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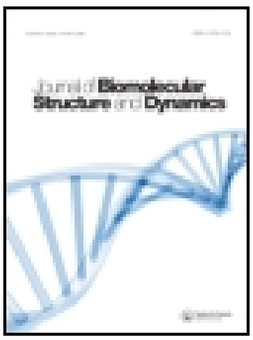

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Identification of new benzamide inhibitor against α-subunit of tryptophan synthase from *Mycobacterium tuberculosis* through structure-based virtual screening, anti-tuberculosis activity and molecular dynamics simulations

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Multi-drug-resistant tuberculosis and extensively drug-resistant tuberculosis has emerged as global health threat, causing millions of deaths worldwide. Identification of new drug candidates for tuberculosis (TB) by targeting novel and less explored protein targets will be invaluable for antituberculosis drug discovery. We performed structure-based virtual screening of eMolecules database against a homology model of relatively unexplored protein target: the α-subunit of tryptophan synthase (α-TRPS) from *Mycobacterium tuberculosis* essential for bacterial survival. Based on physiochemical properties analysis and molecular docking, the seven candidate compounds were selected and evaluated through whole cell-based activity against the H37Rv strain of *M. tuberculosis*. A new Benzamide inhibitor against α-subunit of tryptophan synthase (α-TRPS) from *M. tuberculosis* has been identified causing 100% growth inhibition at 25 μg/ml and visible bactericidal activity at 6 μg/ml. This benzamide inhibitor displayed a good predicted binding score (−48.24 kcal/-mol) with the α-TRPS binding pocket and has logP value (2.95) comparable to Rifampicin. Further refinement of docking results and evaluation of inhibitor-protein complex stability were investigated through Molecular dynamic (MD) simulations studies. Following MD simulations, Root mean square deviation, Root mean square fluctuation and secondary structure analysis confirmed that protein did not unfold and ligand stayed inside the active pocket of protein during the explored time scale. This identified benzamide inhibitor against the α-subunit of TRPS from *M. tuberculosis* could be considered as candidate for drug discovery against TB and will be further evaluated for enzyme-based inhibition in future studies.

Keywords: α-subunit; tryptophan synthase; benzamide; MD simulations; RMSD; RMSF

Abbreviations: α-TRPS – alpha subunit of tryptophan synthase; DMSO – Dimethyl sulfoxide; INH – Isoniazid; logP – Partition coefficient; MD – Molecular dynamics; MIC – Minimum inhibitory concentration; PLP – Pyridoxal phosphate; RMSD – Root-mean-square deviation; RMSF – Root-mean-square fluctuation; TB – Tuberculosis; MDR-TB – Multi-drug-resistant tuberculosis; XDR-TB – Extensively drug-resistant tuberculosis

Introduction

Tuberculosis (TB) ranks second among deadliest contagious diseases causing millions of deaths around the globe. *Mycobacterium tuberculosis* affects major part of world population leading to death of over 1.7 million people worldwide with an estimated more than 9 million cases per year (Avila, Saïd, & Ojcius, 2008; Dye, 2009; Geneva & Organization, 2007; Organization, 2016). Although TB is curable disease and billions of dollars have been spent on TB control programs still it is major cause of mortality and no new anti-tuberculosis drug has been reported in recent decades. Multi-drug resistance developed by micro-organisms poses serious threat to mankind and led to an alarming situation for discovery of new antibiotics. Micro-organisms can develop resistance to drugs through various mechanisms like efflux systems that regulate inner environment of cell by removing toxic substances (Dwivedi et al., 2017; Verma, Maurya, Tiwari, & Tiwari, 2017; Verma, Tiwari, & Tiwari, 2017). These efflux pumps reduces the effect of antibiotics by effecting their transport across cell membrane through various multi-drug-resistant (MDR) transporters (Kesherwani, Michael Gromiha, Fukui, & Velmurugan, 2017). Multi-drug-resistant transporters comprises five families including ATP-binding cassette, major facilitator, resistance-nodulation-cell division, Multidrug and Toxic compound extrusion and small multi-drug resistance family (SMR) family (Bera et al., 2017; Verma, Maurya, et al., 2017). Different in silico studies have been reported previously on these MDR transporter families to identify novel lead compounds for

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antibiotic discovery (Kesherwani et al., 2017). Due to advent of Multi-drug-resistant tuberculosis (MDR-TB), extensively drug-resistant tuberculosis (XDR-TB) and side effects of current drugs there is an urgent need to discover new, safer and compatible drugs having novel mode of action against TB (Crick & Brennan, 2000). Moxifloxacin a well-known antibiotic belonging to fluoroquinolone class has been suggested for cure of MDR-TB, XRD-TB (Pandey et al., 2018). Various lead compounds have been identified as drug candidates against TB but no one emerged for clinical use in recent era (Khedr et al., 2017). Inhibition of unique, unexplored targets that are essential for bacterial survival-like tryptophan biosynthesis found in micro-organisms and plants but not in animals may lead to identification of new candidates with novel mode of action (Zhang et al., 2013).

The tryptophan biosynthetic pathway is genetically validated target for novel antibiotics discovery (Eckert, Kübler, Hoffmann, & Braus, 2000; Wellington et al., 2017). The tryptophan synthase (TRPS) is a bi-enzyme complex that catalyzes conversion of Indole-3-glycerol phosphate (IGP) to tryptophan involving both alpha and beta subunits of TRPS. Although both α and β subunit retain their functions as monomers but activity of $\alpha\beta\beta\alpha$ complex is much higher (Barends et al., 2008; Dunn, 2012; Dunn et al., 1990; Dunn, Niks, Ngo, Barends, & Schlichting, 2008). The α-subunit catalyzes conversion of IGP to indole and glyceraldehyde-3-phosphate while β-subunit is involved in condensation of serine and indole to form tryptophan in PLP-dependent reaction. Both α and β subunits are linked through 25 Å hydrophobic tunnel (Hyde, Ahmed, Padlan, Miles, & Davies, 1988; Miles, 1995; Pan, Woehl, & Dunn, 1997). The substrate channeling in TRPS complex is allosterically regulated by ligand-binding interactions at α-subunit (Weber-Ban et al., 2001). Already reported ligand of α-subunit namely F6, F9, F12, F19 allosterically effect β-subunit reaction and substrate channeling in TRPS complex (Ngo et al., 2007). Identification of prospective inhibitors against α-subunit of TRPS would be an effective approach for lead compounds discovery.

Combined computational and experimental approaches have been considered as more reliable tool for drug discovery as compared to traditional methods (Danishuddin, Mobeen, & Khan, 2017). Structure-based virtual screening utilizes three-dimensional structure of protein target for screening of library through docking and has been proved as successful tool for identification and optimization of new drugs candidates against various protein drug targets (Hou et al., 2012; Moitessier, Englebienne, Lee, Lawandi, & Corbeil, 2008).

This present study attempts the identification of new inhibitors against α-subunit of TRPS from *M. tuberculosis* through structure-based virtual screening approach. We have applied filters over eMolecules database according to Lipinski's rule of five (Lipinski, 2004) and resulting database was subjected to docking to get chemical scaffolds against binding pocket of homology model generated for TRPS α-subunit of *M. tuberculosis*. The top hits were then subjected to experimental validation through whole cell assay for growth inhibition of *M. tuberculosis* that resulted in identification of new benzamide inhibitor, namely 4-(((5-(3-chloro-phenyl)-isoxazol-3-yl)-formylamino)-methyl)-benzamide against H37Rv of *M. tuberculosis*. To investigate the binding mode stability of this benzamide inhibitor (**C2**) inside pocket, its complex with α-TRPS model was subjected to MD simulations studies and analysis was done in terms of Root-mean-square deviation (RMSD), Root-mean-square fluctuation (RMSF) and secondary structure analysis.

Materials and methods

Model generation of TRPS alpha subunit through homology modeling

The homology model was generated for α-subunit of TRPS from *M. tuberculosis*. The amino acid sequence was retrieved from Universal Protein resource (UniProt) (Consortium, 2016) having UniProt ID: P9WFY1. The crystal structure of α-subunit of TRPS from *Salmonella enterica* (PDB: 1TJP) (Kulik et al., 2005) as a template (28% sequence Identity) and model was generated using the homology modeling tool of ICM v3.8-4a or above (Molsoft L.L.C., La Jolla, CA). The binding pocket was identified using ICM pocket finder tool (An, Totrov, & Abagyan, 2005) for binding pocket predictions of model.

Preparation of chemical database for screening

The virtual screening of eMolecules database comprising of over six million compounds against binding pocket of TRPS (α-subunit) was done by applying filters over it. Filters were applied according to Lipinski rule of five-like intrinsic water solubility (logS <4) parameter was less than 4 and partition coefficient (logP <5) was less than 5. Similarly volume available for compound's interaction with active site was in the range of 250–500 and Toxicity score for molecule was below 1. The size of compound was retained below 60 number of atoms.

Docking of top Hit compounds into binding pocket

The docking of chemical database retrieved after filters was performed through ICM DockScan by keeping the number of efforts 3 and generated 5 conformations for each molecule in database having good binding score and stability in binding pocket. This screening resulted in identification of over 1500 compounds having good binding score i.e. below −40 kcal/mol. Top 100 hits were

selected for re-docking into binding pocket in more precise way by increasing number of efforts to 10 and by selection of Grid box to specify area for interaction of compounds in active region.

These compounds were further analyzed by calculating Molecular polar surface area (MolPSA), number of hydrogen bond acceptor (HBA) and Hydrogen bond donor (HBD), molHERG, and drug likeliness. The top seven hits were purchased and subjected to further experimental evaluation.

Whole cell assay for anti-tuberculosis activity

The bactericidal potential of seven candidate compounds was determined by agar dilution method (Kumar, Chaturvedi, Bhatnagar, Sinha, & Siddiqi, 2008; Singh et al., 2015). 5 mg/mL stock solutions and serial dilutions of test compounds were made in DMSO solvent. Middlebrook 7H10 agar medium was prepared by following standard protocol. Isoniazid (INH) a well-known anti-tuberculosis drug (Timmins & Deretic, 2006) was used as positive control while DMSO as negative control. 0.1 ml from different dilutions of candidate compounds, INH and DMSO were mixed with 1.9 mL of agar medium separately in plates and allowed to solidify. A suspension (10^5 bacilli/plate) of *M. tuberculosis* H37Rv was inoculated onto the plate and incubated for 4 weeks at 37°C. The minimum inhibitory concentration (MIC) was taken as lowest concentration at which no visible growth was observed.

Molecular dynamic simulation

The stability of predicted binding mode of candidate compound having best MIC value against *M. tuberculosis* H37Rv was investigated through molecular dynamic simulations using AMBER 14 suite (Duke & Gohlke, 2014) in explicit solvent. Complex system was solvated in cubic box having TIP3P water molecules (Jorgensen, Chandrasekhar, Madura, Impey, & Klein, 1983) with marginal radius of 12 Å and overall system was neutralized by adding counter ions. Initial minimization was done for 200 ps using steepest descent and conjugate gradient method. The optimized protein complex was subjected to heating to 310 K for 500 ps followed by equilibration for another 400 ps in series of steps removing the restraints gradually. Finally, both proteins were allowed to equilibrate for 5 ns after removing restraints. Production run was carried out for 20 ns followed by the analysis of MD trajectory files. The analysis was done in terms of RMSD, RMSF, ligand protein interaction studies, and secondary structure analysis. The Visual Molecular Dynamics (VMD) software has been used for visualization and interpretation of results (Humphrey, Dalke, & Schulten, 1996). Hydrogen bonds between inhibitor and active site residues were analyzed during the course of MD simulation and the cut-off distance during this analysis was set to be ≤3.7 Å.

Results and discussion

Multi-drug-resistant TB (MDR) and extensively drug resistant (XDR-TB) has emerged as global health threat and significant efforts are needed for discovery of new anti-tuberculosis drugs with novel mode of action. Structure-based drug design has been considered as important tool for rational drug design and optimization of lead compounds. We have identified a new benzamide inhibitor against relatively less explored target i.e. α-subunit of TRPS from *M. tuberculosis* through structure-based virtual screening possessing good bactericidal activity against H37Rv strain of *M. tuberculosis*. The absence of biosynthetic pathway for tryptophan in humans and its essentiality for survival of mycobacteria has made these enzymes a viable target for antibiotic discovery (Evans et al., 2014)

Homology model for α- subunit of TRPS from M. tuberculosis

The model of α-subunit of tryptophan synthase from *M. tuberculosis* was built using homology modeling tool of ICM as depicted in Figure 1(A) and (B). The quality of model was good as analyzed through visual inspection, PROCHECK analysis (Laskowski, MacArthur, Moss, & Thornton, 1993) (overall G-factor 1.7, Figure S1) as well as Ramachandran plot which showed most amino acids in favored region as shown in Figure 1(C).

The three binding pockets for protein model were predicted through ICMPocket Finder and the one selected for virtual screening of compounds database has volume 432.7 and Area 403.7 as depicted in Figure 1(B). This binding pocket possesses active site residues necessary for catalytic activity of TRPS alpha subunit particularly Aspartic acid (D68) and Glutamic acid (E57) (Ngo et al., 2007). Further validation of binding pocket was done by comparison with active pocket of reported co-crystal structure of α-subunit of Tryptophan synthase (PDB: 1TJP). The superimposed binding pockets of model and crystal structure has been added in supplementary information (Figure S2)

Virtual screening and molecular docking of chemical database

The screening of chemical database resulted in identification of few hits having good binding energies below −40 kcal/mol and through visual analysis of docking poses of compounds into binding pocket. Finally, top seven compounds (Figure 2) were selected for

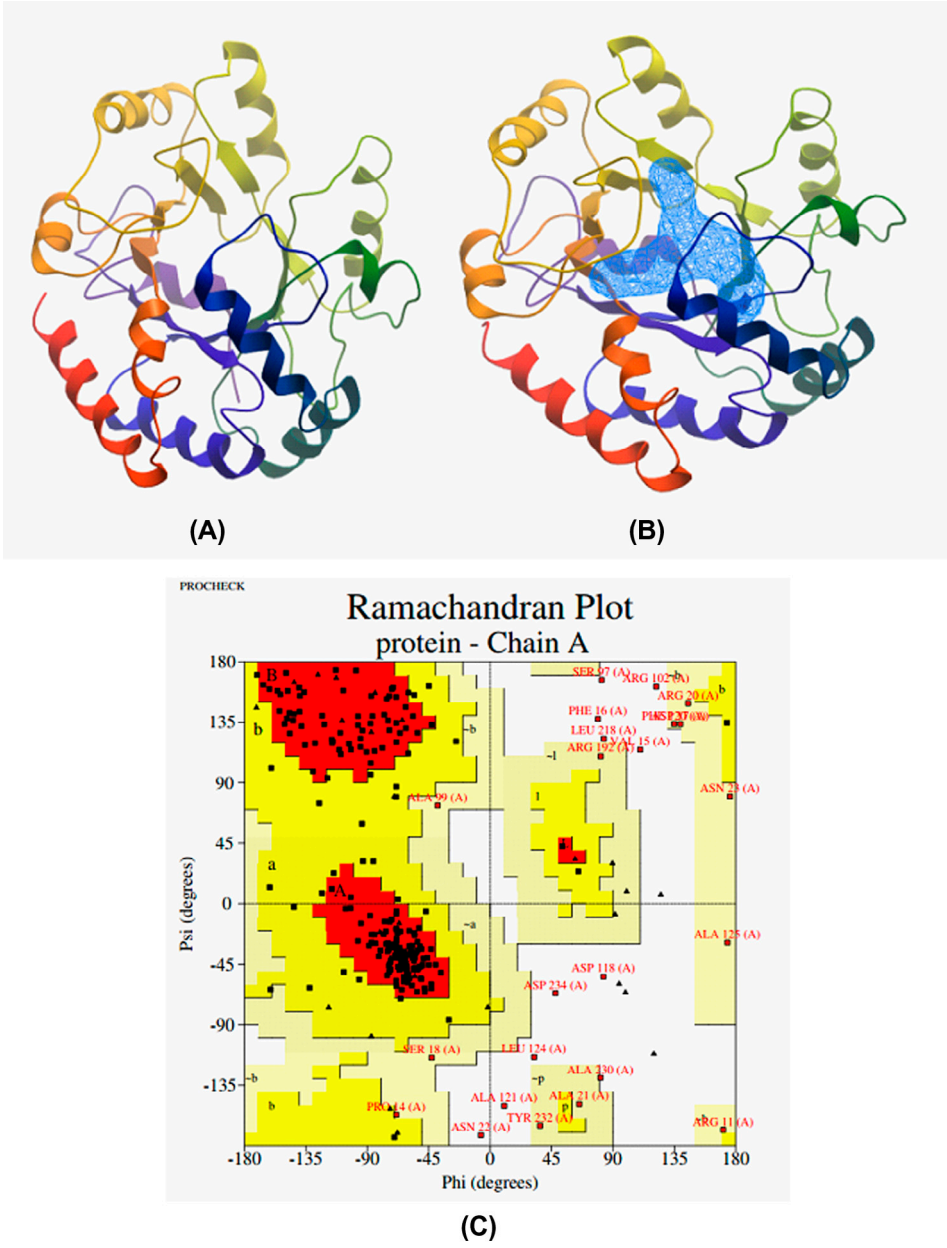

Figure 1. **(A).** 3D structure of TRPS α-subunit (*M. tuberculosis*). (**B).** 3D structure of TRPS α-subunit with pocket. (**C).** Ramachandran plot for homology model generated for TRPS α-subunit.

experimental validation on the basis of good binding energies, their potential to make hydrogen bond with binding pocket residues, active site area coverage and physiochemical properties etc.

The top hit compound **C1** having bromine substituted furan ring showed best binding interactions with active site residues particularly Aspartic acid (D68) one of key amino acid previously reported essential for catalytic activity of *α*-subunit of TRPS as depicted in Figure 3. The toxicity score, partition coefficient, as well as polar surface area values of compound **C**1 are in agreement with Lipinski rule of five (Table 1).

Compound **C2** a benzamide derivative was suggested to be most potent candidate having same logP value 2.95 as Rifampin a well-known anti-tuberculosis drug (Table 1) and falls within the range 2.5–4.0 attributed to most of anti-tuberculosis drugs (Barry, Slayden, Sampson, & Lee, 2000). Physical characteristics of mycobacteria are unique and there is direct relation of hydrophobicity of drug and activity against mycobacteria

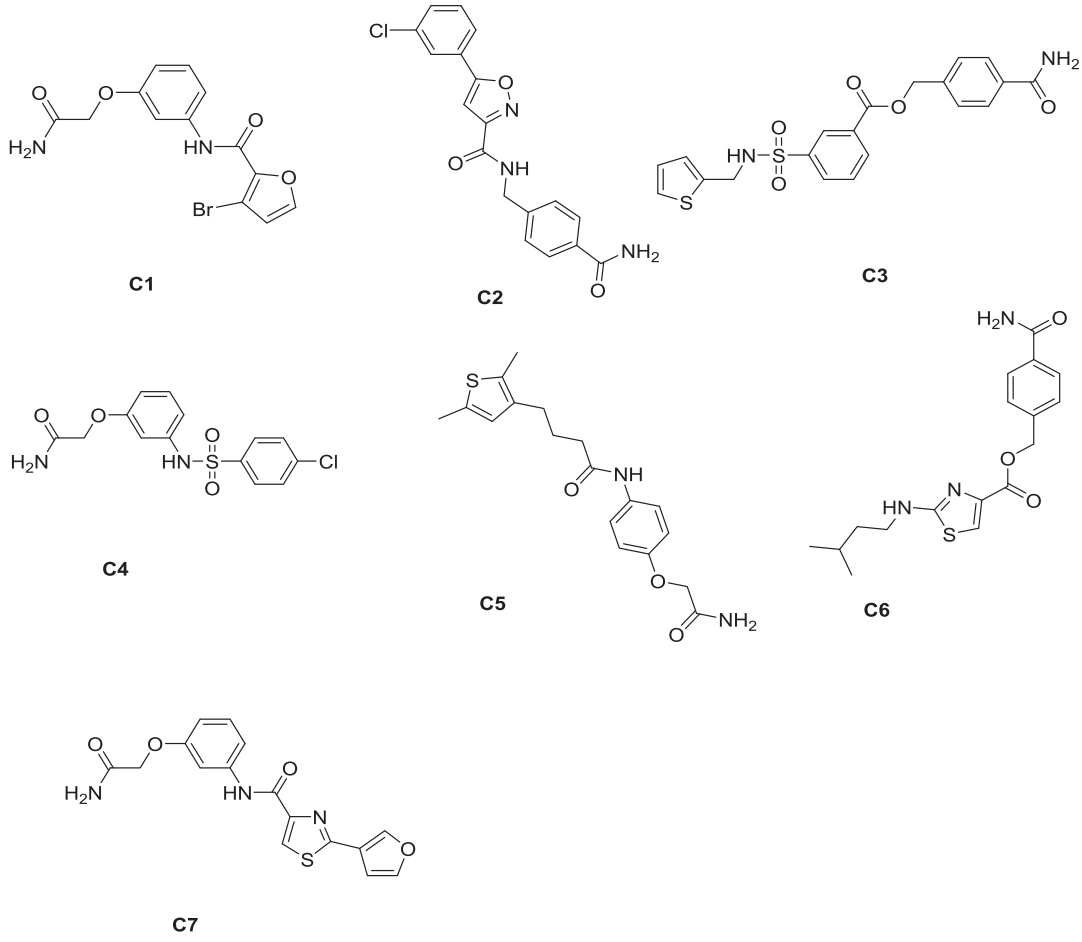

Figure 2. Chemical structures of compounds (**C1–C7**).

(Waisser, Kuneš, Klimeš, Polášek, & Odlerová, 1993). Persistent hydrogen bonds with the time line of >85% were observed between the inhibitor (**C2**) and protein. Val 220 formed a hydrogen bond with amino group of inhibitor with average bond distance of ~2.7 Å. Similarly another hydrogen bond was spotted between inhibitor and Arg 221 having distance averaged around 3.1 Å. Likewise, Ile 237 formed a hydrogen bridge with inhibitor mapped by an avg. distance of 3.1 Å. This benzamide derivative fit best into binding pocket through H-bonding interactions with active site residues and also showed good bactericidal activity against H37Rv *M. tuberculosis*. Similarly Compound **C3** having thiophene moiety also showed some bactericidal activity against H37Rv *M. tuberculosis* that might be related to its logP value of 2.90 comparable to Rifampicin. The binding interaction of **C3** with catalytic residues of active pocket like Aspartic acid (D68) and Arginine (R221) has been depicted in Figure 3.

In addition, the characteristic properties like partition coefficient, solubility, toxicity score, binding score, and binding interactions of compounds **C4**–**C7** with active site of *α*–subunit TRPS model has also been given in Tables 1 and 2.

Whole cell assay for Anti-tuberculosis activity against H37Rv M. tuberculosis

The selected compounds (**C1–C7**) were further screened for their bactericidal potential against H37Rv strain of *M. tuberculosis* using agar dilution method. The minimum inhibitory concentration (MIC) of compounds has been taken as minimum concentration at which compounds showed visible bactericidal effect after incubation as given in Table 3.

The compound **C2** showed considerable bactericidal effect against H37Rv *M. tuberculosis* at concentration of 6 µg/mL and exhibited 100% growth inhibition of *M. tuberculosis* at 25 µg/mL as depicted in Figure 4.

Similarly compound **C3** also showed some bactericidal activity but at much higher concentration as compared to **C2** i.e. 50 µg/mL as depicted in Table 3. The compound **C2** has shown considerable antimicrobial activity and is suggestive to be an effective anti-tuberculosis drug

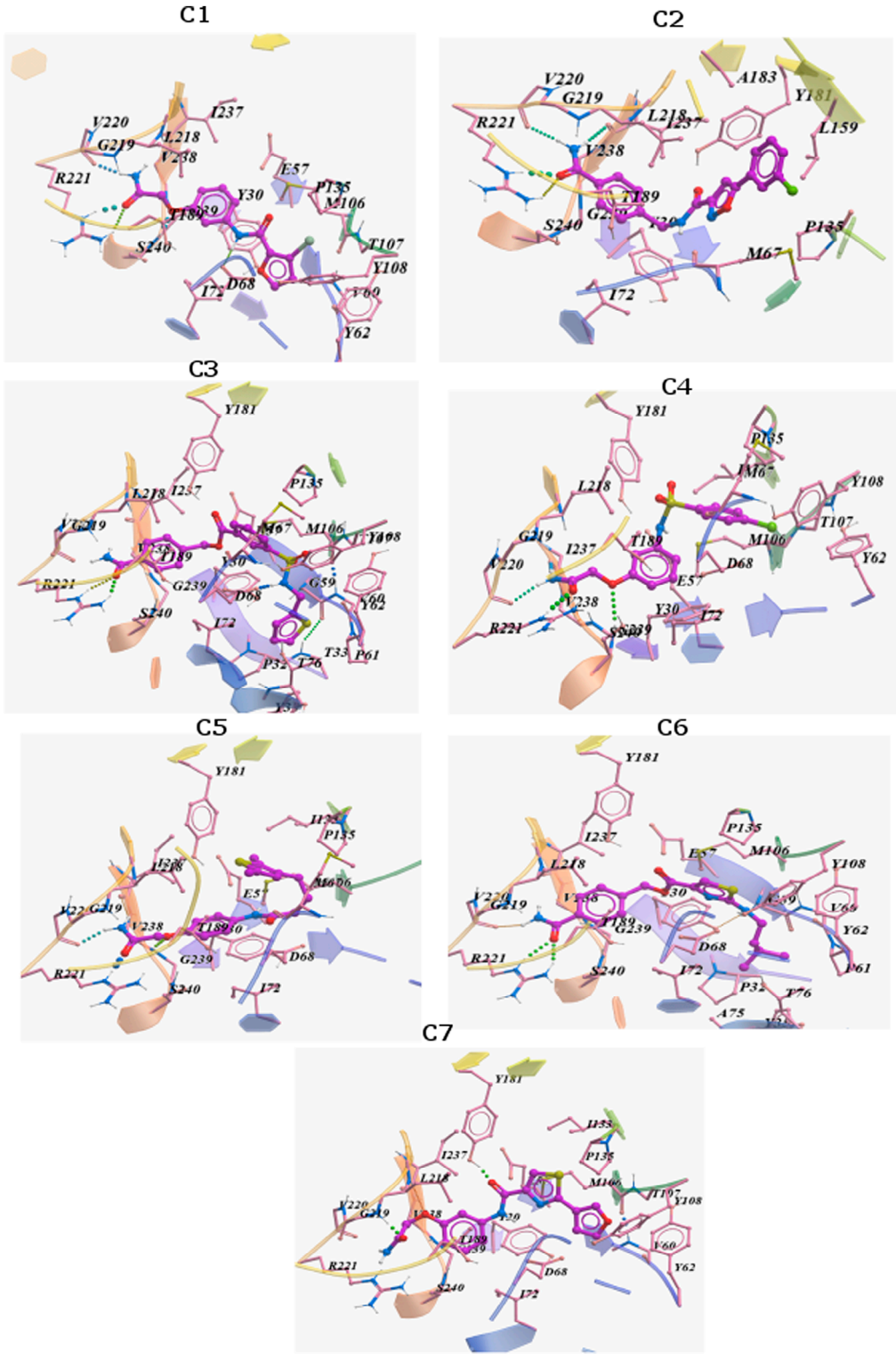

Figure 3. Binding interaction of candidate compounds (**C1–C7**) inside the binding pocket of homology model of α-subunit of TRPS (*M. tuberculosis*).

Table 1. Binding score and physiochemical properties of compounds (**C1–C7**).

Compound	Molecular Formula	Binding score (kcal/mol)	molLogP	molLogS	molPSA	Drug likeliness	Toxicity Score	molHERG
C1	$C_{13}H_{11}BrN_2O_4$	−48.63	1.6	−3.348	73.03	0.4882	0	0.21
C2	$C_{18}H_{14}ClN_3O_3$	−48.24	2.95	−5.039	80.25	0.4597	0	0.26
C3	$C_{20}H_{18}N_2O_5S_2$	−43.04	2.90	−5.226	97.93	0.1755	0	0.29
C4	$C_{14}H_{13}ClN_2O_4S$	−41.83	2.09	−4.724	81.78	0.2861	0	0.25
C5	$C_{18}H_{22}N_2O_3S$	−33.66	2.83	−3.948	65.02	0.6058	0	0.22
C6	$C_{17}H_{21}N_3O_3S$	−29.45	3.66	−5.203	75.55	1.171	0	0.25
C7	$C_{16}H_{13}N_3O_4S$	−20.65	1.84	−5.137	81.75	0.6558	0	0.24

Table 2. Binding interaction of candidate compounds (**C1–C7**) with pocket residues.

Compounds	H-Bonding Residues (Bond Distance Å)	Other interactions
C1	D68 (2.15 Å), V220 (2.06 Å), R221 (2.04, 2.28 Å), S240 (2.04 Å)	I237 (arene-arene interaction)
C2	R221 (3.1 Å), V220(2.7 Å), I237 (3.1 Å)	–
C3	R221 (1.96, 2.14 Å), D68 (1.96 Å),	–
C4	I237 (2.16 Å), V220(1.95 Å), R221 (2.49 Å), S240 (2.46 Å)	Y30 (arene-arene interaction)
C5	G239(1.88 Å), V220(1.96 Å), R221 (1.65 Å)	Y30 (arene-arene interaction)
C6	R221 (2.23, 2.13 Å),	–
C7	Y181 (1.45 Å), L218 (2.27 Å), G219 (1.59 Å)	–

Note: Single letter code used for Amino acids representation.

Table 3. Anti-tuberculosis activity of compounds (**C1–C7**) against H37Rv *M. tuberculosis.*

	Concentration				
Compounds	50 μg/ml	25 μg/ml	12 μg/ml	6 μg/ml	3 μg/ml
C1	–	–	–	–	–
C2	+++	+++	(±)	(±)	–
C3	(±)	–	–	–	–
C4	–	–	–	–	–
C5	–	–	–	–	–
C6	–	–	–	–	–
C7	–	–	–	–	–

Notes: Isoniazid (used as standard) gave 100% growth inhibition at 2 μg/ml.
+++ indicates total growth inhibition.
(±) indicates partial growth inhibition.
– indicates zero growth inhibition.

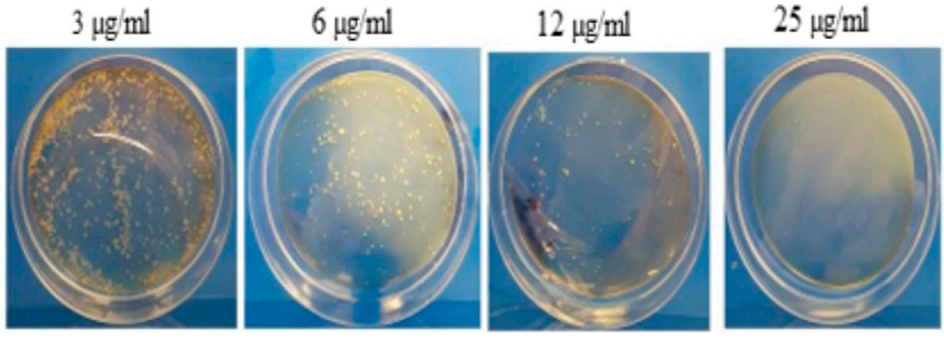

Figure 4. Growth inhibition of *M. tuberculosis* (whole cell assay): Images of plates showing colonies of bacterium at different concentration of Benzamide inhibitor (**C2**).

candidate. The other compounds in the current study did not show any bactericidal activity against H37Rv strain of *M. tuberculosis* as shown in Table 3.

Molecular dynamic simulations

The proposed benzamide inhibitor (**C2**) showed good bactericidal activity against *M. tuberculosis* H37Rv and its complex with protein was subjected to MD simulations studies to investigate its binding mode stability inside the active pocket. The preliminary studies on the MD trajectory involved RMSD and RMSF analysis. The RMSD analysis was done on Cα atoms of protein–ligand complex and was compared against initial minimized structure of protein complex as well as with ligand-free protein. Both bound and free protein showed fluctuations in the range 1.2 and 2.3 Å, respectively, confirming overall stability during the explored time scale, however, the behavior of both bound and free state is quite distinct as evident from RMSD analysis. Presence of ligand induced rigidity into the protein matrix and reduced the amplitude of fluctuation near the active region of protein as depicted in Figure 5(A).

The information about flexibility of Cα atoms of amino acid residues of protein system was obtained through RMSF analysis that showed similar pattern of fluctuations in both proteins (bound and free) in some regions, however, the magnitude of fluctuation varied specifically in the active region marked as loop 6 in Figure 5(B). Protein's region that lies in close vicinity of the active site (shown enclosed in Figure 5(B)) experienced distinct behavior as observed by marked increase in fluctuation in free state proposing the idea that presence of ligand induced rigidity into the protein matrix. RMSF analysis of side chains for both bound and free proteins has been shown in Figure 5(C). MD simulations gave a clear evidence of existence of the ligand inside the active pocket during the explored time scale.

Result of secondary structure analysis coupled with both RMSD and RMSF confirmed that protein in LB form remained stable and did not unfold during the explored time scale.

Despite the fact that TRPS is an allosteric enzyme and ligand binding at α-subunit might cause structural modifications in β-subunit still it has no effect on PLP binding site of β-subunit. Indole produced in α-reaction is transferred to β-subunit through hydrophobic tunnel where it reacts with serine to produce tryptophan and inhibiting α-subunit reaction will ultimately lead to inhibition of tryptophan production necessary for bacterial survival. In this current study, we have focused on inhibitor discovery against α-subunit of TRPS and have predicted a binding pocket for homology model having amino acid residues like Aspartic acid, and glutamic acid reported previously essential for catalytic activity of

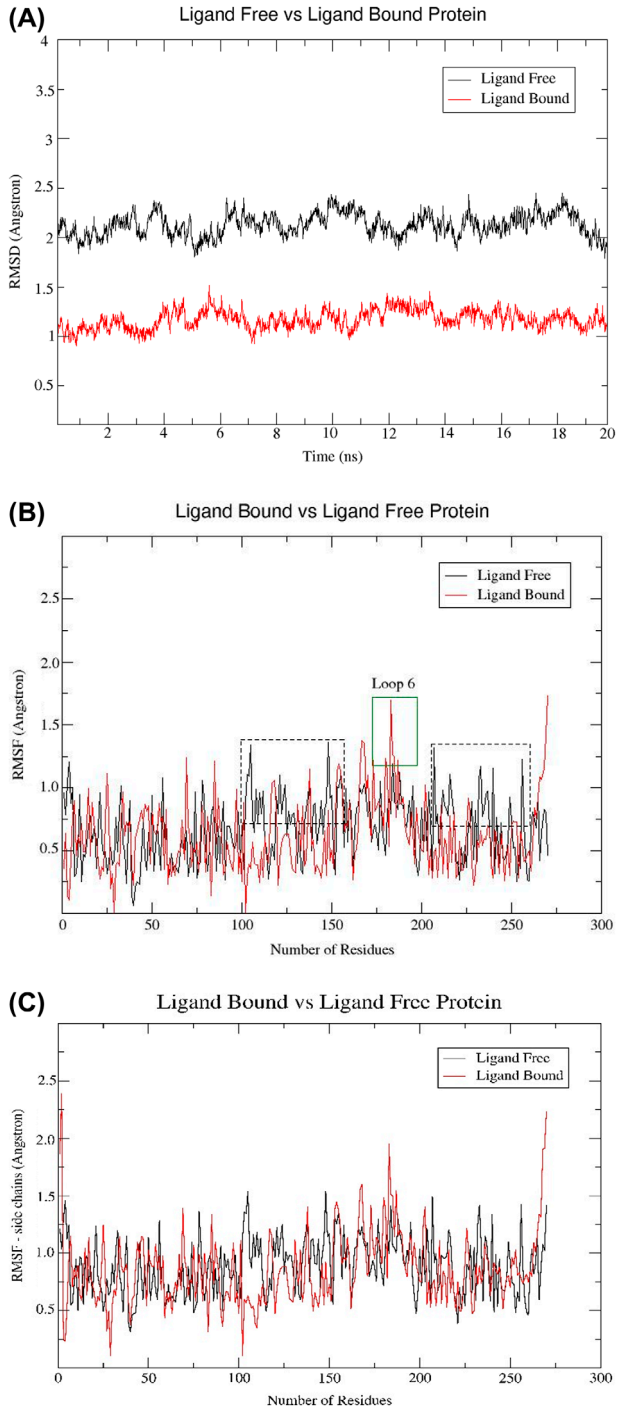

Figure 5. **(A).** RMSD analysis graph of Benzamide-protein model complex. **(B).** RMSF analysis graph showing fluctuation pattern for different regions of Benzamide-protein complex. **(C).** RMSF analysis graph of side chains showing fluctuation pattern for both (ligand bound and free) Protein.

α-subunit. The characteristic properties of proposed benzamide inhibitor like drug likeliness, toxicity score, solubility coefficient, partition coefficient (same as

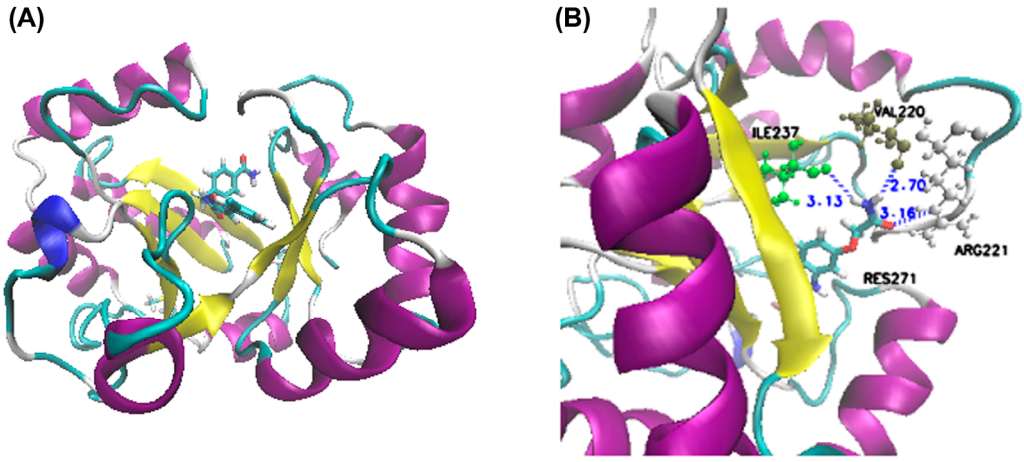

Figure 6. **(A).** Secondary structure representation of Benzamide-protein complex over the explored time scale (MD simulations). **(B).** Residue interaction analysis of Benzamide-protein complex (MD simulations).

Rifampicin), and polar surface area combined with its ability to cause 100% growth inhibition of *M. tuberculosis* H37Rv suggested it to be a good Lead candidate. This bactericidal activity of benzamide inhibitor might be related to its hydrophobicity or logP value found within the range for most anti-tuberculosis drugs (Waisser et al., 1993) that facilitate smooth transfer of compounds through cell wall of slowly growing mycobacteria.

The loop6 and loop2 has been reported important for catalytic activity of *α*-subunit of TRPS that facilitate ligand retention as well as its interaction inside the binding pocket (Khan, Farooq, & Kurnikova, 2016). The RMSF analysis of benzamide inhibitor protein complex showed major fluctuations in loop6 region that help in orientation of active site in order to retain ligand inside binding pocket as depicted in Figure 6(A). Similarly the binding interaction analyzed after docking and MD simulation showed identical binding site suggesting the preference of the inhibitor towards active site as shown in Figure 6(B).

The RMSD, RMSF, and secondary structure analysis further confirmed stability of benzamide inhibitor protein complex over the explored time. Current study focused specifically on *α*-subunit of TRPS for identification of drug candidates against tuberculosis but did not study the effect of proposed inhibitor on *β*-subunit of TRPS. This study can be further extended to enzyme inhibition assay analysis as well as the effect of identified inhibitor on activity of whole protein complex through in silico approaches.

Conclusion

The aim of current study was to identify a new inhibitor against relatively less explored protein target i.e. *α*-subunit of TRPS from *M. tuberculosis* through structure-based virtual screening of compound's database. Seven candidate compounds (**C1–C7**) were selected based on molecular docking and physiochemical properties for antituberculosis activity that resulted in identification of new Benzamide inhibitor. This proposed benzamide inhibitor (**C2**) showed 100% growth inhibition of H37Rv strain of *M. tuberculosis* at 25 µg/ml and considerable bactericidal effect up to 6 µg/ml in whole cell-based activity. The binding stability of benzamide inhibitor inside the binding pocket was further investigated through MD simulation studies involving RMSD, RMSF, and secondary structure analysis that showed no major structural fluctuations in protein structure during the explored time scale. The current study can be further extended for enzyme based assay evaluation of identified benzamide inhibitor and could be considered as candidate for drug discovery against tuberculosis.

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Disclosure statement

The authors declared that there is no conflict of interest.

Supplementary material

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