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Resisting resistant *Mycobacterium tuberculosis* naturally: Mechanistic insights into the inhibition of the parasite's sole signal peptidase Leader peptidase B

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ABSTRACT

Tuberculosis (TB) is the second highest cause of mortality after HIV/AIDS and is one of the leading public health problems worldwide. The growing resistance to anti-TB drugs and the recalcitrant nature of tenacious infections present arduous challenges for the treatment of TB. Thus, the need to develop therapeutics against novel drug targets to help overcome multi-drug resistant TB is inevitable. Leader peptidase B (LepB), the sole signal peptidase of *Mycobacterium tuberculosis* (*MTb*), is one such potential drug target. The present work aims at identifying potential inhibitors of LepB, so as to repress the formation of the functional proteins essential for the growth and pathogenesis of *MTb*. In this study, we screened a large dataset of natural compounds against LepB using a high throughput approach. The screening was directed toward a binding pocket consisting of residues, some of which are critical for the catalytic activity of the enzyme, while others are part of the conserved domains of the signal peptidases. We also carried out molecular dynamics simulations of the two top-scoring compounds in order to study their molecular interactions with the active site functional residues of LepB and also to assess their dynamic behavior. We report herein two prospective non-covalent type inhibitory drugs of natural origin which are active against tuberculosis. These lead molecules possess improved binding properties, have low toxicity and are specific against *MTb*.

1. Introduction

Tuberculosis (TB) is the second greatest killer worldwide next to HIV/AIDS and is caused by the sole infectious agent *Mycobacterium tuberculosis* (*MTb*). It has been reported that 8.7 million people became ill and 1.4 million died from TB in 2011 (World Health Organization – Fact Sheet, 2012). The standard 6 month course for TB treatment includes the prescription of four antimicrobial drugs – rifampin, pyrazinamide, isoniazid and ethambutol. However, the emergence of multi drug resistance (MDR) and extensive drug resistance (XDR) toward standard tuberculosis treatment has resulted in increasing severity of the disease.

Disease caused by the resistant bacteria does not respond to the usual first-line treatments thus resulting in MDR TB. Though this MDR TB is curable with second-line drugs, the treatment opportunity is narrowed by their limited availability and high cost. This treatment requires 2 years of extensive chemotherapy which can be quite costly and can also result in severe side-effects in patients. In 2011, approximately 310,000 worldwide cases of MDR TB were notified among total notified TB patients. Moreover, it has been estimated that about 9% of MDR TB cases were XDR TB. This ever-increasing disease incidence highlights an urgent need to identify novel pathways in the bacteria against which potent therapeutics can be developed.

Current therapeutics target cell wall synthesis, DNA transcription and protein translation [1]. Protein secretion is a key cellular process that exports approximately one-fifth of all bacterial proteins into the extracellular environment. Such proteins play an important role in promoting motility, cell wall biosynthesis, chemotaxis, adhesion to host cells and nutrient uptake [2]. These essential and conserved protein export pathways in *M. tuberculosis* include the secretion (Sec) pathway and a twin-arginine translocation (Tat) pathway. Sec export is a post-translational process devoted to carrying out the export of unfolded proteins, while the Tat system exports proteins that are prefolded in the cytoplasm only [3]. After translocation through the SecYEG [4] and TatABC [5] channels, the signal peptide is removed by a Type I signal peptidase (SPaseI) [1,5]. SPaseI enzymes are a special type of serine proteases which utilize a Ser-Lys or Ser-His catalytic dyad instead of the prototypical catalytic triad formed by Ser-His-Asp residues [6].

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The genome of *MTb* encodes one protein, Leader peptidase B (LepB), with a significant sequence similarity to SPaseI. Signal peptide from the preproteins in *MTb* is cleaved by LepB after translocation, thereby releasing the mature protein into the periplasm [7]. The enzyme LepB fulfills the criteria of being a worthy drug target since it is essential for the survival of the pathogen and is quite distinct from its human homologue. Its structure suggests increased accessibility to ligands as its active site is located on the extracellular surface of the cytoplasmic membrane. Moreover, it offers the possibility for designing highly specific inhibitors since it is known to possess a unique Ser/Lys catalytic dyad at the active site [6]. LepB has been validated as a potent drug target by assessing the effect of a previously described SPaseI inhibitor on the viability of *MTb* [8].

The present study is focused on identifying highly specific natural inhibitors of LepB. The inhibition of LepB would result in repressing the cleavage of the signal peptide from the preproteins which would lead to arresting the folding of proteins which are essential for the growth and pathogenesis of *MTb* into their active mature conformation. In the present study, knowledge of the residues forming the catalytic center (Ser96 and Lys174) of the protein [9] and those present in the conserved domains has been taken into consideration in order to identify the binding pocket and to carry out interaction analysis on the screened molecules with LepB.

2. Methods

2.1. Modeling the LepB structure

A 294 amino acid long protein sequence of LepB from *M. tuberculosis* – H37Rv – was retrieved from NCBI (accession no.: NP_217419.1). The absence of any crystal structure in LepB and the unavailability of any significant homologues demanded the generation of an *ab initio* modeled structure. Therefore, we used a Phyre2 platform [10] along with a new *ab initio* folding simulation called Poing [11] to build a 3D structure of LepB. The quality of the obtained model was assessed on the basis of various structural properties using Verify3D [12] and ERRAT [13]. The model was further refined and stabilized by carrying out molecular dynamics (MD) simulations.

2.2. Protein preparation and dataset

The modeled structure of LepB was stabilized using molecular dynamics (MD) simulations and was then prepared using Schrödinger's protein preparation wizard [14]. Hydrogen bonds were added to the structure and optimized. Other preparation steps involved the removal of bad contacts, optimization of bond lengths, creation of disulfide bonds and capping of protein terminals.

A data set consisting of 169,109 natural compounds was obtained from ZINC [15]. These molecules were prepared using LigPrep's ligand preparation protocol which generated different tautomeric, stereochemical and ionization variants of the small molecules along with energy minimization and flexible filtering. The prepared small molecule dataset was then used for carrying out high throughput virtual screening (HTVS) and molecular docking studies.

2.3. Binding site identification

Q-siteFinder [16] was used to validate the binding pocket of LepB. It calculated the van der Waals interactions of a methyl probe with the protein. The probe sites with favorable energy were then clustered together based on their spatial proximity which was followed by ranking of these clusters based on their total interaction energies.

2.4. High throughput virtual screening and docking studies

A grid was generated in the active site of the prepared LepB structure using Schrödinger's Glide module [17,18]. The prepared data-set of natural compounds was then virtually screened against the prepared protein at the desired grid coordinates using a Glide model HTVS platform [18]. The compounds above the threshold of −7.00 HTVS docking score were then selected and subjected to more refined docking using a Glide XP platform. The top-scoring compounds above a cutoff of −8.50 XP docking score were selected for further analysis using MD simulations.

2.5. Molecular dynamics simulations of ligand-bound LepB complexes

All of the simulations were carried out using the GROMACS package [19]. An Amber FF99SB-ILDN [20] force field was used for the protein. For the ligands, the generalized Amber force field (gaff), using the antechamber program [21] implemented in the AmberTool package [22], was used. The Amber topology files were then converted into GROMACS topology files using the antechamber python parser interface script. The protein–ligand complexes were solvated in a cubic box of TIP3P [23] water molecules and appropriate counter-ions were added to ensure electrical neutrality. The solvated system was minimized for about 10,000 steps using steepest descent and conjugate gradient methods until the force on each atom was below100 kJ/mol/nm. These geometry-minimized systems were then used for carrying out isobaric (constant pressure–temperature NPT) molecular dynamics simulations for up to 26 ns. The system was kept at 300 K temperature and 1 atmospheric pressure coupled with the procedure given by Parrinello–Rahman [24] with a time constant of 5 ps. A 2-fs time step was used for integrating the equations of motion. Periodic boundary conditions were applied throughout, along with the particle mesh Ewald summation method [25] used for calculating electrostatic potential between partial charges on atoms.

3. Results and discussion

3.1. 3D structure of LepB

Supplementary Fig. 1A and B depicts the quality of the modeled structure using Ramachandran and ERRAT plots. To improve the quality of the modeled protein, it was stabilized using MD simulations. An RMSD of 2.974 Å was observed upon alignment of pre- and post-MD simulated structures of LepB (Supplementary Fig. 2A). After the modeled protein was stabilized post MD simulations, it was found that 73.1% of the residues were present in the most favored region while only 1.7% remained in the disallowed regions. None of the residues present in the disallowed regions were part of the active site, nor were they involved in any type of interaction with the functionally critical residues of LepB (Supplementary Fig. 2B). The overall quality factor of the ERRAT plot improved to 73.39 after stabilization of the modeled structure using MD simulations (Supplementary Fig. 2C).

3.2. Binding site identification

It was previously shown by Ollinger et al. [26] that site-directed mutagenesis of LepB carried out on the residues SER 94, SER 96 and LYS 174 made the protein nonfunctional, indicating that these residues are essential for LepB's function. Based on the studies in *E. coli*, they reported that the catalytic center is formed by SER 96 and LYS 174 while SER 94 stabilizes the interaction within the preprotein and the catalytic serine residue. In another study by Paetzel et al. [27] with structural alignments of LepB with SPase I

of other bacterial species, it was found that LepB contained certain conserved regions (Box B, Box C, Box D and Box E) within a short intracellular and a large extracellular domain. These conserved domains play an important role in protein interactions, enzyme activity and other important cellular processes. UniProt confirms SER 96 and LYS 174 as important active site residues in the extracellular topological domain of LepB. Q-SiteFinder reported a potential binding pocket which involved these two critical residues. The volume of this site was reported as 158 Cubic Å and formed by SER 74, LYS 174 and 16 other residues: TYR 90, ILE 92, PRO 93, ILE 130, VAL 131, PHE 132, ARG 133, GLY 134, ASN 170, ASP 171, LEU 172, VAL 173, ALA 244, HIS 249, CYS 250, ILE 269. Most of these residues belong to the conserved domain boxes, thus validating the appropriateness of the selected binding pocket.

3.3. High throughput virtual screening and docking studies

The prepared library of natural compounds was screened against the stabilized structure of LepB using a Glide HTVS platform. Several hundred compounds with a docking score of less than −6.00 were further analyzed for their binding affinity with the more precise and refined Glide XP docking platform. The two top-scoring compounds (ZINC08234345 and ZINC70454376) with an XP score below −8.5 were studied for their detailed interaction with LepB. Molecule 1 (EMP) [6-[2-(3,4-dihydroxyphenyl)ethoxy]-4,5-dihydroxy-2-[(3,4,5-trihydroxy-6-methyl-tetrahydropyran-2-yl)] (Fig. 1A) displayed a docking score of −8.68 while Molecule 2 (PHM), [2-(3, 4-dihydroxyphenyl)-5,8-dihydroxy-7-[(2S,3R,4R)-4-hydroxy-4-(hydroxymethyl)-3-[(2R,3R,4S,5R,6R)-] (Fig. 1B) possessed a docking score of −8.59.

The ligand efficiency score, a measure of the binding efficiency of the ligand with the protein, was found to be −0.2045 for EMP and −0.1973 for PHM. The Glide Emodel score, a combined function derived from Gscore, coulombic, van der Waals and the strain energy of the ligand, has been reported to be a good correlating score between theoretical and experimental binding energies [28]. The low Emodel score of −75.28 and −81.47 for EMP and PHM, respectively, ensured a good binding affinity of these compounds with LepB.

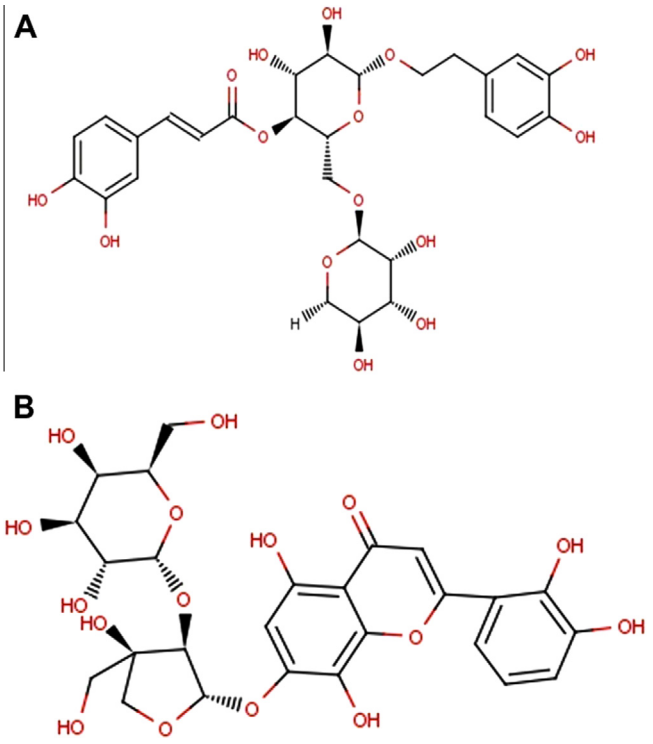

Fig. 1. Structures of screened LepB inhibitors (A) Molecule 1, EMP. (B) Molecule 2, PHM.

3.4. Interaction analysis of the docked compounds

The majority of bacterial proteins with potential roles in growth, invasion and pathogenesis are synthesized as preproteins with an amino terminal extension known as a signal peptide [29]. These signal peptides help in translocating the preprotein across the membranes via secretion pathways. After translocation, SPaseI cleaves the signal peptide from the preprotein and releases the mature protein from the membrane [30]. It has been reported that inhibition of bacterial SPaseI leads to the accumulation of preproteins resulting eventually in the death of the pathogen [31,32]. The presence of this enzyme's active site on the extracellular surface of the cytoplasmic membrane, which involves a unique Ser/Lys catalytic dyad, offers a possibility for the identification and development of highly specific inhibitors. In view of these facts, targeting LepB, the sole type I signal peptidase of *M. tuberculosis*, is a worthy proposition for the development of novel therapeutics to restrict tuberculosis.

3.4.1. EMP: a potential natural inhibitor of conserved domain boxes B and D

In LepB, the catalytic dyad is formed by SER 94 and LYS 174. The serine participates in the signal peptidase hydrolysis reaction by acting as an acylating nucleophile, whereas lysine acts as a general base in both the acylation and deacylation steps of the catalysis. The catalytic dyad is surrounded by structural motifs which form the binding pocket. This binding pocket consists of motifs known as boxes which have remained conserved throughout evolution, from bacteria to humans [6].

As illustrated in Fig. 2A, EMP tightly binds to LepB, forming seven hydrogen bond interactions with the enzyme's crucial residues. These residues include the catalytically important residue, SER 94, and two of the binding pocket residues, ASN 170 and ASP 171. SER 94 helps in stabilizing the substrate intermediate complex during the catalysis mechanism [33]. EMP was found to be forming hydrophobic interactions with the residues TYR 90, ILE 92, PRO 93, LEU 172, ALA 244, GLU 95, TYR 216 and SER 243 of LepB (Fig. 2B). Most of these residues fall into one of the conserved boxes B, D and E. Since these conserved boxes lie near the catalytic domain, these are shown to contribute to the LepB's conserved structure and function [6].

3.4.2. Molecular dynamics simulations of EMP complexed with LepB

To study the dynamical behavior of the interactions of EMP with LepB, MD simulations were performed for a period of 25 ns. The root mean square deviation (RMSD) trajectory for the enzyme–ligand complex is shown in Fig. 4A. Low RMSD values along the trajectory signified that the simulated structure did not deviate much from its initial structure. The steady RMSD trajectory obtained for the protein–ligand complex suggested the stabilization of the complex. A small deviation was observed in the beginning but this stabilized during the remaining simulations run after 16 ns. An RMSD of 2.82 Å was observed when the pre- and post-MD simulated EMP-bound LepB complexes were aligned (Fig. 4B).

An average structure computed using the most stable time frame (20–25 ns) was used for analyzing the interactions between the ligand and the enzyme (Fig. 2C). It was observed that the ligand was bound to LepB, forming two hydrogen bond interactions with ASP 171. In addition to this, EMP formed hydrophobic contacts with the residues ILE 92, PRO 93, GLU 95, LEU 172, TYR 216 and PRO 251 of LepB, most of which are part of the conserved boxes

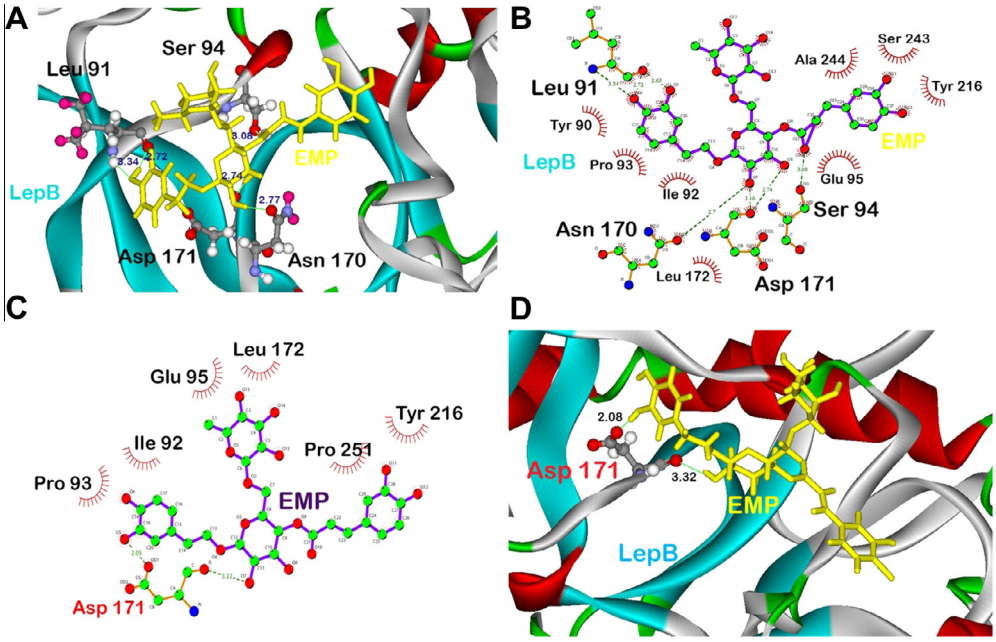

Fig. 2. Interactions of LepB–EMP. (A) Pre-MD complex of LepB–EMP showing various hydrogen bond interactions. (B) Ligplot of Pre-MD LepB–EMP complex showing residues involved in hydrogen bond and hydrophobic interactions. (C) Ligplot of post-MD LepB–EMP complex showing residues involved in hydrogen bond and hydrophobic interactions. (D) Post-MD complex of LepB–EMP showing various hydrogen bond interactions.

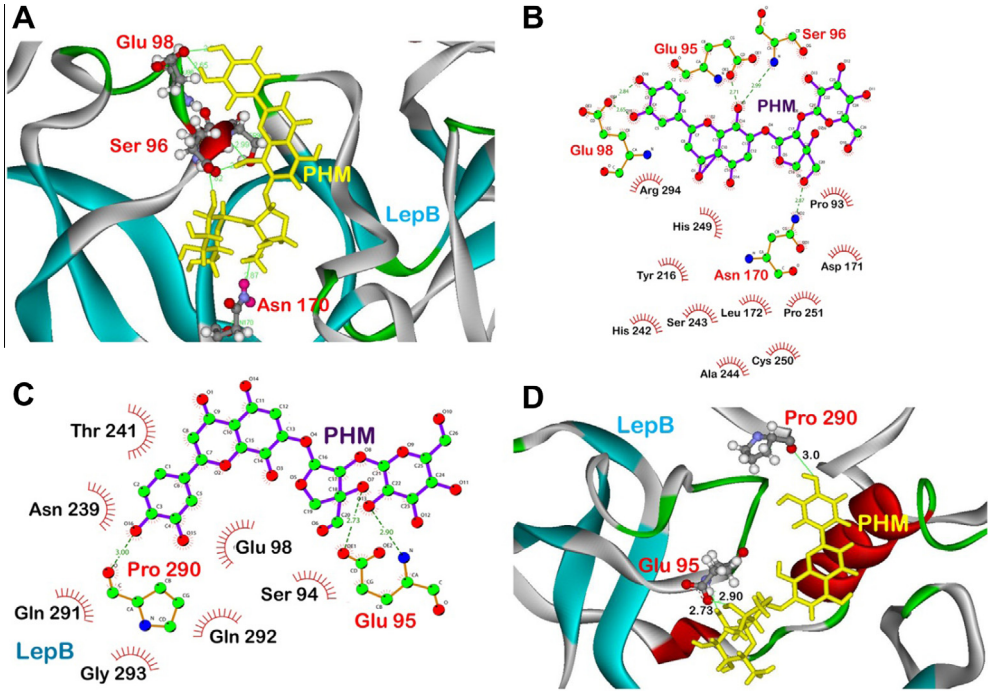

Fig. 3. Interactions of LepB-PHM. (A) Pre-MD complex of LepB–PHM showing various hydrogen bond interactions. (B) Ligplot of pre-MD LepB–PHM complex showing residues involved in hydrogen bond and hydrophobic interactions. (C) Ligplot of post-MD LepB–PHM complex showing residues involved in hydrogen bond and hydrophobic interactions. (D) Post-MD complex of LepB–PHM showing various hydrogen bond interactions.

B and D. Box B contains a structural motif extending from SER 94 to LEU 101. The residues SER 96, MET 97 and PHE 99 are the most highly conserved residues in the group of gram positive bacteria. The conserved serine residue acts as a nucleophile which is activated by the lysine from Box D during the catalytic mechanism of the enzyme. The residues ASP 171 to GLN 182 of the structural motif form the conserved Box D and LYS 174, the general base, followed by the highly conserved arginine residue, plays an important structural role [6]. Fig. 2D shows that EMP tightly binds to LepB in the binding pocket and interacts with the residues belonging to the conserved boxes. This binding of EMP to LepB will hinder the necessary interactions of the catalytically important residues with the signal peptides of preproteins, thus inhibiting the natural cleavage process. Based on these interactions, it can be strongly suggested that EMP is capable of acting as a potential natural candidate for LepB inhibition.

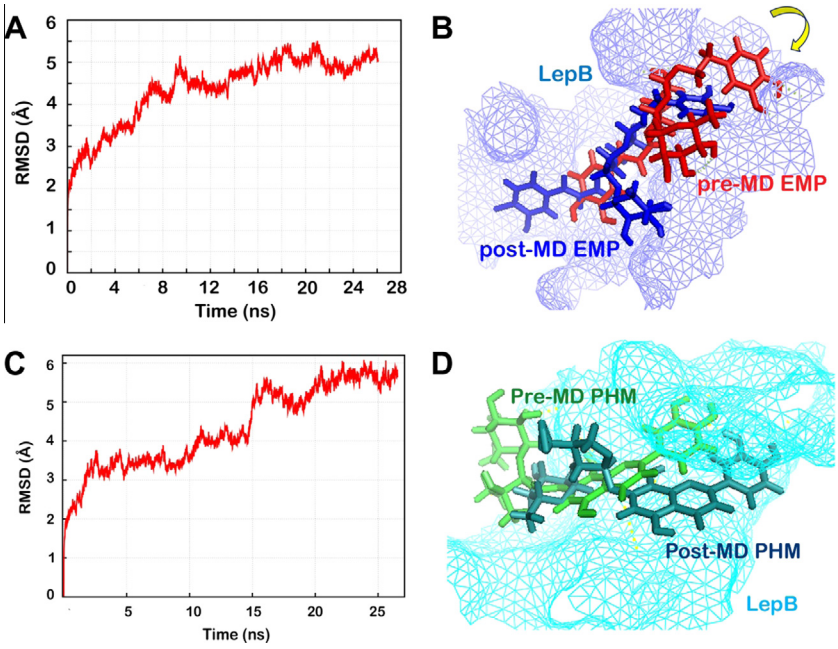

Fig. 4. Analysis of dynamical interactions. (A) RMSD trajectories for LepB–EMP complex. (B) Superimposition of pre-MD (red) and post-MD (blue) complex of LepB–EMP. (C) RMSD trajectory for LepB–PHM complex. (D) Superimposition of pre-MD (green) and post-MD (cyan) LepB–PHM complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4.3. PHM: a potential natural inhibitor of conserved domain boxes B and E

In contrast with EMP, PHM was found binding to LepB in the active site via hydrogen bonding with SER 96 instead of SER 94 as shown in Fig. 3A. Pertaining to the fact that SER 96 acts as an acylating nucleophile in the hydrolysis of the peptide, inhibition of SER 96 would hamper the cleavage of the signal peptide from the preprotein. As shown in Fig. 3B, the Ligplot of LepB, complexed with PHM, shows five strong hydrogen bond interactions among them. The LepB residues involved with H-bond interactions with PHM are: the functional site residue SER 96, residues from the conserved boxes (GLU 95 and GLU 98) and ASN 170 which surrounds the binding pocket. The binding was further coordinated by substantial hydrophobic interactions with the surrounding residues PRO 93, ASP 171, LEU 172, TYR 216, HIS 242, SER 243, ALA 244, HIS 249, CYS 250, PRO 251 AND ARG 294.

3.4.4. Molecular dynamics simulations of PHM complexed with LepB

In order to analyze the time-dependent behavior of the LepB–PHM complex in the presence of virtual *in vivo* conditions, the complex was subjected to MD simulations for 25 ns. The RMSD trajectory for the ligand-bound enzyme complex is shown in Fig. 4C. The backbone of the complex stabilized during the later part of the simulations and remained steady until the end. To identify the changes in the conformations before and after MD simulations, both complexes were superimposed as shown in Fig. 4D and an RMSD value of 3.782 Å was observed between the two structures. An average structure computed from the most stable time frame (20–25 ns), was further analyzed for its interaction pattern and the interactions are shown in Fig. 3D. It was observed that PHM was involved in hydrogen bond interactions with GLU in both the pre- as well as post-MD simulated structures. In the stabilized structure post simulations, the PHM binding involved three hydrogen bond interactions with the residues GLU 95 and PRO 290 of LepB, in addition to hydrophobic interactions with residues SER 94, GLU 98, ASN 239, THR 241, GLN 291, GLN 292 and GLY 293 of LepB. These residues are part of the functionally critical boxes B and E (Fig. 3C). Along with the catalytic SER 96, Box B also consists of residue SER which helps in stabilizing the intermediate complexes formed during the enzyme's catalytic process. This hydrogen bond interaction between GLU 95 and PHM will restrict the flexible interaction of the signal peptide of the preprotein with the nucleophilic center. Thus it will hinder the hydrolysis reaction and prevent it from taking place. Furthermore, the ligand's interaction with SER 94 will weaken the residue's involvement in the process of stabilizing the intermediates formed. Ranging from GLY 237 to ARG 247, Box E contains highly conserved residues. The first serine from the box helps the catalytic lysine to position itself towards the nucleophilic serine. Thus, the interaction of PHM with the residues from Box E will reduce the efficiency of the hydrolysis reaction [6]. This analysis suggests that the binding of PHM to LepB would result in substantially altering the functional properties of the enzyme which would result in the inhibition of LepB.

Many studies have been conducted on many different bacterial species, including *MTb*, reporting loss in viability from the site-directed mutagenesis of LepB. We report here two natural compounds (ZINC08234345 – EMP and ZINC70454376 – PHM) as potential anti-tuberculosis agents targeting LepB signal peptidase, the sole Type I signal peptidase of *MTb*, which also possesses the ability to overcome TB resistance. Both of these molecules were found to bind substantially in the binding pocket of LepB made up of the highly essential conserved domains. The present study provides substantial evidence for considering these screened natural compounds as prospective drugs for the treatment of tuberculosis. Therefore, this will add to the ensemble of drugs currently available – with the non-covalent type inhibitory drugs having improved binding properties and low toxicity to human cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.013.

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