Chapter V

Discussion

To tackle drug-resistant strains of *Mycobacterium tuberculosis* (Mtb), the development of new structures or frameworks with strong antimycobacterial activity is crucial. This involves various procedures such as discovering new compounds with a distinct mechanism of action, identifying inhibitors, and modifying existing medications chemically to create novel compounds. A method, that includes high-throughput screening, was employed to identify potential inhibitors. Using such techniques, many *Mur* enzyme inhibitors from different organisms have already been discovered[1] as well as numerous antitubercular scaffolds presently being assessed in various phases of clinical trials,[4] bedaquiline is an example of such an inhibitor. It prevents Mtb by blocking the *ATP synthase* enzyme.[5] The presence of libraries containing small molecules and advancements in computational techniques have significantly broadened the possibilities for discovering novel molecular scaffolds that target specific proteins. Additionally, if *in-silico* hits were to receive experimental validations, that would be the icing on the cake.

Cell walls of MTB have drawn interest recently as a possible therapeutic target because they are crucial for the pathogen's survival of which *MmpLs* from the outermost layer of mycolic acid biosynthesis are one of the first targets.[2,3,5,20] *MmpLs* are a crucial and newly discovered target for the formulation of Mtb inhibitors.[9] Large *Mycobacterial* membrane proteins, known as *MmpLs*, have crucial functions in the transportation of lipids, polymers, immunomodulators, and the efflux of therapeutic molecules. A twelve-helix transmembrane domain and a periplasmic pore domain

make up *MmpLs*. Several compounds have been shown to have an impact on *MmpLs*, the exclusive transporter of "trehalose monomycolate" in Mtb, an essential component for the development of the MA layer in the cell wall.[20] Multiple screening studies conducted within the last decade have discovered a putative target for numerous small molecules inhibitors, including compounds like AU1235, C215, E11, BM212, DA-5, indolcarboxamides, NITD-349, HC2091, rimonabant, TBL-140, PlPD1, SQ109, and THPP.[4,13,18,174,180-183,188,192,193] Spheroplasts were recently used to demonstrate the direct suppression of *MmpL* by BM212, the first substance discovered to target *MmpL*.[20] Dual "Asp-Tyr" pairings that are primarily situated in this domain seem to be important proton-translocation facilitators. These Asp-Tyr pairings are inhibited by the binding of SQ109,[16] AU1235, ICA38 (Indolcarboxamides38), and rimonabant inside the transmembrane space.[13] This structural information will help *MmpL3* inhibitors become more effective TB medications. Degiacomi and colleagues discovered four classes that target *MmpLs*, "quinoline" (Q1), "amino benzimidazole" (A1), "phenyl-urea" (P1), and "2-(piperidin-1-yl) ethan-1-amine" (E1).[12] Two additional inhibitors, namely SPIRO and NITD-349, bind tightly to the core channel of the TM domains, significantly altering the protein's structure.[14] The binding of NITD-349 with SPIRO involves the interaction between the amide and indole nitrogen of "NITD-349" and the "piperidine nitrogen" of SPIRO, leading to the clamping of Asp 645. The two compounds' structural study suggests that both inhibitors aim to decrease *MmpLs'* action by blocking the proton relay pathway.[14] Adamantyl urease,[15] pyrroles,[18] indole carboxamides,[18] diamines, tetrahydro pyrazolopyrimidines (THPPs),[4] and spiro cycles (Spiros),[173] among others, are anti-*MmpLs* pharmacophores that are effective against drug-resistant strains of Mtb. Additionally,

GSK2623870A and GSK2783100A showed variations in how much they depended on *MmpLs* expression.[16] Through genetic research utilizing transposon mutant libraries and employing diverse elimination methods, it was uncovered that *MmpL* plays a vital role in the survival of *M. tuberculosis*.[15] When *MmpL* was silenced in mice, the lungs and spleens were completely cleared of bacteria, regardless of whether the infection was acute or persistent. These results support the notion that *MmpL* is a desirable therapeutic target and show that *MmpL* inhibitors may shorten the duration of treatment.

The second target from the middle layer is arabinogalactan *GlfT2* (galactofuranosyl transferases). Despite being crucial for AG assembly, a chemical inhibitor of *GlfT2* is much less explored. The first compound to be found to prevent *Mycobacterial* galactan from being synthesized was the pyrrolidine analog of galactofuranose.[175] Only a few exploratory investigations have been described for activity against *GlfT2* enzymes, utilizing *UDP-Galf* or iminopentitol derivatives.[63] The fluorinated ex-glycan analog of *UDP-Galf* revealed an IC_{50} value of 180 µM in the radiometric assay employing the crude cell wall enzyme fraction from *M. smegmatis* and *"O-alkyl β-d-Galf-(1→6)-β-d-Galf"* acceptor. This makes it the most potent compound against the enzyme.[176]

According to recent searches, thiazolidinone derivative was identified as a potential leading candidate for *GlfT2* inhibitors based on analyses of its characteristics using "molecular docking", "3D-QSAR", and *in-silico* "ADMETox" investigations.[177] Although whole-cell screens are commonly used, the efficiency of these methods is constrained by the limited chemical diversity found in commercially available or

proprietary libraries. This observation is particularly evident in the outcomes of experimental and *in-silico* screening conducted on current compound collections. However, optimism persists that advancements in computational and experimental technologies within the realm of developing TB inhibitors will facilitate the discovery of prospective compounds with inhibitory effects on *GlfT2*.

The cell envelope of *Mycobacterium TB* is distinct in its composition and structure, and it contains a layer of peptidoglycan that is crucial for virulence and maintaining cellular integrity. Enzymes responsible for peptidoglycan production, degradation, remodeling, and recycling have regained attention as promising targets for the development of anti-infective compounds.[20] *MurB* (UDP-N-acetylenolpyruvyl-glucosamine reductase) is an appealing target due to its crucial and distinctive function in the development of bacterial cell walls as well as the fact that the *MurB* enzymes from many bacterial species have been characterized both biochemically and structurally.

There have been reports of some inhibitors of *MurB*.[11,20,194] For a more extensive exploration of chemical inhibitors targeting bacterial *Mur* ligases, readers are encouraged to refer to the comprehensive review article authored by Hrast and colleagues. This review encompasses a wide array of broad-spectrum inhibitors.[195] Another fresh investigation by Maitra and colleagues also discusses the use of these inhibitors in treating bacterial infections.[7] Up to this point, only a few inhibitors have been described and most current findings use the CADD approach. Although *MurB* inhibitors' lack of anti-bacterial effectiveness is a long-standing issue, this case shows how successfully structural data may be used to create inhibitors of the *Mur* enzyme.

A recently published study introduced a new series of tetrazole compounds as *MurB* inhibitors, demonstrating potent activity with IC_{50} values in the low micromolar range. The inhibitors were identified using a combination of structure-based pharmacophore modeling and molecular docking techniques. However, no antibacterial activity was observed for these inhibitors.[7] Novel inhibitors of *MurB* were discovered to be a family of 3,5-diozopyrazolidines.[8] In terms of drug development, developing an innovative "one-pot assay" for recognizing pharmacological inhibitors of *Mur* ligases (A-F), and using computational techniques to analyze substrate binding could be advantageous.[17] By employing the recently discovered crystal structure of *MurB Mycobacterium tuberculosis*[186] and a "one-pot assay" that reconstructs the *in-vitro* environment,[187] it may be possible to develop new inhibitors of peptidoglycan biosynthesis in *M. tuberculosis*.

This study undertook an effort to screen compounds from promising accessible databases to find *MmpL*, *GlfT2*, and *MurB* enzyme-specific inhibitors. These enzymes were chosen based on the literature review, their importance in the biosynthesis of MTB cell walls, and the availability of their crystal structures. This study attempts to test 30,417 compounds against the *MmpL*, *GlfT2*, and *MurB* enzymes using Structure-based screening across three databases. A total of 153 compounds were discovered, fifty-one against each target. The top hits of seventy-five were achieved following docking analysis. Additional calculations were made regarding their drug-likeness and ADMET properties. For discovered chemicals to be used as inhibitors, evaluation of their absorption, distribution, metabolism, and excretion is necessary. The pharmacokinetics of a drug can be quickly ascertained using *in-silico* predictions of

these properties. Numerous physiochemical characteristics are considered including solubility, molecular weight, H-bond acceptor and donor, and others. The range for molecular weight (MW) is between 363.49-785.55 g/mol, lipophilicity (Log*P*) lies between -5.74 to 8.66 whereas the range for aqueous solubility is between -1.15 to -6.67 moles/L.

A total of fourteen refined compounds, seven against *GlfT2* and *MurB* were put through molecular simulation studies (MDS). MDS provides insight into the behavior of complex systems that are difficult or impossible to observe experimentally, such as the behavior of the protein-ligand complexes. The stability and binding of the three ligands, DB12424, ZINC000063933734, and ZINC000095092808, were investigated through molecular simulation studies with the *GlfT2* protein.

Analysis of the simulation data revealed that all three ligands exhibited good binding affinity towards the protein. In particular, ligand DB12424 demonstrated good stability and binding throughout most of the simulation, with only slight diffusion observed in the timeframe of 50-70 ns. Strong binding affinity was observed during the timeframe of 70-100 ns, with an RMSD of the proteins at 2.7Å, indicating consistent and stable interactions with *GlfT2*. Similarly, the *GlfT2*-ZINC000063933734 complex demonstrated a stable binding affinity, with the RMSD equilibrating at 0-15 ns and 35-45 ns but fluctuating between 20-30 ns. However, a weak to moderate binding was observed during the 45-100 ns timeframe. Ligand ZINC000095092808 exhibited strong, consistent, and stable binding throughout the 100 ns simulation. Based on the RMSD values and the constancy of the protein-ligand interactions witnessed it appears that ligands DB12424, ZINC000063933734, and

ZINC000095092808 exhibit the most stable and best interactions with the *GlfT2* proteins. Based on these findings, it can be inferred that these ligands hold promise as potential therapeutic agents for further development, specifically targeting *GlfT2*.

In context with *MurB* protein, ligands DB15688, ZINC084726167, DB12983, and ZINC254071113 exhibit different binding behavior, ranging from diffusion to stable and consistent binding interactions with the protein. The strongest binding interactions were observed between 30-100 ns for DB15688, 10-60 ns for DB12983, and 20-80 ns for ZINC254071113. The ligand ZINC084726167 shows binding interaction with the *MurB* proteins between 20-80 ns. However, the ligand shows erratic fluctuations after 80 ns, which may compromise the stability of the ligand-receptor complex and affect its overall efficacy as an inhibitor. However, the diffusion behavior of ZINC254071113 after 80 ns may compromise the stability of the ligand-receptor complex and affect its overall efficacy as an inhibitor. The RMSD values for all ligands within the range of 2.1Å-3.6Å demonstrate a good binding affinity with the *MurB* protein. Further investigation into the underlying factors contributing to the diffusion behavior of ZINC254071113 may be necessary to optimize its binding interaction with the *MurB* protein.

Out of the fourteen compounds identified through *in-silico* screening, four top-scoring compounds, two each against *GlfT2* and *MurB* with consistent stable interaction with the target proteins were chosen for further *in-vitro* experiments based on their predicted properties. Furthermore, the choice of these compounds was predicated on their favourable *in-silico* findings and accessibility, further supporting their potential for future development and utilization. Two compounds ZINC000095092808 (A) and

DB12424 (B), demonstrated effectiveness against *GlfT2*, while compounds ZINC254071113 (C) and DB15688 (D) exhibited promising inhibitory activity against *MurB*. Compound A, and Compound C, among the screened compounds, were found to be the most promising and effective MIC values. According to an analysis of the ligand's binding affinity for ligand ZINC000095002808 (docking value of -12.2 Kcal/mol) and DB12424 (docking value of -12.8 Kcal/mol) as well as contact stability (seen by MD simulation), and *in-vitro* validation, these compounds looked to be a novel potential target of *GlfT2*. Similarly, the Compound with IDs, ZINC254071113 and DB15688 looked to be a potential inhibitor of *MurB*. The compound ZINC254071113 indicates promising MIC values against *MurB*.

A target-specific *in-vitro* study revealed that both identified compounds inhibited the primary target enzyme, *GlfT2*. Similarly, the discovered compounds ZINC254071113 and DB15688 significantly inhibited the major target enzyme, *MurB*. Additionally, Lys 369, Ala 405, Gln 409, Glu 451, Gly 232, Ser 233, Lys 369 (two contacts), Asp 371, and His 413 were found as the main interaction residues for *GlfT2* while Arg 176, Tyr 210, Ser 257, Asn 261, Glu 302, Ala 325, Glu 361, Val 284, His 286 and Gly 298 against *MurB* in *in silico* studies.

Subsequently, *in-vitro* experiments were conducted to assess the activity of the identified compounds against the target enzymes. In this study, a comprehensive evaluation of compounds obtained through *in silico* analysis was conducted for their potential antitubercular activity. *In vitro* validation of screened compounds was assessed by the disc diffusion method and BACTEC radiometric assay. These compounds were initially tested against non-pathogenic *Mycobacterium* strains, *M.*

phlei and *M. smegmatis* because they are fast growers and have similar cell wall compositions to that of pathogenic *M. tuberculosis* strain.[196,197] All four compounds exhibited activity against both *M. phlei* and *M. smegmatis*. Specifically, compounds ZINC000095092808, DB12424, ZINC254071113, and DB15688 demonstrated promising activity against these strains and hence further tested against virulent strains of *Mycobacterium tuberculosis*. This screening was conducted with the BACTEC 460 TB system.[196] The results showed that these compounds effectively inhibited the growth of virulent strains of *M. tuberculosis* strains. Importantly, the assay allowed for the detection of antitubercular activity within a short timeframe, typically within 7-8 days. Additionally, a reduction in the growth index (GI) value of *M. tuberculosis* was observed, indicating the compounds' efficacy in inhibiting bacterial growth.[198] The results of our study suggest that the BACTEC assay is a reliable method for assessing the efficacy of compounds with antitubercular activity. Its sensitivity and ability to detect changes in the growth index make it a valuable tool for tuberculosis drug discovery and development.[196] In conclusion, this study represents the first time reporting of two potent compounds having antituberculosis activity against both pathogenic and non-pathogenic strains of *Mycobacterium* species. Further research and development of these compounds may hold potential for the treatment of tuberculosis, a global health concern. Two important antibiotics used in the treatment of *Mycobacterium tuberculosis* (MTB), are rifampicin and moxifloxacin. Rifampicin is like a precision sniper. It targets a crucial enzyme called RNA polymerase in MTB. This enzyme transcribes genes and produces proteins, vital for bacterial survival and growth. Rifampicin disrupts this process, rendering Mtb powerless.[93] Moxifloxacin, on the other hand, takes a different approach. It focuses on enzymes known as DNA

gyrase and topoisomerase IV, which Mtb uses for DNA replication and repair. By interfering with DNA replication, moxifloxacin inhibits Mtb's ability to multiply.[165]

In the present study, promising inhibitors against the two targets i.e., *GlfT2* and *MurB* were found. These targets are involved in building *Mycobacterium tuberculosis* protective cell wall.[16,18,187,189] Disrupting these enzymes is responsible for combating *Mycobacterium tuberculosis*. The most exciting part is that very less or no studies have tried using compounds of the present study that target *GlfT2* and *MurB* against MTB. We embarked on a scientific journey using computer simulations (*in silico*) and lab experiments (*in vitro*) to see if these compounds could be effective.

Our findings are promising. These compounds have shown significant effectiveness against MTB. This discovery opens new possibilities in the fight against tuberculosis, giving us hope for a brighter future where this disease can be conquered with the power of science. Taking everything into account, the compounds identified against *GlfT2* and *MurB* demonstrate promising growth inhibition in *Mycobacterium tuberculosis* (Mtb). These candidates could serve as a foundation for chemical modifications in medicinal chemistry, aiming to develop a "higher-affinity scaffold" with enhanced inhibitory properties. Moreover, this investigation presents an effective method for evaluating compounds for an anti-tubercular activity that combines *in-silico* “structure-based screening” with laboratory experimental validation.