Chapter III

Materials and Methods

As shown in Figure 3.1, the screening process used in this study for the discovery of hit compounds included *In-silico* and *In-vitro* approaches. Performing *in-silico* screening and molecular docking of compound libraries can significantly decrease both the time and costs linked to experimental testing of extensive compound collections for the identification of potential hits.

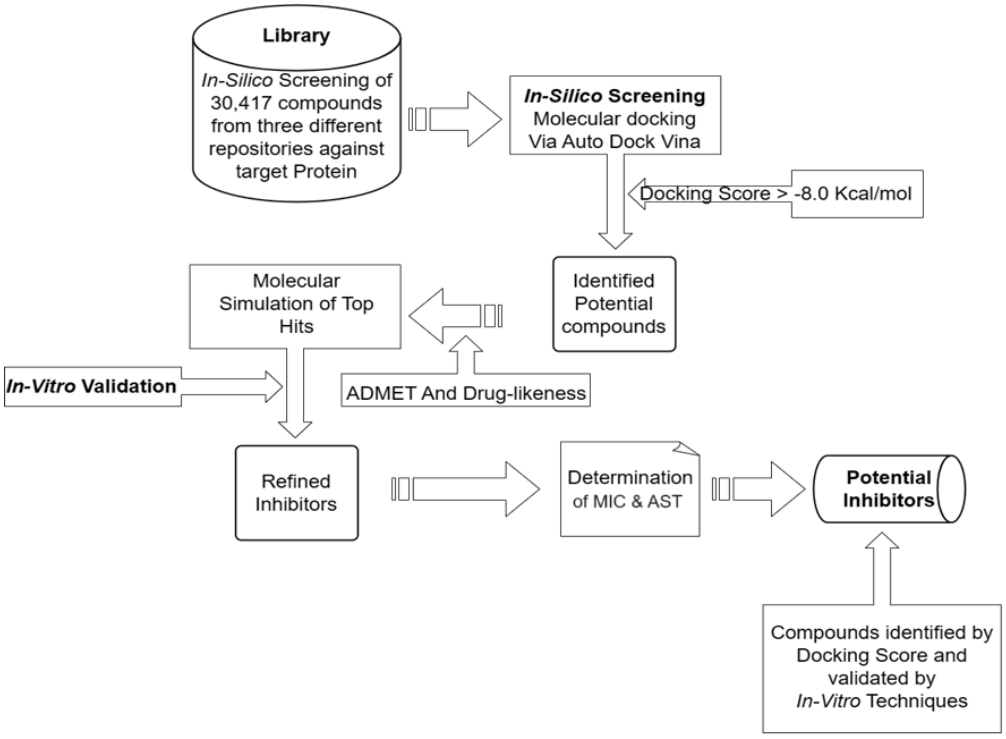


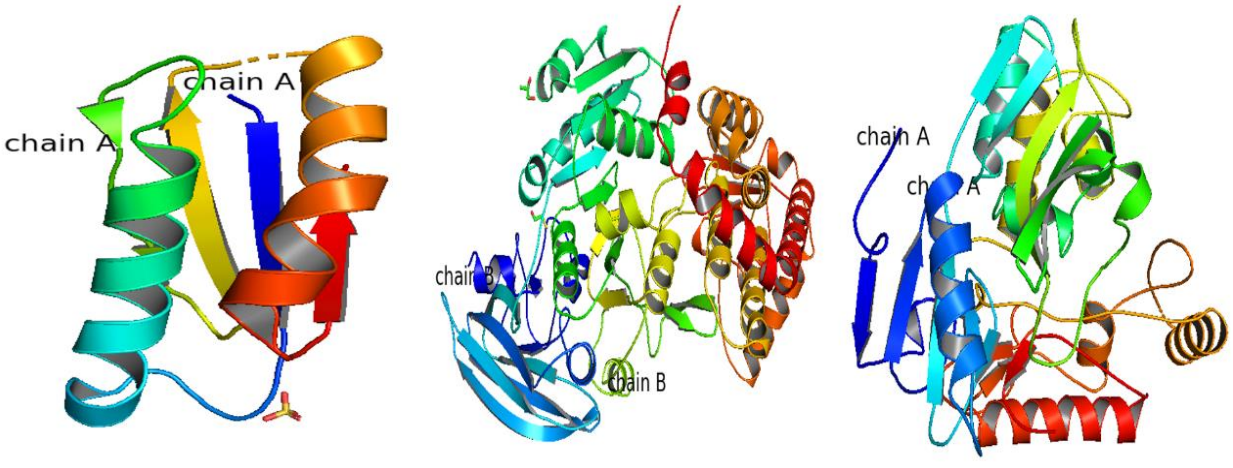
Figure 3.1. Illustration of research methodologies employed in this study for the identification of potential inhibitors against MTB's cell wall proteins.

3.1. *In-silico Screening*

3.1.1. Data collection and target protein preparation

Leveraging the Protein Data Bank (PDB) (https://www.rcsb.org/), three-dimensional crystal structures of three enzymes (Figure 3.2) of MTB's cell wall including *MmpL* 11D2 (PDB ID: 4YOL), *GlfT2* (PDB ID: 4FIX), and *MurB* (PDB ID: 5JZX) were retrieved.

The target proteins (receptors) underwent preparation using the AutoDock tool, specifically version 1.5.6.[173] The preparation involved (1) the elimination of water molecules from the target protein structures, (2) the deletion of hetatms, (3) the fixing of missing residues, (4) the addition of hydrogen and Kollman charges, and then receptor molecule with charge saved in. PDBQT format.



A. *MmpL* 11D2 (4YOL) **B. *GlfT2* (4FIX)** **C. *MurB* (5JZX)**

Figure 3.2. The 3D structure of the targeted proteins (receptors) that were considered in this investigation.

3.1.2. Data collection and preparation of the ligand library

The collection of compounds from "ChemSpider",[174] "Drug Bank" (https://go.drugbank.com),[175] and the "Zinc Database"[176] was used to create the ligand library. The compounds' structures were initially retrieved in SDF-structure data file format and subsequently transformed to the appropriate arrangement (PDBQT) using the "Open Babel" tool (Open Babel.org).[177] A total of 10,000 compounds from "ChemSpider", 9137 from "Drug Bank", and 11280 from the "Zinc Database" were obtained and screened against target receptors. The steps in the ligand preparation produce variations and optimization of the structure, which is then saved as PDBQT.

3.1.3. Grid generation and virtual screening

The AutoDock Vina script[178] was utilized for the docking of multiple compounds, all 30,417 compounds were independently tested against the target protein of MTBs first with *MmpL*, then with *GlfT2*, and lastly with *MurB* receptor.

3.1.4. *MmpL* 11D2 (4YOL)

A docking approach was carried out using the grid's 100,100 and 100 dimensions with 0.500 Å spacing and 9.417x, 39.917y, 11.161z-center was taken into consideration for *MmpL* 11D2 (PDB ID: 4YOL).

3.1.5. *GlfT2* (4FIX)

By the utilization of docking with grid's parameter of 144.08x, 45.029y, 45.473z-center and 100, 100, 100 dimensions with 0.500 Å spacing was selected for *GlfT2* (PDB ID: 4FIX).

3.1.6. *MurB* (5JZX)

Similarly, A grid box was set out with 69.084x, 40.549y, 40.662z-center with 0.500 Å spacing with 100, 100, and 100 dimensions, and docking was performed.

AutoDock Vina was employed to generate various docked conformations and dynamically position ligands within the protein conformational space. This was achieved using the Lamarckian genetic algorithm. With a population size of 150 and a maximum generation of 27000, a total of 10 runs are carried out, and 2,500,000 for the maximum generation evaluation under default parameters. Vina predicted the ligands' modes of binding, and kilocalories per mole (Kcal/mol) were used to measure binding affinity. After the docking process was finished, all results were graded based on their binding affinity. The threshold cut-off score for docking was established based on the target enzymes' scores for substrate binding affinity. *MmpL* 11D2, *GlfT2*, and *MurB* had maximum binding affinities of -9.9 Kcal/mol; -13.5 Kcal/mol; and -13.0 Kcal/mol, correspondingly. Based on these scores, a limit of -10.5 Kcal/mol for *MmpL*; -9.0 Kcal/mol for *GlfT2*; and -9.5 Kcal/mol for *MurB* was established. A total of thirteen compounds are identified against target *MmpL* 11D2, five from ChemSpider, and four compounds are from Drug Bank and Zinc database respectively. Against *GlfT2*, a total of thirty compounds are identified, two compounds from ChemSpider, nineteen from Drug Bank, and nine from the Zinc database with

top hits. With the top binding affinity, thirty-two compounds were discovered, eleven from ChemSpider, ten from Drug Bank, and eleven from the Zinc database against the target *MurB*. The top-ranked compounds with the best binding affinity were chosen for further refinement. Ten distinct poses were generated for each identified compound and their inhibition constant (*KI*) was calculated via the AutoDock tool.[173] The initial "crystal structure of the target proteins", "the root mean square deviation" (RMSD) values, and the "inhibitory constant" (*KI*) of the docked complex were all taken into account. Using the PyMOL program, the receptor-ligand complex was visualized.[179] Protein plus,[180] PLIP tools[181] and all of the aforementioned predictions were combined and used to analyze the receptor-ligand interactions.

3.1.7. Identification of ADMET and Pharmacophores Characteristics

To forecast the drug-like characteristics of lead compounds, ADMET features were investigated by using the tools molsoft L.L.C. (Molsoft L.L.C.: Drug-Likeness and molecular property prediction.), pkCSM (pkCSM (uq.edu.au),[182] and ADME lab 2.0 (ADMETlab 2.0 (scbdd.com),[183] among others. To choose molecules with desirable properties, these compounds underwent additional screening for drug attractiveness, pharmacokinetics, and physicochemical features. Pharmacophoric feature-based geometrical models that were designed and extracted from many potent lead compounds are beneficial in identifying several drug targets.

3.1.8. MDS-Molecular Dynamic Simulation of The Receptor-Ligand Complexes

Utilizing Schrodinger-Maestro v10.4's Desmond 4.4 module[184] and 100 ns molecular dynamics (MD) simulations and on a Linux-powered HP Z2 workstation, the complex

stability of the highest-scoring poses for receptor-ligand complexes was evaluated. The protein-ligand complex MD systems were developed as orthorhombic grid boxes (10 x 10 x 10) buffers. TIP4P water molecules were incorporated into the system builder tools in the Desmond maestro interface to minimize the system in 3000 steps by utilizing the steepest descent techniques, shadowed by a 5000-step "conjugate gradient technique" with 120 Kcal/mol threshold energy. The pressure was kept constant during MD simulations by using "anisotropic diagonal position scaling" on a 0.002 ps time step break. A 20 PS isothermal-isobaric (NPT) ensemble with a target pressure of 1 Atm and a gradual rise in system temperature was also authorized (100 K to 300 K). In addition, the Berendsen algorithm[185] was fixed at 0.2 constant and the "Lennard-Jones" cut-off value was set at 9 Å. Furthermore, all chemical bonds, even those involving hydrogen were subject to "SHAKE" ideal limitations.[185] Finally, each complex's MD simulation was run for 100 ns intervals under the same parameters. The evaluation of simulation interactions was conducted using the simulation diagram tool within the Desmond v4.4 module of Schrodinger Maestro v10.4[184] and then examined the simulation trajectories.

3.2. *In-vitro* Validation

After completing docking, ADMET, and Molecular simulation studies, seven compounds (against each target) were selected against target *GlfT2* and *MurB*. These compounds were CSID541554, CSID67239, DB12983, DB12424, ZINC000043203371, ZINC000063933734, ZINC000095092808 against *GlfT2* while CSID1438694, CSID2166135, DB15688, DB12983, ZINC003975327, ZINC084726167, ZINC254071113 against *MurB*. Out of the total fourteen

compounds, the compounds that showed the best results in MDS were evaluated for their antituberculosis activity against non-pathogenic strains of *Mycobacterium*. These strains (non-pathogenic) were *Mycobacterium smegmatis* and *Mycobacterium phlei*. Four compounds were chosen for *in vitro* validation. The selection of these compounds was guided by both the outcomes of *in silico* analysis and their accessibility.

3.2.1. Bacterial Strain and Growth Conditions

The bacterial strains employed for antituberculosis activity included *Mycobacterium phlei* (MTCC-1724), *Mycobacterium smegmatis* (MTCC-6), *M. tuberculosis* H_{37}Rv (ATCC 27294), *M. tuberculosis* H_{37}Ra (ATCC 25177), and a clinical multidrug-resistant (MDR) strain of *Mycobacterium tuberculosis* SRHU-1. These strains were cultivated on LJ medium (Himedia, India) and Middlebrook 7H9 Broth Base agar (Himedia, India) slants at 37°C.

3.2.2. Anti-mycobacterial Activity Against Non-Pathogenic *Mycobacterium* Species

The bacterial inoculum preparation involved overnight (24 hours) growth of cultures in nutrient broth (Difco) supplemented with tween-80 (0.1% v/v; Merk). The culture's turbidity was adjusted to 0.5 McFarland units, approximately equivalent to 1.2 x 108 CFU mL-1. For spreading the inoculum on agar plates, 100 µl aliquots were used along with a sterile glass spreader. A paper disk with a 5 mm diameter (Whatman filter paper no.3; Millipore) was impregnated with 10 µL (1µg/µL) of the test sample and allowed to dry for 30 minutes. Subsequently, the paper disk was placed on the

bacterial lawn. The negative control consisted of disks containing solvents, while the positive control involved a disk containing the antibiotic rifampicin (5.0 µg disk-1; Sigma, St Louis, Mo).

The Petri dishes were incubated in an incubator at a temperature of 37°C for 48 hours. Following incubation, the diameter of the zone around the disk where bacterial growth was inhibited was measured. This procedure was repeated twice, and the average value from the three experiments was recorded.

3.2.3. Evaluation of MIC Against Non-Pathogenic Strains of *Mycobacterium*

The minimum inhibitory concentration (MIC) of the test compounds was determined using a two-fold micro-dilution technique in sterile flat-bottom 96-well polystyrene microtiter plates from Axgen, CA, USA. The compounds were diluted in a series of two-fold dilutions using nutrient broth supplemented with 0.1% Tween-80 (v/v). Microtiter plates were inoculated with the bacterial culture and incubated at 37°C for 48 hours. Bacterial growth was assessed by measuring absorbance at 600 nm with a spectrophotometer. To determine MIC, a tetrazolium salt indicator, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) from Merck, was used. Viable bacteria reduce the tetrazolium salt indicator, resulting in a blue-purple color formation. For the MIC assay, 40 µl of 0.2 mg/ml-1 MTT was added to each well, and after incubation at 37°C for 1 hour, control wells displayed a purple color. MIC values were determined based on concentrations where a noticeable decrease in color formation occurred, indicating inhibition of bacterial growth.

3.2.4. Antimycobacterial Activity Using BACTEC Radiometric Assay (Virulent and Clinical strains)

To evaluate the anti-tuberculosis activities of the identified compounds against *M. tuberculosis*, the BACTEC assay was utilized. The BACTEC 460 TB system, which is a radiometric susceptibility testing platform designed for slow-growing *Mycobacterium* species, was employed for this purpose. The virulent strains of Mtb H_{37}Rv and the avirulent strain H_{37}Ra were retrieved from a freezer set at -80 °C. These strains were then cultured on LJ medium slant for a period of 21 days. The bacterial cells were harvested and subsequently transferred to BACTEC diluting fluid, where they were homogenized using glass beads, 2mm in diameter, and allowed to stand briefly for sedimentation of any bacterial clumps. Before testing, the homogeneous suspension that was obtained was amended to McFarland standard 1.0 using diluting fluid.

To initiate the BACTEC assay, a 0.1 ml suspension of the bacterial culture obtained from the initial inoculum culture vial, containing approximately 500 growth index (GI), was introduced into a BACTEC 12B vial. Test samples were prepared using DMSO, and a 0.1 ml portion of the bacterial suspension from the primary inoculum culture vial was injected into the test samples using a 1.0 ml insulin syringe. Following the 1% proportion method, 0.1 ml of the primary inoculum was mixed with 9.9 ml of BACTEC diluting fluid, resulting in a 1:100 dilution. The diluted culture was then added to the 12B vial along with the control solvent. Daily GI testing was conducted by incubating the vials at 37°C, with GI monitored every 24 hours. Once the control vial with a 1:100 dilution reached a GI of 30, the GI values of the test vials

containing the compounds were compared to those of the control vials based on the difference in growth (DGI).

The interpretation of the results depended on the variation in growth (DGI) between the current GI and the previous day's GI. If the DGI of the test sample vial was lower than the DGI of the control (1:100) vial for the corresponding periods, the test compounds were deemed effective against MTB. Conversely, if the DGI of the test sample vial was higher than the DGI of the control vial, the test compound was considered inactive.