Proteomic and Morphologic Evidence for Taurine-5-Bromosalicylaldehyde Schiff Base as an Efficient Anti-Mycobacterial Drug^S

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Mycobacterium tuberculosis, a causative pathogen of tuberculosis (TB), still threatens human health worldwide. To find a novel drug to eradicate this pathogen, we tested taurine-5-bromosalicylaldehyde Schiff base (TBSSB) as an innovative anti-mycobacterial drug using *Mycobacterium smegmatis* as a surrogate model for *M. tuberculosis*. We investigated the antimicrobial activity of TBSSB against *M. smegmatis* by plotting growth curves, examined the effect of TBSSB on biofilm formation, observed morphological changes by scanning electron microscopy and transmission electron microscopy, and detected differentially expressed proteins using two-dimensional gel electrophoresis coupled with mass spectrometry. TBSSB inhibited mycobacterial growth and biofilm formation, altered cell ultrastructure and intracellular content, and inhibited cell division. Furthermore, *M. smegmatis* adapted itself to TBSSB inhibition by regulating the metabolic pathways and enzymatic activities of the identified proteins. NDMA-dependent methanol dehydrogenase, NAD(P)H nitroreductase, and amidohydrolase AmiB1 appear to be pivotal factors to regulate the *M. smegmatis* survival under TBSSB. Our dataset reinforced the idea that Schiff base-aurine compounds have the potential to be developed as novel anti-mycobacterial drugs.

Keywords: *Mycobacterium*, tuberculosis, taurine-5-bromosalicylaldehyde Schiff base, morphology, cell wall, two-dimensional gel electrophoresis

Introduction

Mycobacterium tuberculosis, a causative pathogen of tuberculosis (TB), has evolved to multidrug-resistant/rifampin resistant (MDR/RR)- or extensively drug-resistant (XDR)-*M. tuberculosis*, and remains a major threat to human health worldwide. According to the 2018 WHO global TB report, 3.5% of new TB cases (an estimated 10 million of new TB cases in 2017) and 18% of previously treated TB cases were estimated to be MDR-TB/RR-TB globally, and the treatment success rates for the cases with MDR-TB/RR-TB were much lower (only approximately 55%) based on the current anti-TB treatment regimens [1]. In addition, TB treatment required long-term medication. *M. tuberculosis* infection therefore imposes a heavy economic burden on the global economy. On the other hand, the increasing rise

of MDR/RR-*M. tuberculosis* strains has promoted a significant worldwide effort to discover innovative anti-TB drugs for their eradication.

As therapeutic agents, Schiff base complexes have been intensively investigated due to their antifungal, anticancer, and antibacterial properties [2–5]. Schiff bases, named after the chemist Hugo Schiff [6], contain a functional double bond between carbon and nitrogen which can mimic the structural features of the biological regulator's active sites. In addition, Schiff base complexes derived from salicylaldehyde and amino acids have recently attracted great attention. Taurine, a sulfur-containing amino acid, is essential to human beings as it has a variety of physiological functions such as modulation of neurotransmitters, hormone release regulation, and neuronal excitability [2, 7]. In this study, taurine-5-bromosalicylaldehyde Schiff base (TBSSB), a taurine

Fig. 1. Taurine-5-bromosalicylaldehyde Schiff base (TBSSB) in complex with potassium.

Schiff base complex with potassium, was synthesized from the condensation of the amine group of taurine and the active aldehyde group of the aromatic compound (Fig. 1), and its structure was confirmed by spectral infrared radiation (IR) and single-crystal X-ray diffraction [8].

Our previous studies demonstrated that TBSSB is an effective and attractive drug against planktonic MRSA [8]. However, little is known about the roles of TBSSB in mycobacteria. Based on the results in planktonic MRSA, it was estimated that TBSSB could also be effective against *M. tuberculosis* because they are all gram-positive bacteria. However, it is known that the walls of mycobacteria cells usually prevent drugs from entering the bacteria. In this study we investigated the antibacterial activity of TBSSB against *M. smegmatis*, commonly used in mycobacterial research as a surrogate model for the pathogenic and slower-growing *M. tuberculosis*, to reveal the influence of TBSSB on mycobacterial survival and the regulation mechanism of mycobacteria grown under TBSSB. Our results provide the evidence to support the use of TBSSB for anti-TB drug design and underscore the significance of TBSSB as an anti-mycobacterial drug.

Materials and Methods

Strains and Chemicals

M. smegmatis mc²155 cells from the ATCC were routinely cultivated in Luria-Bertani (LB, Oxoid Ltd., England) broth with 0.05% Tween 80 or on LB agar at 37°C. TBSSB was synthesized and confirmed as described previously [8].

Growth Monitoring of the *M. smegmatis*

A single colony of wild-type *M. smegmatis* was inoculated into 5 ml LB broth for 48 h. Then 20 µl of the culture was added to 20 ml LB broth containing 0.05% Tween 80, and TBSSB was added at a gradient with final concentrations of 0, 25, 40, 50, 60, and 75 µg/ml. The growth of *M. smegmatis* was determined by monitoring the optical density (OD) at 600 nm of cultures at intervals of 12 h using a Multiskan FC microplate reader (Thermo

Scientific, USA). Growth curves were plotted with OD_{600nm} versus incubation time, and the optimal inhibition factors were determined from the incubation time and TBSSB concentration.

Morphologic Observation by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

M. smegmatis cells treated with TBSSB were harvested after 40-h incubation followed by fixation in 2.5% glutaraldehyde in 100 mM PBS (pH 7.4). Fixed *M. smegmatis* cell pellets were further fixed in 100 mM (pH 7.4) with 1% osmium tetroxide (OsO₄), and dehydrated in a gradient series of 20%, 40%, 60%, 70%, 80%, 90%, and 100% ethanol. For SEM analysis, dehydrated cell pellets were resuspended in 100% ethanol, applied to a silicon wafer slide, and gold-coated to a thickness of 5 nm. The cells were examined with a JSM-6500F scanning electron microscope using an accelerating voltage of 15 kV. For TEM analysis, the dehydrated cells were embedded using an Epon 812 embedding kit and cut into ultrathin sections. The sections were then stained with both uranyl acetate and lead nitrate, and the cell morphology was observed using a JEM-2000EX TEM (JEOL, JAPAN) [9]. *M. smegmatis* cells not treated with TBSSB were used as the control.

Biofilm Assays

M. smegmatis cells inoculated in LB broth and incubated at 37°C to 0.5 of OD_{600 nm} were transferred to M63 medium (0.5% Casamino acids, 1 mM MgSO₄ 0.7 mM CaCl₂) at a dilution rate of 1:1000. TBSSB was added into the medium at a gradient of final concentrations of 0, 25, 40, 50, 60, and 75 µg/ml. A 150 µl culture was added to each well in a 96-well polystyrene microtiter plate (Costar, USA). The plates were incubated at 30°C for 5 days without shaking. After incubation, the wells were rinsed three times with 200 µl physiological saline to remove unattached bacteria, and stained with 150 µl of 1% crystal violet (Sigma-Aldrich, USA) for 15 min at room temperature. The plates were rinsed with water three times to remove the excess dye. Stain was resolubilized with 150 µl of 95% ethanol and the OD_{595 nm} was read in a microplate reader (Thermo Scientific). The test of biofilm formation was performed in triplicate.

Two-Dimensional Electrophoresis and Image Analyses

Soluble protein was extracted from the harvested *M. smegmatis* cells treated with TBSSB and processed by a Cleanup Kit (Bio-Rad, USA) according to the manufacturer's instructions. Processed protein pellets were dissolved in 50 µl IEF buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, and 30 mM Tris pH 8.0), and the total soluble protein was quantified using a BCA Protein Kit assay (keyGEN BioTECH, China). Fifty micrograms of total soluble protein was subjected to bidimensional (2D) electrophoresis analysis (Protein IEF Cell, Bio-Rad). The subsequent 2D electrophoresis analysis and the image analyses were performed as described previously [10]. Proteins were separated in the first dimension using Immobiline DryStrips (11 cm, pH 4–7, Bio-Rad), and in the second dimension using 12% SDS-PAGE gels. The gels

were stained using a ProteoSilver Plus SilverStain Kit (Sigma-Aldrich). Gel image analysis and statistical quantification were conducted using PDQuest software. Student's *t*-test was used to assess the statistical significance of the differentially expressed proteins based on average spot volume ratio. The protein spots with a fold-change (FC) ≥ 2 or ≤ 0.5 at 95% confidence level (Student *t*-test; $p < 0.05$) were selected for further mass spectrometry (MS) identification. The spots located near the gel borders and the small or faint spots were excluded from protein identification. *M. smegmatis* cells not treated with TBSSB were used as the control.

Trypsin Digestion, MS/MS Analysis and Data Acquisition

The selected protein spots were excised manually from the gels, and the subsequent decolorization using 100 mM NH_4HCO_3 /30% ACN with $\text{K}_3\text{Fe}(\text{CN})_6$ and $\text{Na}_2\text{S}_2\text{O}_3$, and in-gel trypsin digestion using 100 mM NH_4HCO_3 containing 12.5 ng/ μl sequencing-grade trypsin for 20 h at 37°C were conducted as described previously [11]. MS identification was conducted using a 5800 MALDI-TOF/TOF MS (Applied Biosystems, USA) in positive ionization mode and automatic data acquisition mode. The following optimized source/gas parameters were used: the instrument contained a Nd:YAG laser; the mass analyzer scan was 800–4,000 Da m/z ; ions were accelerated with a voltage of 2 kV, and CID in the off setting. Data were acquired using Mascot 2.2 software. MS spectra were searched against the NCBI database. Confidence in peptide identifications was assessed based on the MASCOT sequence assignment score and visual inspection of the molecular mass and *pI* values of the selected spots in the gels.

Bioinformatics Analysis

Analysis on functional classification of proteins was performed for the differentially expressed proteins identified by tandem mass spectrometry. The 14 gene symbols in *M. tuberculosis* with corresponding homology to the proteins identified in *M. smegmatis*

were entered into the PANTHER database (<http://www.pantherdb.org/>) for functional classification. The 20 gene symbols in *M. smegmatis* were input to STRING (<https://string-db.org/cgi/input.pl>) for protein network construction.

Results

Determination of TBSSB-Dose Dependent Growth

The effect of TBSSB was assayed on *M. smegmatis* cultures. Growth curves were plotted as $\text{OD}_{600\text{ nm}}$ versus cultivation time. A TBSSB-dose dependent growth curve of *M. smegmatis* was established by the gradient of 0–75 $\mu\text{g/ml}$ TBSSB concentrations. Fig. 2A shows that $> 60 \mu\text{g/ml}$ TBSSB completely inhibited *M. smegmatis* growth, whereas $< 40 \mu\text{g/ml}$ TBSSB did not have an obvious inhibitory effect on growth, and 50 $\mu\text{g/ml}$ TBSSB showed an optimally inhibitory growth. The optimally inhibitory growth was determined with 50 $\mu\text{g/ml}$ TBSSB for 40 h.

Decreased Biofilm Formation of *M. smegmatis* Inhibited by TBSSB

To further investigate the effect of TBSSB on mycobacterial growth, inhibition by TBSSB of biofilm formation by *M. smegmatis* was assessed. As shown in Fig. 2B, a TBSSB-dose dependent inhibition in biofilm formation was revealed. Even though the growth curve did not display an obvious inhibition with 40 $\mu\text{g/ml}$ TBSSB, a significant decrease in the biofilm formation was detected in *M. smegmatis* grown at a lower TBSSB concentration. Statistical analysis of the crystal violet staining showed that TBSSB had a dose-dependent significant inhibition on *M. smegmatis* biofilm formation.

Fig. 2. Influence of TBSSB on growth and biofilm formation of *M. smegmatis*.

(A) *M. smegmatis* grown under TBSSB. Cell growth was monitored at 600_{nm} with values plotted from triplicate experiments. Final TBSSB concentrations included: 0 $\mu\text{g/ml}$ (dark blue), 25 $\mu\text{g/ml}$ (red), 40 $\mu\text{g/ml}$ (green), 50 $\mu\text{g/ml}$ (purple), 60 $\mu\text{g/ml}$ (light blue) and 75 $\mu\text{g/ml}$ (orange). (B) Statistical analysis of biofilm formation. *M. smegmatis* cells were exposed to TBSSB concentrations ranging from 0 to 75 $\mu\text{g/ml}$. Bars represent the mean $\pm$ SEM; $p < 0.05$ * was considered significant.

Fig. 3. Representative SEM micrographs of *M. smegmatis*.

M. smegmatis cells exposed to TBSSB at 50 µg/ml (A–C) and 0 µg/ml (D–F). Magnification: A and C, 2,000×; B and E, 8,000×; C and F, 20,000×.

Morphological Alterations of *M. smegmatis* Inhibited by TBSSB

Morphology of *M. smegmatis* grown under 50 µg/ml TBSSB was observed by TEM and SEM using non-TBSSB-treated *M. smegmatis* as the control. SEM images are shown in Fig. 3. Non-TBSSB-treated *M. smegmatis* cells had a clear cell shape, and appeared as straight, thin rods with a smooth cell surface. In contrast, the TBSSB-treated *M. smegmatis* cells displayed a warped cell morphology without a defined and clear cell borderline and appeared to be fused together as shown in Figs. 3A, 3B, and 3C. No independent cells and integrated cell wall were found in SEM images. In addition, compared with the morphological micrographs obtained in planktonic MRSA, TBSSB showed a higher destruction of the cell wall in *M. smegmatis* than planktonic MRSA.

TEM images are displayed in Fig. 4. Compared with the images from non-TBSSB-treated *M. smegmatis* cells, alteration of the cytosolic content was observed in TBSSB-treated *M. smegmatis* cells based on electron density. Non-TBSSB-treated *M. smegmatis* had filamentous DNA and clear nucleoid region visible within cells, whereas TBSSB-treated *M. smegmatis* displayed a rearrangement of the intracellular contents including an invisible nucleoid region and concentrated cytosolic materials represented by high electron-dense bodies. Additionally, a dramatic increase in numbers of Z-ring, a typical marker of cell division, was observed in TBSSB-treated *M. smegmatis* (Fig. 4B). Based on this observation, TBSSB appears to block *M. smegmatis* cell

division to further inhibit its growth. Furthermore, more *M. smegmatis* cells displaying a longitudinal sectional view were found when inhibited by TBSSB. *M. smegmatis* cells usually have a firm and rigid cell envelope to protect them against adverse conditions, and it is hard to obtain wild *M. smegmatis* cells with longitudinal sectional view using the TEM analysis. All this evidence pointed to the fact that TBSSB-treated *M. smegmatis* cells have an attenuated cell envelope and were easily cut using the TEM technique.

Identification of Differentially Expressed Proteins by 2D-Gel Electrophoresis Coupled with Mass Spectrometry

To search for differentially expressed proteins, we performed a pairwise comparison of TBSSB-treated and non-TBSSB-treated *M. smegmatis* cells. Proteome changes in gel were automatically analyzed by PDQuest software, and the spots corresponding to the most prominent changes in terms of fold-change (ratios < 0.05 and > 2) and statistical significance ($p < 0.05$) were selected. Fig. 5 shows the paired 2D-gel images manually labeled according to their numbers obtained from the PDQuest analysis. Considering the spot intensities and their locations on the 2D-gel images, 20 spots with significantly differential expression (Table 1) were extracted for further MS identification. Of the 20 spots, seven spots showed a ≥ 2 -fold up-regulation, whereas 13 showed a ≤ 0.5 -fold down-regulation in TBSSB-treated compared to non-TBSSB-treated *M. smegmatis* cells.

Seven proteins (alanine dehydrogenase, Myo-inositol 2-dehydrogenase, hemerythrin HHE cation-binding protein,

Fig. 4. Representative TEM micrographs of *M. smegmatis* cells grown with TBSSB at 50 µg/ml (A–D) and 0 µg/ml (E–H). Magnification: A and E, 10,000×; B and F, 20,000×; C and G, 40,000×; D and H, 60,000×. High electron-dense bodies indicated by arrows in D; filamentous DNA and clear nucleoid region indicated by arrows in F and G; *M. smegmatis* cells with longitudinal sectional view indicated by arrows in A and E; Z-rings indicated by arrows in B.

3-beta hydroxysteroid dehydrogenase, molecular chaperone DnaK, mannose-binding lectin, and pyridoxal 5'-phosphate synthase lyase subunit PdxS) were up-regulated in TBSSB-treated *M. smegmatis* cells. Fourteen proteins including glycerate kinase, bifunctional 5,10-methylene-tetrahydro-

folate-dehydrogenase/5,10-methylene-tetrahydrofolate cyclohydrolase, aldehyde dehydrogenase, isochorismatase hydrolase, hypothetical protein MSMEG_1680, eptc-inducible aldehyde dehydrogenase, forkhead-associated protein, NAD(P)H nitroreductase, oligopeptide transport ATP-

Fig. 5. Representative maps of differentially expressed proteins identified by 2D-gel electrophoresis analysis. A pairwise comparison was performed between TBSSB-treated *M. smegmatis* cells (A) and TBSSB non-treated cells (B). Gels were visualized after silver staining. Numbers labeled manually according to SSP numbers by PDQuest analysis.

Table 1. Properties of the differentially expressed proteins identified by MS.

SSN	TBSSB/ WT Ratio	Protein Name	NCBI Accession No.	No. in <i>M. smegmatis</i>	No. in <i>M. tuberculosis</i>	Protein MW	Protein PI	Pep. Count	Protein Score	Protein Score C. I. %
2805	14.49	Alanine dehydrogenase	Q8KY18.1	MSMEG_2659	Rv2780	38931	5.56	15	74	99.704
3401	10.73	Myo-inositol 2-dehydrogenase	CKI19118.1	MSMEG_4650		35303.6	4.93	15	108	100
3104	0.12	glycerate kinase	WP_058125940.1	MSMEG_2528	Rv1437	37686.7	4.49	14	64	97.478
4804	0.14	bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/5,10-methylene-tetrahydrofolate cyclohydrolase	YP_886023.1	MSMEG_1647	Rv3356c	29674.7	5.9	12	61	94.482
204	0.18	aldehyde dehydrogenase	CKG90698.1	MSMEG_0309	Rv0223c	52014.4	4.65	17	72	99.541
6106	0.24	isochorismatase hydrolase	YP_889227.1	MSMEG_4976		20686.6	4.67	8	72	99.519
6307	0.45	hypothetical protein MSMEG_1680	YP_886056.1	MSMEG_1680		23529.9	4.74	4	71	99.732
1501	0.45	epc-inducible aldehyde dehydrogenase	YP_885925.1	MSMEG_1543	Rv0458	55944.1	5.01	4	117	100
8405'	5.56	hemerythrin HHE cation-binding protein	YP_886755.1	MSMEG_2415		21247.8	4.82	10	182	100
6607'	4.51	3-beta hydroxysteroid dehydrogenase	AIU08609.1	MSMEG_3470	Rv3785	31062.9	5.14	9	307	100
5510'	3.06	pyridoxal 5'-phosphate synthase lyase subunit PdxS	WP_011728705.1	MSMEG_2937	Rv2606c	32082.3	5.11	8	119	100
5109'	2.89	mannose-binding lectin	AIU08795.1	MSMEG_3662		21909	4.54	8	62	97.924
1307'	2.44	molecular chaperone DnaK	YP_885116.1	MSMEG_0709	Rv0350	66606.2	4.71	33	539	100
9402'	0.3	forkhead-associated protein	AIU06864.1	MSMEG_1634	Rv3863	20435.1	4.86	7	63	98.312
5601'	0.27	NAD(P)H nitroreductase	AIU10280.1	MSMEG_5246	Rv2032	36198.5	5.19	12	106	100
4508'	0.18	oligopeptide transport ATP-binding protein OppD	YP_885050.1	MSMEG_0640	Rv1281c	36035.5	5.05	14	68	99.466
4711'	0.22	tetrahydropicolinate succinylase	YP_889353.1	MSMEG_5104		32430.7	5.38	12	61	97.067
4701'	0.23	Amidohydrolase AmiB1	YP_886055.1	MSMEG_1679	Rv3306c	40703.4	5.39	15	125	100
3702'	0.41	NDMA-dependent methanol dehydrogenase	WP_011731151.1	MSMEG_6242	Rv3692	46262	5.36	19	240	100
2703'	0.45	methylmalonate-semialdehyde dehydrogenase	YP_885880.1	MSMEG_1498	Rv0753c	54269.7	5.31	21	173	100

TBSSB/WT Ratio>1, up-regulated; TBSSB/WT Ratio<1, down-regulated. The number with a prime represented the data from the second batch of MS identification.

binding protein OppD, tetrahydropicolinate succinylase, amidohydrolase AmiB1, N-nitrosodimethylamine (NDMA)-dependent methanol dehydrogenase, and methylmalonate-semialdehyde dehydrogenase were significantly down-regulated in TBSSB-treated *M. smegmatis* cells (Table 1).

Discussion

In this study we found that TBSSB affects *M. smegmatis* cells to attenuate the cell envelope. The mycobacterial cell wall consists of a cell wall core with an inner plasma

membrane. The core structure of the cell wall (mAGP, mycolic acids-arabinogalactan-peptidoglycan) is composed of three covalently linked substructures: peptidoglycan (PG), arabinogalactan (AG) and mycolic acids. The innermost layer consists of PGs covalently attached to the middle layer of AG (a polysaccharide of arabinose and galactose sugars) via a phosphoryl-*N*-acetylglucosaminosylrhamnosyl linkage. AG is esterified with the outer layer of mycolic acids containing long-chain fatty acids (C60-C90) [12–14]. Both AG and PG are hydrophilic, but mycolic acids are highly hydrophobic. This relatively impermeable cell wall usually serves as a natural barrier against harmful compounds and factors that inhibit growth, and is essential for mycobacterial survival. Most anti-mycobacterial drugs have been designed to target the mycobacterial cell envelope, especially the enzymes involved in cell wall biosynthesis [15–17]. In this study we found that TBSSB efficiently targeted the mycobacterial cell envelope and led to cell wall destruction. Moreover, TBSSB displayed a more efficient inhibition on *M. smegmatis* cells than on planktonic MRSA. For these reasons, TBSSB has significant potential to be developed into anti-mycobacterial drug.

Morphological images obtained in this study displayed a vast majority of *M. smegmatis* cells fused together to form complex communities where they lived and died. Recently, a number of intriguing observations have suggested that microorganisms likely evolved programmed cell death

(PCD) in response to damage, antibiotics, environmental stimuli or other harmful conditions [18]. PCD is a genetically encoded process that leads to cell death. It plays a critical role in many developmental processes and homeostasis in bacteria [19]. Damaged bacteria undergoing PCD possibly are of benefit to the whole microbial community because they donate nutrients to neighboring cells for repairing them instead of draining resources from their neighbors. This phenomenon is noted as altruistic cell death accompanied by autolysis of mother cells. Autolysis is a self-digestion of the cell wall catalyzed by peptidoglycan hydrolases [18]. It was interesting to note that the amidohydrolase AmiB1, one important autolysin, had been detected using proteomic techniques in this study. AmiB plays an important role in cell division and cell lysis [18, 20, 21]. In this study, we found that AmiB (MSMEG_1679) down-regulated altruistic behavior of microorganisms by forming a regulation network with MSMEG_1680 and DapD (MSMEG_5104) (Fig. 6A), and that downregulation of this network plays a pivotal role in protecting live *M. smegmatis* cells from autolysis.

To reveal a possible in vivo regulation mechanism on *M. smegmatis* cells exposed to TBSSB, the proteomic analysis on soluble proteins was carried out by 2D-gel electrophoresis coupled with tandem mass spectrometry analysis. As a consequence, 20 proteins were identified to be significantly regulated. Of the 20 identified proteins, seven were

Fig. 6. Bioinformatic analysis of the differentially expressed proteins identified by tandem mass spectrometry. (A) Protein-protein interaction networks analysis using STRING database, (B) and (C) protein classification analysis using PANTHER database. The representative pie diagram displayed the involvement percentage for differently functional categories of the proteins. (B) Proteins categorized into groups according to the biological process; (C) proteins categorized into groups according to the protein class.

upregulated and 13 were downregulated. To gain functional information of these identified proteins in the dataset, we searched their corresponding homologous proteins in *M. tuberculosis* for functional classification in PANTHER. The pie diagram of Figs. 6B and 6C displays graphically the involvement percentage of the identified proteins for different functional categories. Proteins with high involvement were in the oxidoreductase class (54.5%); other functional categories were hydrolase (18.2%), transferase (18.2%), and transporter (9.1%) (Fig. 6C). They respond to biological processes including metabolic (50.0%), cellular (35.7%), response to stimulus (7.1%) and localization (7.1%) processes (Fig. 6B). Thus, cell survival was regulated via altering the metabolic pathway and enzymatic activities of the identified protein.

Interestingly, NDMA-dependent methanol dehydrogenase (MSMEG_6242) and NAD(P)H nitroreductase (MSMEG_5246) were identified as differentially expressed proteins in this study; they had been identified in *M. smegmatis* grown under DdIA deficiency as well. DdIA is a ligase that catalyzes the dimerization of two D-alanine molecules yielding D-alanyl-D-alanine, which is an indispensable substrate for peptidoglycan biosynthesis [10]. MSMEG_5246 has been documented previously as an NAD(P)H nitroreductase [22], proteins which have a remarkable ability to catalyze reduction of nitroaromatic compounds to their corresponding hydroxylaminoaromatics via ionic or radical mechanisms [23]. Nitroreductases have been proved as targets for eradication of various pathogens by widely used antibiotics such as nifurtimox, nitrofurantoin and metronidazole [24]. NDMA-dependent methanol dehydrogenase (MSMEG_6242) allows Gram-negative microorganisms to generate energy from methanol oxidation and synthesize compounds with carbon-carbon bonds from methanol assimilation [25]. In this study, MSMEG_6242 was found to interact directly with MSMEG_0309, MSMEG_1543, MSMEG_1498 to form a network that also showed a strongly positive interaction with the other five identified proteins including MSMEG_1647, MSMEG_0409, MSMEG_2528, MSMEG_4650 and MSMEG_3470 to regulate *M. smegmatis* survival (Fig. 6A). Thus, MSMEG_6242 and MSMEG_5246 play a pivotal role in regulating *M. smegmatis* survival under adverse conditions and have potential as an anti-mycobacterial drug target.

In this study, *M. smegmatis* mc²155 was used as the surrogate model for pathogenic and slow-growing *M. tuberculosis* [26–30]. Compared to *M. tuberculosis*, *M. smegmatis* has a variety of advantages: (1) It grows faster [30] since its

doubling time is around 5 h against 30 h for *M. tuberculosis* [31, 32]; (2) it is non-pathogenic [1]; (3) has a unique cell wall similar to that of *M. tuberculosis* which plays a crucial role in cell survival and growth under adverse environmental conditions or drug resistance [16, 17]; (4) it is closely related to *M. tuberculosis* in species and genetics according to phylogenetic tree analysis. Therefore, it is applicable for *M. smegmatis* to be utilized as the surrogate host of *M. tuberculosis* in vivo.

Taken together, we report here that TBSSB inhibits mycobacterial growth and biofilm formation, attenuated cell division and cell wall ultrastructure, and altered the intracellular mycobacterial content. Additionally, metabolic pathways were regulated to adapt *M. smegmatis* to TBSSB inhibition. Among the 20 differentially expressed proteins identified, NDMA-dependent methanol dehydrogenase, NAD(P)H nitroreductase, and amidohydrolase AmiB1 were found to be critical in regulating *M. smegmatis* survival exposed to TBSSB. Our study reinforces the idea that Schiff base-aurine compounds have great potential to be developed as novel anti-mycobacterial drugs.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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