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Identification and validation of L-asparaginase as a potential metabolic target against Mycobacterium tuberculosis

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Abstract
Multidrug-resistant Mycobacterium tuberculosis (Mtb) has emerged as a major health challenge, necessitating the search for new molecular targets. A secretory amidohydrolase, L-asparaginase of Mtb (MtA), originally implicated in nitrogen assimilation and neutralization of acidic microenvironment inside human alveolar macrophages, has been proposed as a crucial metabolic enzyme. To investigate whether this enzyme could serve as a potential drug target, it was studied for structural details and active site-specific inhibitors were tested on cultured Mycobacterial strain. The structural details of MtA obtained through comparative modeling and molecular dynamics simulations provided insights about the orchestration of an alternate reaction mechanism at the active site. This was contrary to the critical Tyr flipping mechanism reported in other asparaginases. We report the novel finding of Tyr to Val replacement in catalytic triad I along with the structural reorganization of a β-hairpin loop upon substrate binding in MtA active site. Further, 5 MtA-specific, active-site–based inhibitors were obtained by following a rigorous differential screening protocol. When tested on Mycobacterium culture, 3 of these, M3 (ZINC 4740895), M26 (ZINC 33535), and doxorubicin showed promising results with inhibitory concentrations (IC₅₀) of 431, 100, and 56 µM, respectively. Based on our findings and considering stark differences with human asparaginase, we project MtA as a promising molecular target against which the selected inhibitors may be used to counteract Mtb infection effectively.

KEYWORDS
homology modeling, inhibitors, L-asparaginase, molecular docking, molecular dynamics simulations, Mycobacterium, tuberculosis

1 | INTRODUCTION

L-Asparaginases belong to the amidohydrolase family of enzymes that catalyzes the conversion of L-asparagine into L-aspartic acid and ammonia. The antileukemic property of this enzyme has been explored comprehensively. Moreover, for intracellular pathogens, this enzyme has been proposed as a potential molecular target. L-Asparaginase of Mycobacterium tuberculosis (Mtb), although identified and proposed as crucial for the survival of pathogen, has not yet been exploited. Unlike Escherichia coli where 2 types of asparaginases, encoded by two different genes, (cytoplasmic and periplasmic) have been reported, Mtb has a single gene that encodes a periplasmic
1-asparaginase (MtA).\(^5,6\) It is therefore considered to serve a key metabolic function. Mtb is an intracellular parasite and the causative agent of tuberculosis (TB), which is historically the second most deadly infection in humans.\(^7,8\) TB has been present and infecting human hosts since ancient times. With the urbanization of human civilization, a rapid increase in TB infections has been witnessed. Mtb is a highly evolved pathogen that can even survive inside the hosts in a latent phase for years, with only a few members of the infected population exhibiting full-blown symptoms. Although Mtb can infect a variety of host tissues, such as lungs, brain, and spine, but to continue survival it escapes from the host immune system and replicates inside phagosome of professional phagocytic macrophages.\(^9\) It has been reported that Mtb releases certain virulence factors to escape the host immune system that provides survival benefits inside macrophages.\(^10,11\) In addition, the pathogen is able to prevent acidification of phagosome by restricting its fusion with acid-carrying vesicles, which otherwise are the parts of the maturation pathway in the macrophages.\(^12,13\) For drug-susceptible Mtb infection, available therapy includes a cocktail of 4 drugs, viz, rifampicin, isoniazid, pyrazinamide, and ethambutol.\(^14\) Despite having an effective treatment, some strains of this bacillus acquire multidrug resistance that has been designated as a global threat and provided an urgent incentive to search for novel drug targets against this pathogen.\(^15,16\) Of late extreme drug resistance and recent findings of total drug resistance have evoked serious health threats.\(^17,18\)

Comprehensive and comparative genome and proteome analysis can facilitate identification of potential metabolic targets. Recently, asparaginase metabolism and asparaginase itself has been found providing significant benefits to Mtb inside the host and consequently has been anticipated as a novel and potential drug target. A report stated that MtA helps in prevention of the acidification of phagosome and provides a nutritional advantage to the pathogen by producing ammonia and aspartate, respectively.\(^5\) Therefore, no phagolysosomal complex is formed and pathogen easily replicates inside the host macrophages. Similar conditions have been observed in case of Leishmania donovani, and inhibitors against its 1-asparaginase were successful in their intended purpose.\(^19,20\)

As there are no studies conducted targeting MtA as an inhibitor of pathogenesis, this study was undertaken. The primary objective was to gain structural insights and identify small molecule inhibitors against MtA. To achieve this, a 3-dimensional structural model of MtA was generated and compared with known bacterial and human 1-asparaginases. The results highlight characteristic similarities between catalytic site compositions of MtA with known 1-asparaginases from other pathogens. However, there were significant differences with human 1-asparaginase, projecting the former as a safe molecular target against Mtb. Subsequently, the Traditional Chinese Medicine (TCM) database, ZINC database, and Food and Drug Administration (FDA)–approved drug database were selected for identification of potential inhibitors. Active site composition and stability of MtA-inhibitor complexes were verified through molecular dynamics (MD) simulations. For ex vivo correlations, selected inhibitors were tested on Mycobacterium culture. Interestingly, 3 of them displayed potent antimycobacterial activity at conditions similar to the pathogen’s residing environment inside the host (pH 5.5 with asparagine as sole nitrogen source). Overall, this study highlights the crucial role of 1-asparaginases in the survival of this pathogen in a host-like niche and pave the path for designing effective inhibitors.

## 2 | MATERIALS AND METHODS

### 2.1 | Amino acid sequence retrieval and alignment

All amino acid sequences of asparaginases from *E. coli* I (Uniprot: P0A962) and II (Uniprot: P00805), *M. tuberculosis* (Uniprot: R4M6S3), *L. donovani* (Uniprot: E9BC85), *Pyrococcus horikoshi* (Uniprot: O57797), *Mycobacterium smegmatis* (Uniprot: 17FDG6), and *Homo sapiens* (Uniprot: Q7L266) were retrieved from UniProtKB, and multiple sequence alignment was performed by Clustal Omega to identify key residues involved in the formation of catalytic site.\(^21,23\)

### 2.2 | Homology modeling and validation

The 3-dimensional structure of MtA was generated through SWISS-model server (http://swissmodel.expasy.org/) using *P. horikoshi* 1-asparaginase as a structure template (sequence identity 27%). All the predicted models were matched with known protein structures submitted in RCSB Protein Data Bank, and C-scores were assigned based on relative clustering structural density and consensus significance. Based on C-score, the best model was chosen for further analysis. The selected model was further validated by assessment of phi/psi angles using Ramachandran plot and PROCHECK.\(^24-26\) ProSA program was used for energy criterion comparison.\(^27\)

### 2.3 | Virtual screening of inhibitors against MtA

Three drug libraries, namely, TCM database, ZINC database, and FDA-approved database were screened against defined coordinates of MtA active site.
2.3.1 | ZINC database

A composite receptor-ligand based approach was used for virtual screening of the compounds. Initially, 14 million compounds were screened from ZINC database based on physicochemical properties of L-asparaginase substrate, L-asparaginase. AutoDock Vina docking engine (http://mgtools.scripps.edu), adopted for a receptor-based virtual screening via i-Dock modification, was used for further screening of these selected compounds.\textsuperscript{28-30} The docking grid was defined to include the active site of M\textsubscript{t}A (residues 23, 62, 92, 95, 96, and 168 in model structure). A range of ligand poses were obtained and ligands were ranked on the basis of their binding scores. The top 100 compounds with high binding score were sorted on the basis of Lipinski’s rule of five.\textsuperscript{31}

The pharmacokinetics and putative clinical effectiveness of drug candidates was evaluated by absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles. The admetSAR server (http://lmmd.ecust.edu.cn/admetsar1/) was used to predict toxicity, and top 20 potential inhibitors were retained for further analysis.\textsuperscript{32}

2.4 | Redocking and free energy calculation

Redocking of selected compounds from all the libraries (filtered through ADMET) with M\textsubscript{t}A and human L-asparaginase (HLA) was performed by Molecular Operating Environment v2009.10 (MOE)\textsuperscript{33} Details of active site grid parameterization and free energy calculations are described elsewhere.\textsuperscript{19}

2.5 | TCM and FDA-approved drug database

Natural products and drugs were screened from TCM database (TCM Database@Taiwan containing 37,170 compounds) and FDA-approved drug database (downloaded from ZINC database, containing 3714 compounds), respectively. The receptor-based screening method was adopted for initial screening provided catalytic site information to the iScreen server for TCM database, whereas AutoDock Vina tool was used for screening of compounds from FDA database. Out of these, the top 100 compounds were subjected to redocking using MOE and top 5 compounds from each database were selected on the basis of their binding energy.

2.6 | Molecular dynamics simulations

GROMAC package v5.1 (http://www.gromacs.org/) was used for MD simulations using CHARMM27 force field for apo and holoenzyme as well as enzyme-inhibitor complexes.\textsuperscript{34-36} The topologies of ligands were generated by SwissParam (http://www.swissparam.ch/). In a separate set of simulations, systems were placed in cubical boxes, equidistantly at 15 Å from box edges. Hydrogen atoms, added using pdb2gmx module, were constrained using LINCS algorithm. With periodic boundary conditions applied in all 3 dimensions, the protein was explicitly solvated using simple point charge model extended (SPCE) water system and appropriate counter ions to maintain electroneutrality and 0.15 mM NaCl. System was first energy minimized by steepest descent followed by conjugant gradient method. In separate steps of 1 ns each, the systems were equilibrated in an normal volume and temperature (NVT) ensemble followed by an normal pressure and temperature (NPT) ensemble. Particle mesh Ewald method was used to treat long-range electrostatic interactions with cutoff radius of 10 Å. The system temperature of 310 K was kept constant by modified Berendsen coupling. Leap frog integration was used for velocity generation with 2 fs time step. Structural coordinates were recorded at 10 ps intervals for a total of 50 ns simulations. Trajectory analysis was done using standard GROMACS tools (http://www.gromacs.org/) and images were created by PyMol (Schrodinger, New York, NY)\textsuperscript{37-39}

2.7 | Antimycobacterial activity assay and imaging

The effect of top-scored inhibitors was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on M. smegmatis mc\textsubscript{2}155. This is a fast-growing mycobacterium strain which served as a model organism for preliminary drug testing. Moreover, M\textsubscript{t}A and M. smegmatis asparaginase share full conservation in catalytic residue and a high degree of overall amino acid sequence identity (63%). Mycobacteria were grown at 37°C in Sauton-modified medium, containing 0.05% Tween-80, 0.5 g/L KH\textsubscript{2}PO\textsubscript{4}, 0.5 g/L MgSO\textsubscript{4}, 2 g/L citric acid, 10 g/L glycerol, and 5 mM asparagine prepared in double-distilled water and neutralized to pH 7.0 or 5.5 with 10 N NaOH.\textsuperscript{5} Here, Sauton-modified medium contains asparagine as a sole nitrogen source. Cultures were prepared in triplicate in 96-well plate (100 µL each), and bacterial growth was monitored by measuring turbidity (OD\textsubscript{560}) and growth kinetics were studied at 2 different pHs: (i) pH 7—a physiological pH and (ii) pH 5.5—mimicking phagosomal pH. Sterility of culture was checked by acid-fast bacilli (AFB) staining kit (Himedia, Pennsylvania, PA). Top 6 compounds, namely, M3, M26, M29, M31, and doxorubicin (Dox), were selected for ex
vivo testing. All compounds were purchased from Molport (Latvia) except Dox (Sigma-Aldrich, St. Louis, MO). Fifty millimolar of stock of each compound was prepared in dimethyl sulfoxide and diluted to 10, 50, 100, 200, 500, and 1000 μM working concentrations. Mycobacterium grown in medium without inhibitor served as control and medium only served as sterility control. Evaporation is a common problem in 96-well plate; therefore, 200 μL of sterile water was added to all surrounding wells to reduce evaporation. The growth of treated and untreated mycobacteria was monitored at different time points by measuring OD600 till 48 hours.

Bacteria were stained with AFB staining kit (Himedia) and examined under ×100 magnification of differential interference contrast mode using inverted research microscope (ECLIPSE Ti; Nikon, Nikon Instruments Inc., New York, NY).

Atomic force microscopy (AFM) images were also taken using the Bioscope Catalyst AFM (Bruker Corporation, Billerica, MA) having a Nanoscope V controller. The samples were prepared by pouring 10 μL of untreated and compound-treated (IC50) M. smegmatis cells on freshly peeled mica surface immediately followed by drying under nitrogen flow. All images were taken using ScanAsyst mode in air at room temperature using standard silicon nitride cantilevers with nominal spring constant of 0.6 N/m were used. A scan rate of 0.5 Hz with 256 samples per line was used for imaging followed by image analysis using NanoScope analysis v1.4 (Bruker Inc., Massachusetts, MA).

Cell viability and IC50 values were determined by standard MTT assay.42 After 48 hours of bacterial growth, 10 μL of 12 mM solution of MTT (Himedia) in phosphate-buffered saline was added to each well and allowed it to grow for 4 hours at 37°C. Then, 50 μL of dimethyl sulfoxide was added to each well to dissolve formazan crystals and absorbance was measured at 540 nm using a multiscan FC microplate photometer (Thermo Fisher Scientific, Massachusetts, MA). However, sometimes MTT results are not reliable as it is an absorbance-based assay. Therefore, inhibitory potential of the screened compounds was reassessed through resazurin microtiter assay (REMA), a fluorescence-based method.43 Resazurin solution changes color from blue (nonfluorescence) to pink (fluorescence), indicating reduction and therefore bacterial growth. Resazurin sodium salt powder (Sigma-Aldrich) was prepared (0.01%; wt/vol) in distilled water and filter sterilized. Mycobacterium were grown in 96-well plate at 37°C, in presence and absence of inhibitors as followed for MTT (stated above). After 48 hours of incubation, 30 μL of resazurin solution was added to each well, incubated 4 hours at 37°C and fluorescence was monitored (λex, 520 to 560 nm; λem, 590 nm) in a 1-cm path length cuvette using a LS 55 fluorescence spectrometer (PerkinElmer Life Sciences, Inc., Waltham, MA).

3 | RESULTS AND DISCUSSION

3.1 | Homology modeling and structural analysis

The 3D model generated using SWISS-model server was validated through Ramachandran plot analysis where it showed 92.9% residues in the most favored region, 4.5% in the allowed region, and 2.6% in the disallowed region (Figure 1 and Supplementary Figure 1A). Furthermore, the model was validated using PROCHECK and ProSA (Supplementary Figure 1B). Overall structural analysis showed the similarity of MtA with other known L-asparaginases. In accord with previously known crystal structures of bacterial L-asparaginases, it showed 2 distinct α/β domains connected through an unstructured linker (Figure 1).

The larger N-terminal domain (magenta) is composed of 8 β-pleated sheets and 4 α helices along with a rigid β-hairpin at the active site loop (residues 26 to 36 in modeled structure). This is followed by a 20-residue long linker region (green; residues 191 to 210 in modeled structure) composed of a β-sheet that extends to the C-terminal domain containing 3 β-pleated sheets and 2 α helices (yellow).

3.2 | Active site and sequence alignment studies

Mtba genome expresses the single gene for putative L-asparaginase (uniport: P63627), present on Rv1538c locus. Multiple sequence alignment of MtA with asparaginases from other organisms yielded crucial insights of active site composition with significant overlaps in the conserved catalytic residues (blue) (Figure 2). In general, L-asparaginases have been categorized into 3 major families: type I,
type II, and type III. While types I and II are mostly bacterial in origin, the type III is usually found in mammals and plants. The type III has a distinct mode of catalysis in contrast to bacterial L-asparaginase.

The crystal structures of most of the previously known L-asparaginases depict the involvement of 2 catalytic triads (I and II) at each active site. Triad I contains a nucleophile (Thr), a base (Tyr), and an acidic moiety (Glu/Asp) constituting a flexible active site loop (Figure 3A). Likewise, triad II also contains a nucleophile (Thr) that activates a water molecule along with a basic (Lys) and an acidic residue (Glu/Asp) (Figure 3A). These 2 triads work in a concerted manner and convert the substrate asparagine into aspartate and ammonia in the presence of a water molecule (acting as the second nucleophile).

Primary sequence alignment and structural model of MtA revealed key differences in the composition of the catalytic residues. The first nucleophile in triad I (Thr23) along with the entire triad II (Asp96, Thr95, and Lys168) was fully conserved (red). However, crucial Tyr (acting as a base in EcAI/EcAII and P. horikoshi L-asparaginase; Figure 2; arrow) was found replaced with a Val (residue 33 on active site loop) at a similar position in MtA. Previous studies on archael asparaginase have shown that flipping of this Try residue inside and outside the plane is responsible for activating the nucleophile Thr, which in turn attacks the substrate. Hence, finding a Val replacement at this position in MtA creates a certain degree of disparity on the mechanism of action of this enzyme.

To understand this, we did a rigorous sequence and structure analysis of the simulated MtA structure. We observed that there was another Tyr (Tyr282) from oppositely placed monomeric unit laying within the plane of the triad I (Figure 3B). This prompted us to propose that this Tyr might act as a base to complete triad I. This was indeed strengthened through analysis of distance variation of this Tyr from other catalytic triad partners (Figure 3C). During most of the simulation period, Tyr282 positioned itself in the close proximity of Thr23 and Asp62 (0.5 to 0.6 nm) of triad I, upholding its intimate association in catalytic activity. Here, Tyr282 after getting deprotonated by acidic Asp62 activates the first nucleophile Thr23, which in turn attacks the substrate L-asparagine. Interestingly, when compared with L-asparaginase from some other pathogens such as Helicobacter pylori and Leishmania donovani identical Try to Val/Ala replacements were observed, suggesting the possibility of a similar catalytic mechanism operating in these pathogens (Supplementary Figure 3).

Our observations were strengthened with the simulations of MtA in the presence of its substrate asparagine (holoenzyme). Interestingly, in the presence of asparagine, the β-hairpin guarding the active site underwent structural reorganization into a flexible loop (Figure 3D). The flexibility of loop was proposed to regulate the accessibility of substrate to the active site in various other asparaginases. Though our in silico analysis presents new reaction mechanism of substrate hydrolysis by MtA, attempts of crystallization are being undertaken to obtain further insights.
3.3 | Virtual screening of compounds against MtA

Virtual screening is an extensively used method for drug discovery and lead optimization because it can be used to screen large datasets against defined coordinates of the active site. The full screening protocol is summarized in Figure 4.

3.3.1 | ZINC database

For ZINC database, initially, 11,457 potentially drug-like compounds were selected based on their similarity with L-asparagine and then were subjected to receptor-based screening. The system, presubjected to Lipinski’s rule of five, yielded top 1000 compounds which possessed higher binding energies than L-asparagine (>-5.1 kcal/mol, the score for L-asparagine). Next, these compounds were screened on the basis of their toxicity profile and only top 100 compounds were selected based on their lower toxicity levels (LD_{50} < 2.55 mol/kg). This was followed by free energy calculations and comparative binding studies of the selected compounds with MtA and HLA using MOE. Finally, 5 compounds were selected which exhibit significantly higher binding energies with MtA compared to HLA (Table 1; Supplementary Figure 4). These compounds were M3 (ZINC 4740895), M15 (ZINC 91668201), M26 (ZINC 33535), M29 (ZINC 12363043), and M31 (ZINC 12428170) (Figure 5A).

The ADMET profile of these 5 compounds showed significant probabilities for intestinal absorption (>0.97) and low permeability for blood-brain barrier (>0.7) (Table 2). The Caco-2 permeability scores are in the range of 0.5 to 0.6, indicating moderate paracellular movement of compounds across the intestinal monolayer. P-glycoprotein pumps are responsible for drug efflux. The P-glycoprotein nonsubstrate scores are in the range of 0.5 to 0.7 have moderate probability of the drug to be efflux. CYP2D6 enzymes may lead to higher excretion of drug. A value between 0.7 and 1.0 indicates high chances of CYP2D6 induction. These parameters of the screened compounds show suitable pharmacokinetics to act as a drug-like molecules.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Binding energy (kcal/mol)</th>
<th>Molecular weight (g/mol)</th>
<th>x log P</th>
<th>HBD</th>
<th>HBA</th>
<th>PSA (Å²)</th>
<th>MOE score</th>
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<td>134</td>
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</table>

HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; PSA, polar surface area; x log P, partition coefficient.
3.3.2 | TCM and FDA-approved drug database

For identification of inhibitors of MtA, direct receptor-based screening was done using TCM and FDA-approved drug library and top 100 compounds were selected. Redocking and docking scores of top 100 compounds against MtA and HLA was similarly calculated using MOE. Top 5 compounds from each library, showing the lower binding (docking) free energy to MtA compared to HLA were finally retained (Supplementary Figure 5). After extensive screening, compounds, viz, N6 (ZINC02093563), N7 (ZINC02123619), N24 (ZINC02138567), N25 (ZINC02160915), and N29 (ZINC0213082), from TCM database (Figure 5B) and montelukast (F1; ZINC03831150), silybin (F2; ZINC02566164), cefatrizine (F3; ZINC03830401), cortisone acetate (F4; ZINC03830604), and Dox (F5; ZINC3918087) from FDA-approved database were selected as potential inhibitors (Figure 5C). Their chemical properties and details on binding mode are summarized in Table 3. All these screened compounds showed higher binding energy than l-asparagine. Detailed H-bond network of all identified inhibitors are given in Figure 6 and Supplementary Table 1.

3.4 | MD simulations and the overall stability of the structure

The stability of enzyme-inhibitor complexes was validated through MD simulations, carried up to 50 ns and measured in terms of backbone root-mean-square deviation (RMSD), Cα-Rg (gyration radius) and root mean square fluctuation (RMSF) in residues. RMSD is a measure of the difference between an initial and final conformation of the backbone of a protein. The stability of the protein is governed by deviation generated during simulations and larger deviation projects to less stable protein conformation. In the presence of ligands, the backbone RMSD stabilized after 10 ns, indicating the formation of a stable docked complex (Figure 7A). The folding architecture can be assessed through studying changes in the radius of gyration of an enzyme in the absence and presence of a ligand over the simulation period. For both apo- and ligand-bound MtA, the radius of gyration varied between 2.5 and 2.65 nm (Figure 7B) suggesting that the association of ligands to MtA does not affect the original protein architecture. In physiological conditions, enzymes are dynamic in nature, which confer flexibility to the residues to carry specific functions. The RMSF of an enzyme-ligand complex reveals the flexible regions of the complex. The protein has several secondary structures like helix, sheet, turns, etc, in which helix and sheets display lesser fluctuations than loop, coil, and turns. Low RMSF value shows the tightly organized architecture of the enzyme-ligand complex. RMSF analysis of Cα atoms averaged over last 10 ns of simulations revealed overall lower fluctuations in MtA-ligand complexes compared to apo form (Figure 7C).
TABLE 2  ADMET profiles of screened inhibitors using ZINC database

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Blood-brain barrier (+)</th>
<th>Intestinal absorption (+)</th>
<th>Caco-2 permeability (−)</th>
<th>P-glycoprotein substrate</th>
<th>CYP450 2D6 inhibitor</th>
</tr>
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<tr>
<td>M3</td>
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<td>0.8039</td>
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ADMET, absorption, distribution, metabolism, excretion, and toxicity.

TABLE 3  Physicochemical properties and binding free energies of the substrate and screened inhibitors using Traditional Chinese Medicine and Food and Drug Administration–approved drug database

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Binding energy (kcal/mol)</th>
<th>Molecular weight (g/mol)</th>
<th>x log P</th>
<th>HBD</th>
<th>HBA</th>
<th>PSA (Å²)</th>
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<tr>
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<td>544</td>
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HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; PSA, the polar surface area.

FIGURE 6  Protein ligand interactions from ZINC, TCM, and FDA-approved drug database. (A) Molecular interactions of inhibitors from ZINC database within the active site are shown. (B) Inhibitors from TCM database (N6 in red and active site residues in orange). (C) Inhibitors from FDA-approved drug database (Dox in green). Dox, doxorubicin; FDA, Food and Drug Administration; TCM, Traditional Chinese Medicine
**FIGURE 7** Backbone and tertiary structure variations of MtA upon binding to different ligands. (A) Changes in backbone RMSD, (B) gyration radius and (C) RMSF, in apo and holo form of 1-asparaginase of Mtb and in the presence of inhibitors along the simulation trajectory. Binding of inhibitors clearly brings stability to MtA as shown by lowering of trajectories in each case compared to the apo form. RMSD, root mean square deviation; RMSF, root mean square fluctuation.

**FIGURE 8** Effect of selected inhibitors on Mycobacterial culture. (A) Dose-response curve of *Mycobacterium smegmatis* to Dox, M3, and M26 obtained using standard MTT assay. Normalized values are plotted as mean and standard deviation from 3 replicates. IC$_{50}$ (µM) corresponding to drug concentrations compromising 50% survival of the bacteria was derived from each curve. (B) Resazurin microtiter assay-based cell survival estimation in control and compound-treated cells. For all 3 compounds (Dox, M3, and M26), IC$_{50}$ concentrations showed significant decline in cell viability (*P < 0.05; **P < 0.01). (C) Microscopic images show normal, healthy rod-shaped bacilli in control whereas distorted cell shape and cell debris were observed in cells grown with selected compounds (black arrows). (D) Atomic force microscopy images showing cell death accompanying gross changes in cellular morphology in *M. smegmatis* cells in the presence of selected compounds (Dox, M3, and M26). Scale bars in all images is 2 µm. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide
Based on strong hydrogen bonding interactions at the active site groove retained throughout the MD simulation trajectory, these 6 identified compounds showed excellent stability. Analysis showed the persistence of strong H-bond network (~2 to 6 H bonds) between MtA and screened ligands along with Dox. This indicated stable complex formation by various small molecules within the active site pocket of MtA (Supplementary Figure 6).

3.5 | Antimycobacterial activity assay and morphological assessment

Within the current scope of the study, we investigated the antimycobacterial potential of screened inhibitors against *Mycobacteria* at conditions close to the phagosomal environment. Initially, the purity of culture was confirmed through AFB staining (Supplementary Figure 7A) and growth kinetics of this bacilli were studied at 2 different pHs 7.0 and 5.5. Compromised growth kinetics was observed at pH 5.5 reflected by an extended lag phase and reduced log phase when compared to growth at pH 7 (Supplementary Figure 7B). As the pathogen is known to express MtA to resist acid stress at pH 5.5, the inhibitory potential of M3, M26, M29, M31, and Dox was determined at this pH. Survival analysis showed that out of these 5 compounds, only M3, M26, and Dox inhibited the growth of bacterium at pH 5.5 with an observed IC₅₀ of 431, 100.9, and 56.13 μM, respectively (Figure 8A). The data were substantiated by REMA and preliminary microscopic analysis. REMA was performed after 48 hours on *M. smegmatis* grown in presence of selected inhibitors. The normalized fluorescence results showed similar percentage survival of bacteria as observed through MTT assay (Figure 8B). Simultaneously, treated mycobacterial cells were stained with AFB and observed under a microscope (Figure 8C). Not only the cells were significantly reduced in number but also showed distorted morphology, indicating poor survival.

Further, a comparative topological assessment of cells was also done to understand morphological differences in untreated and compound-treated cells using AFM (Figure 8D). The control *M. smegmatis* cells showed characteristic bacilli-like morphology with an average height of 269.8 nm. On the other hand, the Dox and M3-treated *M. smegmatis* cells showed a gross loss in cellular morphology, indicating cell death. This was also evident by drastic reduction in cell heights (Dox, 23.1 nm and M3, 5.6 nm) in both cases. Interestingly, the M26-treated cells still showed bacilli structure but with mutilated surface features (enlarged section; Figure 8D). In this case, roughly 3-fold reduction (85.6 nm) in surface height of *M. smegmatis* cells as compared to control, untreated cells were observed. Overall, the AFM data demonstrated the cytotoxic potential of these 3 compounds and validated their inhibitory effect on *M. smegmatis* cells.

4 | CONCLUSIONS

Our findings highlight the potential of MtA as a novel therapeutic target to combat TB. Sequence and structure analysis revealed the unique composition of the active site in MtA compared to other bacterial counterparts. The characteristic differences with human asparaginase also lend credence to visualize MtA as a nonharmful specific target. The structural variations in terms of critical residues involved in catalysis were novel findings. These were exploited to obtain active site of MtA and also select inhibitors. The large TCM library, FDA-approved drug database, and synthetic compounds were screened for this purpose. The top inhibitory compounds, which show strong binding to MtA active site, were selected using an integrated approach that involves homology modeling, docking, and MD simulations. The comprehensive analysis of all MtA-inhibitor docked complexes exposed the presence of a strong network of intermolecular hydrogen bonds encompassing the active site residues. To the best of our knowledge, based on scientific and medical literature review, this is the firsthand structure-based report of projecting l-asparaginase as a novel drug target and its inhibitors as potential therapeutic of Mtb.

The major conclusions of this study can be summarized as: (i) The catalytic triad I of MtA is constituted of a different set of residues (Tyr282 as base, Asp62 as acid, and Thr23 as nucleophile), not reported in other pathogenic l-asparaginases; (ii) in the presence of substrate l-asparagine, the rigid active site loop of MtA gets unstructured, thus enhancing the possibility of substrate accessibility; (iii) the inhibitors selected here showed high binding affinity to MtA (indicated by the binding score metric in silico analyses) and not to HLA, indicating selective action; (iv) the heterocyclic nature of the identified compounds provides spatial advantage enabling stabilization of the active site groove and establishment of hydrogen bonding with the surrounding residues; (v) M3 (ZINC 4740895), M26 (ZINC 33535), and Dox inhibited the growth of *mycobacteria* in culture with the IC₅₀ in the micromolar range. In brief, the findings of this study provide the mechanistic insights of l-asparaginase function in intracellular pathogen and its role in their survival within the host, thereby paving the way for novel therapeutic strategies. Further, the inhibitors may work on the broader set of pathogenic organisms like *Salmonella typhi*, *H. pylori*, and *L. donovani*, where the catalytic triad display similar (Val/Ala in place of Tyr) arrangement of catalytic residues.
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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