. PLoS One 2011;6:e28177.

[24] Vincent IM, Ehmann DE, Mills SD, et al. Untargeted metabolomics to ascertain antibiotic modes of action. Antimicrob Agents Chemother 2016;60:2281–91.

[25] Belenky P, Jonathan DY, Porter CB, et al. Bactericidal antibiotics induce toxic metabolic perturbations that lead to cellular damage. Cell Rep 2015;13:968–80.

[26] Reiß S, Pané-Farré J, Fuchs S, et al. Global analysis of the *Staphylococcus aureus* response to mupirocin. Antimicrob Agents Chemother 2012 Feb 1;56(2):787–804.

[27] Wang Z-Y, Zhu Q, Zhang H-Y. Metabolite concentration as a criterion for antibacterial discovery. Curr Comput Aided Drug Des 2013;9:412–6.

[28] Okada BK, Wu Y, Mao D, et al. Mapping the trimethoprim-induced secondary metabolome of Burkholderia thailandensis. ACS Chem Biol 2016;11:2124–30.

[29] Lobritz MA, Belenky P, Porter CB, et al. Antibiotic efficacy is linked to bacterial cellular respiration. Proc Natl Acad Sci 2015;112:8173–80.

[30] Zampieri M, Zimmermann M, Claassen M, et al. Nontargeted metabolomics reveals the multilevel response to antibiotic perturbations. Cell Rep 2017;19:1214–28.

[31] Maifiah MHM, Creek DJ, Nation RL, et al. Untargeted metabolomics analysis reveals key pathways responsible for the synergistic killing of colistin and doripenem combination against acinetobacter baumannii. Sci Rep 2017;7.

[32] Kirwan JA, Weber RJ, Broadhurst DI, et al. Direct infusion mass spectrometry metabolomics dataset: a benchmark for data processing and quality control. Scientific Data 2014;1:140012.

[33] Maifiah MHM, Creek DJ, Nation RL, et al. Untargeted metabolomics analysis reveals key pathways responsible for the synergistic killing of colistin and doripenem combination against acinetobacter baumannii. Sci Rep 2017;7:45527.

[34] Han M-L, Zhu Y, Creek DJ, et al. Alterations of metabolic and lipid profiles in polymyxin-resistant Pseudomonas aeruginosa. Antimicrob Agents Chemother 2018 May 25;62(6) (AAC. 02656–17).

[35] Begley TP, Kinsland C, Mehl RA, et al. The biosynthesis of nicotinamide adenine dinucleotides in bacteria. Vitam Horm 2001;61:103–19.

[36] Ly NS, Bulitta JB, Rao GG, et al. Colistin and doripenem combinations against Pseudomonas aeruginosa: profiling the time course of synergistic killing and prevention of resistance. J Antimicrob Chemother 2015;70:1434–42.

[37] Bergen PJ, Bulman ZP, Landersdorfer CB, et al. Optimizing polymyxin combinations against resistant gram-negative bacteria. Infect Dis Ther 2015;4:391–415.

[38] Morello KC, Wurz GT, DeGregorio MW. Pharmacokinetics of selective estrogen receptor modulators. Clin Pharmacokinet 2003;42:361–72.

[39] Butts A, Koselny K, Chabrier-Roselló Y, et al. Estrogen receptor antagonists are anticryptococcal agents that directly bind EF hand proteins and synergize with fluconazole in vivo. MBio 2014;5 (e00765–13).

[40] Miguel DC, Zauli-Nascimento RC, Yokoyama-Yasunaka JK, et al. Tamoxifen as a potential antileishmanial agent: efficacy in the treatment of Leishmania braziliensis and leishmania chagasi infections. J Antimicrob Chemother 2008;63:365–8.

[41] Dolan K, Montgomery S, Buchheit B, et al. Antifungal activity of tamoxifen: in vitro and in vivo activities and mechanistic characterization. Antimicrob Agents Chemother 2009;53:3337–46.

[42] Johansen L, Brannan J, Delos S, et al, Olinger GG. FDA-approved selective estrogen receptor modulators inhibit Ebola virus infection. Sci Transl Med 2013;5 (190ra79).

[43] Hochner-Celnikier D. Pharmacokinetics of raloxifene and its clinical application. Eur J Obstet Gynecol Reprod Biol 1999;85:23–9.

[44] Taras TL, Wurz GT, Linares GR, et al. Clinical pharmacokinetics of toremifene. Clin Pharmacokinet 2000;39:327–34.

[45] Hancock RE, Chapple DS. Peptide antibiotics. Antimicrob Agents Chemother 1999;43:1317–23.

[46] Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis 2001;1:156–64.

[47] Cho K, Salton M. Fatty acid composition of bacterial membrane and wall lipids. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid. Metabolism 1966;116:73–9.

[48] Mansilla MC, Cybulski LE, Albanesi D, et al. Control of membrane lipid fluidity by molecular thermosensors. J Bacteriol 2004;186:6681–8.

[49] Wiseman H, Quinn P, Halliwell B. Tamoxifen and related compounds decrease membrane fluidity in liposomes: mechanism for the antioxidant action of tamoxifen and relevance to its anticancer and cardioprotective actions? FEBS Lett 1993;330:53–6.

[50] Custódio JA, Almeida LM, Madeira VM. The anticancer drug tamoxifen induces changes in the physical properties of model and native membranes. Biochimica et Biophysica Acta (BBA)-Biomem 1993;1150:123–9.

[51] Zhang Y-M, Rock CO. Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 2008;6:222.

[52] Kusumoto S, Fukase K, Shiba T. Key structures of bacterial peptidoglycan and lipopolysaccharide triggering the innate immune system of higher animals: chemical synthesis and functional studies. Proc Jpn Acad Ser B 2010;86:322–37.

[53] Cronan Jr JE, Vagelos PR. Metabolism and function of the membrane phospholipids of Escherichia coli. Biochimica et Biophysica Acta (BBA)-Rev Biomemb 1972;265:25–60.

[54] Arroyo LA, Herrera CM, Fernandez L, et al. The pmrCAB operon mediates polymyxin resistance in Acinetobacter baumannii ATCC 17978 and clinical isolates through phosphoethanolamine modification of Lipid A. Antimicrob Agents Chemother 2011 Aug;55(8):3743–51 (AAC. 00256-11).

[55] Emiola A, Andrews SS, Heller C, et al. Crosstalk between the lipopolysaccharide and phospholipid pathways during outer membrane biogenesis in Escherichia coli. Proc Natl Acad Sci 2016;113:3108–13.

[56] Olaitan AO, Morand S, Rolain J-M. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 2014;5:643.

[58] Raetz CR. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles. Escherichia Coli Salmonella 1996;1:1035–63.

[59] Van Heijenoort J. Murein synthesis. Escherichia Coli Salmonella Typhimurium 1996:1025–34.

[60] Plumbridge J, Vimr E. Convergent pathways for utilization of the amino sugars N-acetylglucosamine, N-acetylmannosamine, and N-acetylneuraminic acid by Escherichia coli. J Bacteriol 1999;181:47–54.

[61] Mengin-Lecreulx D, Van Heijenoort J. Copurification of glucosamine-1-phosphate acetyltransferase and N-acetylglucosamine-1-phosphate uridyltransferase activities of Escherichia coli: characterization of the glmU gene product as a bifunctional enzyme catalyzing two subsequent steps in the pathway for UDP-N-acetylglucosamine synthesis. J Bacteriol 1994;176:5788–95.

[62] Mäki M, Renkonen R. Biosynthesis of 6-deoxyhexose glycans in bacteria. Glycobiology 2004;14:1R–15R.

[63] Dörries K, Schlueter R, Lalk M. Impact of antibiotics with various target sites on the metabolome of Staphylococcus aureus. Antimicrob Agents Chemother 2014;58:7151–63.

[64] Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 2003;67:593–656.

[65] Bugg TD, Braddick D, Dowson CG, et al. Bacterial cell wall assembly: still an attractive antibacterial target. Trends Biotechnol 2011;29:167–73.

[66] Green DW. The bacterial cell wall as a source of antibacterial targets. Expert Opin Ther Targets 2002;6:1–20.

[67] Barna J, Williams D. The structure and mode of action of glycopeptide antibiotics of the vancomycin group. Annu Rev Microbiol 1984;38:339–57.

[68] Dijkmans AC, Zacarías NVO, Burggraaf J, et al. Fosfomycin: Pharmacological, clinical and future perspectives. Antibiotics 2017;6:24.

[69] Matthaiou DK, Michalopoulos A, Rafailidis PI, et al. Risk factors associated with the isolation of colistin-resistant gram-negative bacteria: a matched case-control study. Crit Care Med 2008;36:807–11.

[70] Taylor PL, Blakely KM, De Leon GP, et al. Structure and function of sedoheptulose-7-phosphate isomerase, a critical enzyme for lipopolysaccharide biosynthesis and a target for antibiotic adjuvants. J Biol Chem 2008;283:2835–45.

[71] Brooke JS, Valvano M. Molecular cloning of the Haemophilus influenzae gmhA (lpcA) gene encoding a phosphoheptose isomerase required for lipooligosaccharide biosynthesis. J Bacteriol 1996;178:3339–41.

[72] Gourlay LJ, Sommaruga S, Nardini M, et al. Probing the active site of the sugar isomerase domain from E. coli arabinose-5-phosphate isomerase via X-ray crystallography. Protein Sci 2010;19:2430–9.

[73] Murima P, McKinney JD, Pethe K. Targeting bacterial central metabolism for drug development. Chem Biol 2014;21:1423–32.

[74] Oyedotun KS, Lemire BD. The quaternary structure of the Saccharomyces cerevisiae succinate dehydrogenase homology modeling, cofactor docking, and molecular dynamics simulation studies. J Biol Chem 2004;279:9424–31.

[75] Mercado-Lubo R, Gauger EJ, Leatham MP, et al. A Salmonella enterica serovar typhimurium succinate dehydrogenase/fumarate reductase double mutant is avirulent and immunogenic in BALB/c mice. Infect Immun 2008;76:1128–34.

[76] Cook GM, Greening C, Hards K, et al. Energetics of pathogenic bacteria and opportunities for drug development. Adv Microb Physiol 2014:1–62.

[77] Boshoff HI, Xu X, Tahlan K, et al. Biosynthesis and recycling of nicotinamide cofactors in mycobacterium tuberculosis an essential role for nad in nonreplicating bacilli. J Biol Chem 2008;283:19329–41.

[78] Rodionova IA, Schuster BM, Guinn KM, et al. Metabolic and bactericidal effects of targeted suppression of NadD and NadE enzymes in mycobacteria. MBio 2014;5 (e00747–13).