

Novel Structural Insights of Glutamate Racemase from *Mycobacterium tuberculosis* through Modeling and Docking Studies

Jayashree Ramana

Abstract—An alarming emergence of multidrug-resistant strains of the tuberculosis pathogen *Mycobacterium tuberculosis* and continuing high worldwide incidence of tuberculosis has invigorated the search for novel drug targets. The enzyme glutamate racemase (MurI) in bacteria catalyzes the stereoconversion of L-glutamate to D-glutamate which is a component of the peptidoglycan cell wall of the bacterium. The inhibitors targeted against MurI from several bacterial species have been patented and are advocated as promising antibacterial agents. However there are none available against MurI from *Mycobacterium tuberculosis*, due to the lack of its three-dimensional structure. This work accomplished two major objectives. First, the tertiary structure of MtMurI was deduced computationally through homology modeling using the templates from bacterial homologues. It is speculated that like in other Gram-positive bacteria, MtMurI exists as a dimer and many of the protein interactions at the dimer interface are also conserved. Second, potent candidate inhibitors against MtMurI were identified through docking against already known inhibitors in other organisms.

Keywords—Glutamate racemase, homology modeling, docking, drug resistance.

I. INTRODUCTION

TUBERCULOSIS is a major health problem worldwide with high rates of mortality and morbidity [1]. This is largely attributed to the escalating resistance of the causal bacterium *Mycobacterium tuberculosis* (Mtb) to the currently available drugs, precipitating in the emergence of Multi-Drug Resistant (MDR) (resistant to isoniazid and rifampicin) and Extensively Drug Resistant (XDR) (resistant to isoniazid and rifampicin as well as fluoroquinolone and second line injectable agents) strains. This has intensified the search for novel drug targets in Mtb for chemotherapeutic intervention with the objective of expanding on the armamentarium against this deadly disease. In this context, I explore the candidature of the enzyme glutamate racemase from Mtb (MtGluR or MtMurI) as a potential drug target.

The bacterial cell wall is composed of peptidoglycan (also called murein) which is a continuous cross-linked structure and provides much of the strength and rigidity of bacterial cell wall. It consists of alternating N-acetyl glucosamine and N-acetyl muramic acid where the latter is linked to a tetrapeptide through its D-lactate moiety [2]. This tetrapeptide is usually L-Ala.D-Glu.L-Lys.D-Ala, in which the L-Lys is linked to D-Ala (directly in Gram-negative and through a pentaglycine

bridge in case of Gram-positive bacteria) of the adjacent peptidoglycan unit. The synthesis of the peptidoglycan polymer involves the action of multiple enzymes of the Mur pathway. In the light of the significance of this pathway for the bacteria, it is not surprising that many antibiotics function by inhibiting different steps of this pathway [3]. However as discussed above, Mtb has developed resistance against these antibiotics, necessitating the need to search novel drug targets. This pathway is still a viable source of drug targets because of its indispensability for the pathogen as well as its absence in the human host.

The bacterial enzyme glutamate racemase (MurI) is a co-factor independent enzyme which catalyzes the interconversion of L-glutamate and D-glutamate, which is required for peptidoglycan synthesis. Glutamate racemase has long been considered an important drug target for its integral role in bacterial cell wall synthesis and its inhibitors have been patented as antibacterial agents in different species including *Helicobacter pylori* [4] and *Streptococcus pneumonia* [5]. However there are no inhibitors available against glutamate racemase from *Mycobacterium tuberculosis* owing to the lack of a resolved structure in Protein Data Bank (PDB). This work is inspired by the seminal experiments of Sengupta et al. in 2006 and 2008 whereby glutamate racemase from Mtb was cloned, expressed and assayed [6], [7]. In this work, several novel structural insights about this enzyme were gained by harnessing the currently available experimental data. The tertiary structure of MtMurI was modeled followed by the dissection of the interactions at the dimer interface as well as between the substrate and the enzyme. Potent competitive inhibitors were identified from the already known inhibitors from other organisms. These findings would address some of the hitherto unresolved issues about the biology of this enzyme and could be translated into clinical medicine to provide an innovative approach to counteract this deadly pathogen.

II. METHODS

A. Sequence Analysis

The sequence for MtMurI was retrieved from NCBI Protein database (<http://www.ncbi.nlm.nih.gov/protein>) with accession number NP_215854. A multiple sequence alignment was performed using ClustalW2 [8] using homologues from other

bacterial species (retrieved from Swissprot, *expasy.org/sprot/*) in order to visualize the conserved regions in the protein across different species.

B. Homology Modeling and Analysis of Dimer Interface and Active Site

To create a homology model for MtMurI, BLAST [9] search was first performed using the MtMurI sequence as query and the PDB database (<http://www.pdb.org/>). 3HFR (MurI from *Listeria monocytogenes*) and 2JFP (MurI from *Enterococcus faecalis*) were chosen as templates to model MtMurI in the free and the substrate-bound conformation respectively. Initially the two templates were aligned using version 9v8 of the software MODELLER [10]. Then this alignment was used as a profile in ClustalW2 to align the target sequence MtMurI to each of the templates without disturbing the original alignment. This alignment was provided as input to MODELLER to perform homology modeling. For each case, 50 all atom models were generated and the top ten were chosen on the basis of low molecular objective function and DOPE score. The stereochemical quality of the models was assessed using PROCHECK [11]. The side chains were optimized followed by energy minimization of the obtained structure.

The interactions at the interface of the two monomers were analyzed using the program InterproSurf [12]. The interactions between D-Glu and MtMurI were studied using Ligplot [13].

C. Protein-Inhibitor Docking

The structures of already known inhibitors for MurI I in other organisms were retrieved from NCBI PubChem Bioassay database. 46 inhibitors were obtained from the Bioassay ID 208942. These represent competitive inhibitors as these are 4-substituted D-glutamate analogues and were docked to the D-glutamate binding site using the substrate-bound homology model (after removing the D-glutamate). The docking was performed only for one of the monomers in the model since it does not require the dimer interface. The docking experiments were executed using the CDOCKER program in the Discovery Studio package

III. RESULTS

A. Sequence Analysis

Fig. 1 displays the sequence alignment of MtMurI (NP_215854) with MurI homologs from different bacterial species. It is seen that MtMurI shares high degree of similarity with its homologs and there are several conserved blocks in the alignment. The residues in these regions were found to be present in the catalytic site as well as the interface of two MurI monomers (described in the next section).

B. Homology Modeling and Analysis of Dimer Interface and Active Site

Homology models were obtained for MtMurI using the dimeric templates in the substrate-free as well as substrate-bound form (Fig. 2). The best models chosen according to the lowest values of objective function and DOPE score were

further evaluated by PROCHECK. For both the models, all the residues were found to lie in the favourable regions. The RMSD (root mean square deviation) of the free and bound models with their respective templates stood low at 0.30 and 0.25 Å respectively (Fig. 3).

The active site of MtMurI (Fig. 4) consists of the residues His 187, Thr 186, Asn 76, Ser 77, Gly 45, Tyr 44 involved in hydrogen bonding interactions with D-Glu. All these residues were conserved as seen in the MSA (Fig. 1).

The InterproSurf analysis was performed for the template 3HFR (which is a crystal structure) as well as the homology model (unbound) of MtMurI to understand the interaction at the dimer interface. A number of interacting residues were conserved (Table I) amongst the two and included Q24/Q27, P26/29, R84/87, P90/93, R100/103, E208/211, E213/215 (where a/b signifies that residue is from 3HFR while b is from the model). The evolutionary conservation of the interface residues as well as the large buried solvent accessible surface area (SASA) support the assumption of MtMurI existing as a dimer though it still remains to be answered whether it exists exclusively as a dimer as in *Enterococcus faecalis*, *Helicobacter pylori* and *Staphylococcus aureus* or in equilibrium between the monomer and dimer states as in *Bacillus subtilis* and *Aeromonas pyrophilus*.

C. Protein-Inhibitor Docking

The most potent ligands were identified on the basis of Lig score 2.

IV. DISCUSSION

MtMurI is essential for bacterial growth and lacks a human homolog which makes it an attractive drug target. Analysis of the structure of MtMurI would shed light on its mechanism of action and lead to the identification of potent inhibitors.

These findings would expedite the pace of further experimental studies in this direction and pave the way to demystify a number of hitherto unresolved issues about the biology of this enzyme. Overall, this may provide an effective and robust strategy to counteract the tuberculosis pathogen.

<i>M.tuberculosis/1-271</i>	1 MNSPLA F V B V F D S G V G G L T V A R A I I D Q L F D E D I V Y V G D T G N G P Y G P L T I P E I R A H A L A I G D D L V G - R G V K A L V I A 74	
<i>C.diphtheriae/1-265</i>	1 M N N - - A P I G I F D S G V G G L T V A R V I M E Q L F N E S V I Y I G D T A N S P Y G P K P I A Q V R E L S L A I G E E L V R - R G C K M I V I A 72	
<i>B.subtilis/1-272</i>	1 M L E - - Q P I G V I D S G V G G L T V A K E I M R Q L F K E N I I Y V G D T K R C P Y G P R P E E V L Q Y T W E L T N Y L L E N H H I K M L V I A 73	
<i>S.aureus/1-266</i>	1 M N - - K P I G V I D S G V G G L T V A K E I M R Q L F N E T T I Y L G D I G R C P Y G P R P E E Q V K Q Y T V E I A R K L M E - F D I K M L V I A 71	
<i>S.pneumoniae/1-264</i>	1 M D N - - R P I G F L D S G V G G L T V Y R E L M R Q L F H E E I V Y I G D S A R A P Y G P R P A E Q I R E Y T W Q L V N F L L T - K D V K M I V I A 72	
<i>L.fementum/1-268</i>	1 M D N - - R P I G V M D S G L G G L S V Y R V I Q Q K L F N E E V T F V G D Q G H F P Y G T K D Q A E V R Q L A L S I G A F L L K - H D V K M M V V A 72	
<i>L.acidophilus/1-267</i>	1 M D N - - R P I G L L D S G F G G L S V A K K V I E K L F N E S T I F I G D N A H I P Y G D R T R E D I I N L T R R S V K F L L E - K N V K L I V I A 72	
<i>A.pyrophilus/1-254</i>	1 - M K I G F I D S G V G G L T L K A I R N R Y R K V D I V Y L G D T A R V P Y G I R S K D T I I R Y S L E C A G F L K D - K G V D I I V V A 69	
<i>H.pyloii/1-255</i>	1 - - M K I G V F D S G V G G F S V L K S L L K A R L F D E I I Y G D S A R V P Y G T K D P T T I K G F G L E A L D F F K P - H E I E L L I V A 69	
<i>V.cholerae/1-265</i>	1 - - M S S P S F P R - - V L I F D S G V G G L S Y V R E I E A R L Q L N Y I Y L F D N A A Y P Y G E L T Q E T L I A R V D T L V T R M V E Q E R I D L V I A 76	
<i>E.coli/1-285</i>	1 - M A T K L Q D G N T P C L A A T P S E R P T V L V F D S G V G G L S Y D E I R H L L P D L H Y I A F D N V A F P Y G E K S E A F I V E R V V A I V T A V Q E R Y P L A L A V V A 91	
<i>P.aeruginosa/1-265</i>	1 - M A V E S A A V G F D S G V G G L S V L R E I R A R L P S E S L L Y A D N A H V P Y G E K S A E Y I R E R C E R I G D F L L E Q G - A K A I V I A 74	
<i>C.perfringens/1-260</i>	1 - M Q D D L K N A P I G F D S G L G G L S L R K A L E M M N E N Y I Y G D S K H A P Y G E K T P Q E I R S L S F N A I E F L I K G G - A K A I V I A 76	
<i>M.tuberculosis/1-271</i>	75 C N S A S S A C L R D A R E R Y Q V P V E V I L P A V R R V A A T - - R N G R I G V I G T R A T I T S H A Y Q D A F A A A - R D T E I T A V A C P R F V D F V E R G V T S G R Q V L 163	
<i>C.diphtheriae/1-265</i>	73 C N T A T S A A L R D L R E R F D V P V L G V I L P A V R R A V S T T - - R N G I G V I G T E G T I K S G A Y Q E L F A A S - P S V E V H A Q A C P S F V S F V E R I T S G R Q I L 161	
<i>B.subtilis/1-272</i>	74 C N T A T A I A L D D I Q R S V G I P V V G V I Q P G A R A A I K V T - - D N Q H I G V I G T E N T I K S N A Y E E A L L A L N P D L K V E N L A C P L L V P F V E S G K F L D K T A D 163	
<i>S.aureus/1-266</i>	72 C N T A T A V A L E Y L Q K T L S I P V I G V I E P G A R T A I M T T - - R N Q N V L V I G T E G T I K S E A Y R T H I K R I N P H V E V H G V A C P G F P L V E Q M R Y S D P T I 161	
<i>S.pneumoniae/1-264</i>	73 C N T A T A V V W E E I K A Q L D I P V L G V I L P G A S A I K S S - - G G G K I G V I G T P M T V Q S D I Y R Q K I H D L D P D L Q V E S L A C P K F A P L V E S G - A L T S V T 161	
<i>L.fementum/1-268</i>	73 C N T A T A A A L P A L Q A A L I P V I G V I E P G A R A A L Q D - - K K G P I G V I A T T A T T A G A Y P A T I E R L A P G T P V I A K A T P M E I V E H G - Q T G T A K A 161	
<i>L.acidophilus/1-267</i>	73 C N T A T A V A M P T M Q K E V E Q Q I G V I Q S G A L A A R T T - - K N K N V A V A T N V T V A S H A Y Q K E I K F R D P E I K V T E L A A F K L A P L V E A - - Q K D Y A T N 160	
<i>A.pyrophilus/1-254</i>	70 C N T A S A Y A L E R L K K E I N V P V F G V I E P G V K E A L K K S - - R N K K I G V I G T P A T V K S G A Y R K L E E - - G G A D V F A K A C L F P L A E E G - L L E G E I T 156	
<i>H.pyloii/1-255</i>	70 C N T A S A L A L E E M Q K Y S K I P I V G V I E P S I L A I K R Q V E D K N A P I L V L G T K A T I Q S N A Y D N A L K Q Q - G Y L N I S H L A T S L F P L I E E S - I L E G E L 159	
<i>V.cholerae/1-265</i>	77 C N T A S T I V L P V L R A K L - T I P V V G V V P A I K P A S - L I A - S K A I G L I A P T A T V K R Q Y T Q E L I R D F S A N K N V E L L G S T R L N M A E E K - L R G K P L D 163	
<i>E.coli/1-285</i>	92 C N T A S T V S L P A L R E K F - D F P V V G V V P A I K P A A R L A T - - N G R I V G L A T R G T Y K R S Y T H E L A R F A N E C Q I E M L G S A E M E L A B A K - L H G E D T S 179	
<i>P.aeruginosa/1-265</i>	75 C N T A T A A A A E L R E R Y P Q V L V M E P A V K P A A A A T R - - M R V I G V L A T T G L K A R F A A L D R F A S D V Q V F T Q P C P G L V E R I E A G - D L Y G P Q T 163	
<i>C.perfringens/1-260</i>	77 C N T A T S A A A H D L R E Y Y K D I P I G I E P A L K P A I K L H E - - T G S V I V M A T K A T L T Q E K F K N L M D K Y G E H R E V I P L C P R G L E F I E A G - N L E G E E V 165	
<i>M.tuberculosis/1-271</i>	164 G - L A Q G Y L E P - - L Q R A E V D T L V L G C T H Y P L L S G L I Q L A M G - - E N V T L V S S A E E T A K E V R V L T E I D L L R P H D A P P A T R I F E A T G D P E A F T K L 250	
<i>C.diphtheriae/1-265</i>	162 G - V A Q G Y V E P - - L Q A A G V D T L V L G C T H Y P L L T G V I Q L A M G - - D R M T L V S S A E E T A K D V F K T L S M T D M L A S E D S T P - V R T F E S T G D P V L F A Q L 247	
<i>B.subtilis/1-272</i>	164 E I V K T S - L Y P - - L K D T S I D S L I L G C T H Y P I L K E A I Q R Y M G - - E H N I I S S G D E T A R E V S T I L S Y K G L L N Q S P I A P - D H Q F L T T G A R D Q F A I 249	
<i>S.aureus/1-266</i>	162 S I V I H Q T L K R - - W R N S E S D V I L G C T H Y P L L Y K P I Y D F G - - G K K T V I S S G L E T A R E V S A L L T F S N E H A S Y T E P - D H R F F A T G D P T H I T N I 248	
<i>S.pneumoniae/1-264</i>	162 K K V Y Y E T L R P - - L V G - K V D S L I L G C T H Y P L L R P I I Q N V M G - - P K Q L I D S G A E C V R D I S V L N Y F E I N R G R D A G P L H R R F Y T A S S Q S F A Q I 248	
<i>L.fementum/1-268</i>	162 Q E V Y S E Q L M T - - F K E H P V K T L I M G C T H P F L A P E I S K A V G - - P T A L V D P A K E T V A T A K S W L E Q H Q A M G N H A H P N - - Y H L Y S T G N L P D L R A G 247	
<i>L.acidophilus/1-267</i>	161 L K V Y K E S L A P - - L M G K E F D T L V L G C T H Y P L I Q E E F E E A I N N - K E V T I L D P A D Q V A Q Y T F N V M R R D G L F S D S E K A V - - H E Y Y T T G N S E T F D K L 247	
<i>A.pyrophilus/1-254</i>	157 R K V Y E H Y L K E - - F K G - K I D T L I L G C T H Y P L L K K E I K K F L G D V E V D S S E A L S L S L H N F I K D D G S S S L E L F T D L S P N L Q F L I K - I I L G R D Y P 244	
<i>H.pyloii/1-255</i>	160 E T C M H Y F T P - - L E I - L P E V I I L G C T H P L I A K K I E G Y F M G H F A L P T P P L I H S G D A I V E Y L Q Q K Y A L K N N A T P K V E F H A S G D V I W L E R 248	
<i>V.cholerae/1-265</i>	164 L E E L A S I L Q P - - L K N T I D V A V L G C T H P L I K E E I Q Q V L G - - E Q V Q L I D S G L A I A R R V Q E L L G I E Q - - A V G A K Q K - - H R I Y A S A P P W E E S A 245	
<i>E.coli/1-285</i>	180 L D A L K R I L R P W L R M K E P P D T V Y L G C T H P L L Q E E L L Q V L P - - E G T R L Y D S G A A I A R R T A W L L E H E A P D A K S A D A N - - I A F C M A M T P G A E Q L 266	
<i>P.aeruginosa/1-265</i>	164 R A L L E R L L A P - - I L E Q G C D T L I L G C T H Y P F Y K P L A E L I P - - A E M A V I D T G A A V A R Q L E R V L S A R A L L A S G Q A A T - - P R F W T S A L P E E M E R I 249	
<i>C.perfringens/1-260</i>	166 K N F L R E K L N P - - Y M D R E I S S I V L G C T H Y P F Y K D V I Q D I V G - - E K V D I D G S S G T I R E L K R R L E E N M M Q S E L K K K G - - D L D I F N S L - - E D N K I 249	
<i>M.tuberculosis/1-271</i>	251 A A R F L G P V L G G V Q P V H P S R I H - -	271
<i>C.diphtheriae/1-265</i>	248 A E R F L G P H V T N V E K F A G M - - - - -	265
<i>B.subtilis/1-272</i>	250 A D D W F G H E V G H V E C I S L Q E P I K R	272
<i>S.aureus/1-266</i>	249 I K E W L N L S V N - V E R I S V N D - - - - -	266
<i>S.pneumoniae/1-264</i>	249 G E E W L E K E I H - V E H V E L - - - - -	264
<i>L.fementum/1-268</i>	248 V N K W L L S G H F D L G T A Q I E E G D - -	268
<i>L.acidophilus/1-267</i>	248 A R T F M D D D T L T S K H V D T E N Y - - - -	267
<i>A.pyrophilus/1-254</i>	245 V K L A E G V F T H - - - - -	254
<i>H.pyloii/1-255</i>	249 A K E W L K L - - - - -	255
<i>V.cholerae/1-265</i>	246 L N I K L E Q L G F N P V Q P F L H P I - - - -	265
<i>E.coli/1-285</i>	267 L P V - L Q R Y G F E T L E K L A V L G - - - -	285
<i>P.aeruginosa/1-265</i>	250 L P I L W G S - - P E S V G K L V V - - - - -	265
<i>C.perfringens/1-260</i>	250 L E L S K K L - - I E I K - - - - -	260

Fig. 1 Multiple sequence alignment of MtMurI with different bacterial homologues

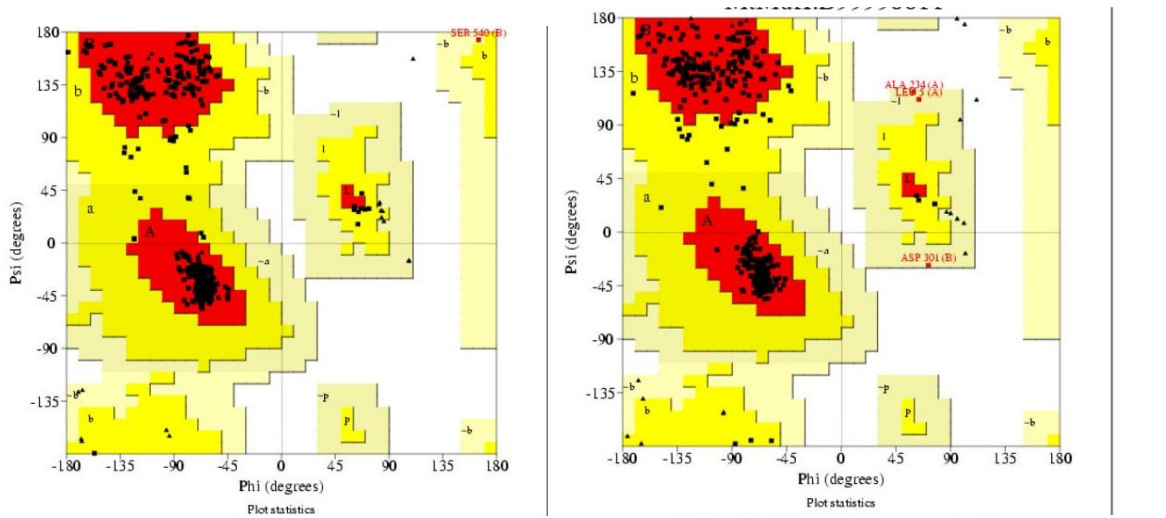


Fig. 2 Ramachandran plots for models generated for substrate-bound and free forms of the enzyme MtMurI

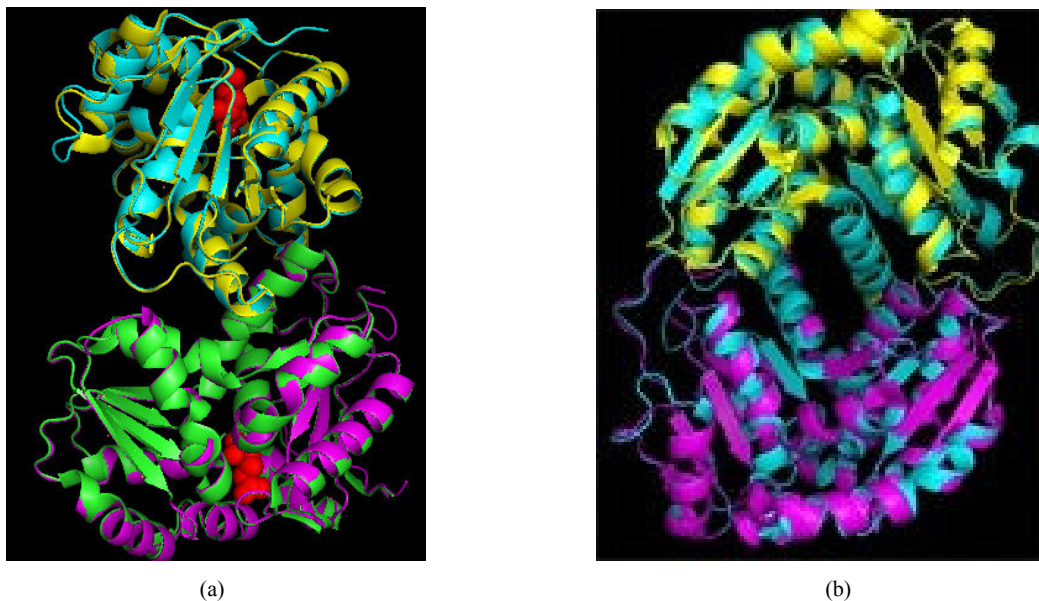


Fig. 3 Superimposition of the models for substrate-bound and free MtMurI with their templates (a) 3HFR and (b) 2JFP

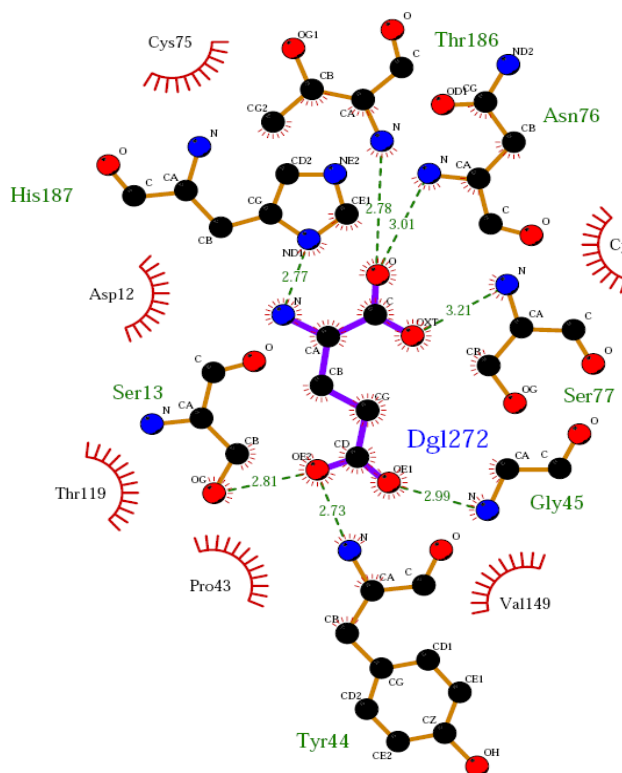


Fig. 4 Interactions between MtMurI and D-Glu

TABLE I
THE RESIDUES PRESENT AT DIMER INTERFACE OF MTMURI, OBTAINED BY
INTERPROSURF ANALYSIS

AMINO ACID RESIDUE	RESIDUE NUMBER
D	26
Q	27
L	28
P	29
D	30
R	84
R	87
P	93
V	95
L	99
R	103
V	106
A	107
A	133
A	134
A	135
R	136
D	137
E	210
E	211
K	214
E	215
V	217
R	218
V	219
T	221
E	222
L	226
D	230

TABLE II
THE TOP NINE COMPETITIVE INHIBITORS FOR MTMURI ON THE BASIS OF
LIGSCORE 2

10960522	6.87
10872317	6.77
10960486	6.71
10916364	6.68
11729651	6.63
10870708	6.45
10925032	6.41
11056087	6.32
10870708	6.23

REFERENCES

- [1] Zumla A, Nahid P, Cole ST: Advances in the development of new tuberculosis drugs and treatment regimens. *Nat Rev Drug Discov* 2013, 12:388-404.
- [2] Garret RH, Grisham CM: Membranes and Cell Surfaces. In *Biochemistry*. 2nd edition; 1999: 279-281.
- [3] Silver LL: Does the cell wall of bacteria remain a viable source of targets for novel antibiotics? *Biochemical Pharmacology* 2006, 71:996-1005.
- [4] de Jonge BLM, Kutschke A, Uria-Nickelsen M, Kamp HD, Mills SD: Pyrazolopyrimidinediones Are Selective Agents for *Helicobacter pylori* That Suppress Growth through Inhibition of Glutamate Racemase (MurI). *Antimicrobial Agents and Chemotherapy* 2009, 53:3331-3336.
- [5] de Dios A, Prieto L, MartÃ-n JA, Rubio A, Ezquerro J, Tebbe M, LÃ³pez de Uralde B, MartÃ-n J, SÃ¡nchez A, LeTourneau DL, et al: 4-Substituted d-Glutamic Acid Analogues: The First Potent Inhibitors of Glutamate Racemase (MurI) Enzyme with Antibacterial Activity. *Journal of Medicinal Chemistry* 2002, 45:4559-4570.
- [6] Sengupta S, Ghosh S, Nagaraja V: Moonlighting function of glutamate racemase from *Mycobacterium tuberculosis*: racemization and DNA gyrase inhibition are two independent activities of the enzyme. *Microbiology* 2008, 154:2796-2803.
- [7] Sengupta S, Shah M, Nagaraja V: Glutamate racemase from *Mycobacterium tuberculosis* inhibits DNA gyrase by affecting its DNA-binding. *Nucleic Acids Research* 2006, 34:5567-5576.
- [8] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al: Clustal W and Clustal X version 2.0. *Bioinformatics* 2007, 23:2947-2948.
- [9] Altschul S. F, Madden T. L, SchÃ¤ffer A. A, Zhang J, Zhang Z, Miller W, Lipman D. J: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997, 25:3389-3402.
- [10] Sali A, Blundell T. L: Comparative Protein Modelling by Satisfaction of Spatial Restraints. *Journal of Molecular Biology* 1993, 234:779-815.
- [11] Laskowski RA, Rullmann JA, MacArthur MW, Kaptein R, Thornton JM: AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J Biomol NMR* 1996, 8:477-486.
- [12] Negi SS, Schein CH, Oezguen N, Power TD, Braun W: InterProSurf: a web server for predicting interacting sites on protein surfaces. *Bioinformatics* 2007, 23:3397-3399.
- [13] Wallace AC, Laskowski RA, Thornton JM: LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng* 1995, 8:127-134.