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HOMOLOGY MODELING AND DRUG DESIGNING OF 18KDA ANTIGEN IN MYCOBACTERIUM LEPRAE

G.Sai Ramalinga Reddy[1]., B.V.Ramana Naidu[2]., B.Suneetha[3]., P.Seshapani[4]., J.Pramoda kumari[5]., D.Jayasimha Rayalu*[6] .

[1]Department of Microbiology, S.S.B.N Degree and P.G college, Anantapur, Andhra Pradesh,India.

[2]Department of Botany, S.S.B.N Degree and P.G College, Anantapur, Andhra Pradesh, India

[3]Department of Bioinformatics, Global Institute of Biotechnology, Himayat nagar, Hyderabad-29, Andhra Pradesh, India.

[4,5]Department of Microbiology, Sri Venkateswara University Tirupathi, Andhra Pradesh, India.

[6]Department of Biotechnology, Akshaya Biological Corporation, Himayat nagar, Hyderabad-29, Andhra Pradesh, India.

Email: jaimscbiotech2007@gmail.com

ABSTRACT : Most of the antigens of *Mycobacterium leprae* that have been identified are members of stress protein families. 18kDa antigen of *M. leprae* is an important antigen in the immune response to leprosy. This protein antigen of *M. leprae* is related to the family of small heat shock protein. To predict the structure of 18kDa antigen and to understand the mechanisms of inhibitors interaction, a three-dimensional model was generated based on the Crystal Structure and assembly of eukaryotic small heat shock protein (PDB: 1GME) by using MODELLER7v7. The structure having a least modeller objective function was used as a starting point for picoseconds-duration molecular dynamics simulations. With the aid of the molecular dynamics and minimization methods, the final refined model was obtained and was further assessed by ERRAT, WHATCHECK and PROCHECK, which suggested that the refined model was reliable. Docking studies were performed by using the models with 2-mercaptoethanol and 3-amino-5-methylhexanoic acid inhibitors. The results indicate that the 3-amino-5,5-diphenylpentanoic acid has more affinity than the other drug derivatives. The docking studies also suggest that MET-03, ARG-04, ASP-31, ALA-32, TRP-33, ARG-34, GLU-35 ARG-89, GLN-90 LEU-91 and VAL-92 are important determinant residues in binding with ligands. From the docking studies, we also suggest that GLU-35, in 18kDa protein domain is an important residue in binding.

Key words: 18 kDa antigen, Homology modelling, Molecular Dynamics, Docking.

INTRODUCTION

Mycobacterium leprae, also known as Hansen's bacillus, is the bacterium that causes leprosy (Hansen's disease) (Ryan KJ et al 2004). *M.leprae* is a gram positive, aerobic rod shaped bacillus surrounded by the characteristic waxy coating unique to mycobacteria. Leprosy can be diagnosed on the basis of clinical signs and symptoms most commonly. Patients with this chronic infectious disease are classified as having paucibacillary (tuberculoid leprosy), multibacillary Hansen's disease (lepromatous leprosy), or borderline leprosy. More recently, several newer, more-potent drugs and immuno modulators have been introduced in the treatment of leprosy. Long courses are necessary to decrease severity of deformities and disabilities (Vanessa Ngan 2007). This has increased the scope for further improvement in the treatment of the disease. Meanwhile, the sequencing of the genome of *Mycobacterium leprae* has been achieved.

The molecular information on the nature of the genome with the full catalogue of genes will give a direction to new options for the treatment of leprosy. In leprosy, antigen specific and MHC class II restricted T cells are responsible for both protection and immunopathology. So it is therefore important to understand the pathological conditions associated with diseases and to develop improved diagnosis and subunit vaccines (Mustafa S.K.E et al 2000). Since the discovery of T cell subpopulations with specialized regulatory activities, mechanisms of anti-inflammatory T cell regulation are studied very actively and are expected to lead to the development of novel immunotherapeutic approaches, especially in chronic inflammatory diseases. In leprosy, the lepromatous form of the disease is associated with specific unresponsiveness to the antigens of *Mycobacterium leprae*. Most of the antigens of *Mycobacterium leprae* that have been identified are members of stress protein families (Shabaana K et al 2003). Heat shock proteins (Hsp) are possible targets for regulatory T cells due to their enhanced expression in inflamed tissues and there is evidence that Hsp induce anti-inflammatory immunoregulatory T cell responses. Immune reactivity to Hsp was found to result from inflammation in various disease models. Hsp perform important functions in the folding and unfolding or translocation of proteins, as well as in the assembly and disassembly of protein complexes. Because of these helper functions, Hsp have been termed molecular chaperones. Hsp represent prominent antigens in the humoral and cellular immune response mediated by antibodies and T cells respectively. This 18 kilo Dalton antigen of *M. leprae* is an important antigen in the immune response to leprosy. This protein antigen of *M. leprae* is related to the family of small heat shock protein (Desai. S et al 2000). The gene encoding this immunologically important antigen has been sequenced, and the amino acid sequence of the antigen has been reduced. This 18kDa antigen is strikingly similar in size and sequence to a family of eukaryotic heat shock protein (Audun H.Nerland et al 1988). It is reported to be a major T-cell antigen **(**Desai et al 1982). Out of the six protein antigens, in *M. leprae* the 18kDa antigen was the first antigen demonstrated to stimulate *M.leprae* specific T-cell clones. The protein is predicted to contain five epitopes capable of stimulating T cells. The 18kDa antigen gene is highly specific for *M. leprae,* although genes with limited sequence homology were reported in *M. avium*, *M. scrofulaceum*, *M. gordonae*, *M. chelonei*, *M. intracellular* and *M. habana* based on Southern hybridization (Chakrabartty, et al 1999 Mathur and J. S et al 1999). The 18kDa Hsp belongs to the family of *a*-crystallin, low molecular mass HSPs. Relatively little is known about this protein. The cellular function of this protein is not yet known. We have initiated structural studies of the 18kDa antigen in an effort to understand the molecular basis for this affinity and to study the role of inhibitors in affecting the receptor. The structure also serves as a basis for understanding the interaction of 18kDa antigen protein with its physiological ligands.

MATERIALS AND METHODS

3D model building:

The MODELLER software was used to build the initial model of 18kDa antigen.

The first step is searching a number of related sequences to find a related protein as a template by the BLAST program (Altschul, *et al*, 1990; 1997). The high sequence identity between 18kDa and the reference protein 1GME is 41%, which allowed for rather straight forward sequence alignment. In the second step, the backbone coordinates of the residues in 18kDa were generated with the MODELLER software; a program for comparative protein structure modeling optimally satisfying spatial restraints derived from the alignment and expressed as probability density functions (pdfs) for the features restrained. The pdfs restrain Cα- Cα distances, main-chain N-O distances, main-chain and side-chain dihedral angles. The structurally conserved regions (SCRs) were determined by multiple sequence alignment, which is based on the Needleman and Wunsch Algorithm (Needleman and wunch, 1970), and the coordinates of SCRs in 18kDa were generated by copying from 1GME. The structure having the least modeller objective function, obtained from the modeller was improved by molecular dynamics and equilibration methods using NAMD 2.5 software (Kale, *et al*, 1999) using CHARMM27 force field for lipids and proteins (Schlenkrich, *et al*, 1999) along with the TIP3P model for water (Jorgensen, *et al*, 1983).

The energy of the structure was minimized with 10,000 steps. A cutoff of 12 Å (switching function starting at 10 Å) for van der Waals interactions was assumed. No periodic boundary conditions were included in this study. An integration time step of 2 fs was used, permitting a multiple time-stepping algorithm (Grubmuller, *et al*, 1991) to be employed in which interactions involving covalent bonds were computed every one time step and short-range non bonded interactions were also computed in every two time steps, and long-range electrostatic forces were computed every four time steps. The pair list of the non bonded interaction was recalculated in every ten time steps with a pair list distance of 13.5 Å. The short-range non bonded interactions were defined as van der Waals and electrostatic interactions between particles within 12 Å. A smoothing function was employed for the van der Waals interactions at a distance of 10 Å. CHARMM27 force-field parameters were used in all simulations in this study. The equilibrated system was simulated for 1 ps with a 500 kcal/mol/Å2 restraint on the protein backbone under 1 atm constant pressure and 310 K constant temperature (NPT) and the Langevin damping coefficient was set to 5 ps unless otherwise stated (Mackkerelle, *et al*, 1992). Finally, the structure having the least energy with low RMSD (Root Mean Square Deviation) was used for further studies. In this step, the quality of the initial model was improved.

The final structure obtained was checked by Ramachandran's map using PROCHECK (Programs to check the Stereo chemical Quality of Protein Structures) (Brunger, 1992) and environment profile using ERRAT graph (Structure Evaluation server) (Laskoswki, *et al*, 1993). This model was used for the identification of active site and for docking of the substrate with the protein.

Binding-site analysis:

The Bindig-site of 18kDa antigen was identified using CASTP server (Carpena, et *al*, 2003). A new program, CAST, for automatically locating and measuring protein binding pockets and cavities, is based on precise computational geometry methods, including alpha shape and discrete flow theory.

Docking of derivatives to 18kDa protein:

By means of the 3D structures of 2-mercaptoethanol and 3-amino-5-methylhexanoic acid inhibitors, which were built through the Chemsketch program, the automated molecular docking was performed by using FRED (OpenEye Scientific Software, Santa Fe, NM). The relevant stereo isomers of the compounds were minimized with the MMFF force field in the Openeye package. Conformation and minimization of the compounds was performed using Omega (OpenEye Scientific Software, Santa Fe, NM). FRED requires a set of input conformers for each ligand. The conformers were generated by Omega and stored in a single binary file and the output file was used for docking.

Docking calculations were performed with FRED version 1.1 for efficient handling of large compound databases.

RESULTS AND DISCUSSION

Homology modeling of 18kDa antigen:

A high level of sequence identity should guarantee more accurate alignment between the target sequence and template structure. In the results of BLAST search only two reference proteins, 1GME A (Chain A, Crystal Structure and assembly of eukaryotic small heat shock protein) and 2BYU A (Chain A, structure of the small heat shock protein Acr1 from Mycobacterium tuberculosis), have a high level of sequence identity and the sequence identities of these two reference proteins identity with 18kDa protein are 41% and 34%, respectively. In order to define SCRs of the protein family, multiple sequence alignment based on the structural conservative was used to superimpose the reference structure, and the SCRs were determined as shown by Fig.1.

```
18kd    ------------MLMRTDPFRELDRFAEQVLGTSARPAVMP--MDAWREG-EEFVVEFDL 45
1GME    SIVRRSNVFDPFADLWADPFDTFRSIVPAISGGGSETAAFANARMDWKETPEAHVFKADL 60
                     : :***  :  :.  : * .:..*.:.     *:*  * .*.: **

18kd    PGIKADSLDIDIER-NVVTVRAERPGVDPDR--EMLAAERPRGVFNRQLVLGENLDTERI 102
1GME    PGVKKEEVKVEVEDGNVLVVSGERTKEKEDKNDKWHRVERSSGKFVRRFRLLEDAKVEEV 120
        **:* :.:.:::*  **:.* .**.  . *:  :   .**. * * *:: * *: ..*.:

18kd    LASYQEGVLKLSIPVAERAKPRKISVDRGNNGHQTINKTAHEIIDA 148
1GME    KAGLENGVLTVTVPKAEVKKPEVKAIQISG---------------- 150
         *. ::***.:::* **  **.  ::: ..
```

Fig 1: sequence alignment of 18kDa protein from *M.leprae* with Structure of eukaryotic small heat shock protein (PDB code 1GME) done using clustalw server that was subsequently submitted to MODELLER. The conserved regions are indicated by '*'.

Fig 2: The final 3D structure of 18kDa protein. The structure is obtained by energy minimizing the average conformation over the last 1000 femto seconds of molecular dynamics simulation. The α-helix is represented by blue cylinders and β-sheet by red arrows.

Then, the Needleman Wunsch algorithm with the identity matrix was used to align the amino acid sequence of 18kDa antigen to the SCRs. Because of long variable regions alignment, it is difficult to assign coordinates based on 2BYU. In the following study, we reject 2BYU and choose 1GME as reference for modeling the target protein. After assigning coordinates from the reference protein (1GME) to the SCRs, we further assign coordinates from the reference loop region to VRs, and coordinates of N-termini and C-termini. All the side chains of the model protein were set by rotamers. With this procedure, the initial model was generated. The options used for running the MODELLER are shown in Tab. I.

Tab. I: Options used in MODELLER program

INCLUDE	#include the predefined TOP routines
SET ALNFILE	= 'alignment.ali'
SET KNOWNS	= '1GME'
SET SEQUENCE	= 'query'
SET HETATM_IO	= on
SET WATR_IO	= off
SET HYDROGEN	= off
SET STARTING_MODEL	= 1
SET ENDING_MODEL	= 20
CALL ROUTINE	= 'model'

This model was refined by MM optimization and MD simulations, and the final stable structure of 18kDa protein was obtained, as shown in Fig.2.

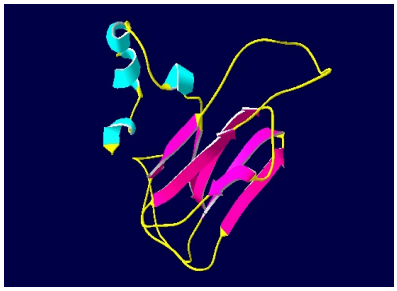


Fig 2: The final 3D structure of 18kDa protein. The structure is obtained by energy minimizing the average conformation over the last 1000 femto seconds of molecular dynamics simulation.

The α-helix is represented by blue cylinders and β-sheet by red arrows.
From Fig.2 we can see that this protein has 4 helices and 7 sheets. An analysis by Procheck shows that there is no significant difference between the calculated values of the bond lengths and bond angles and that of the known proteins for the total residues. The final structure was further checked by Verify-3D and the results are shown in Fig.3.

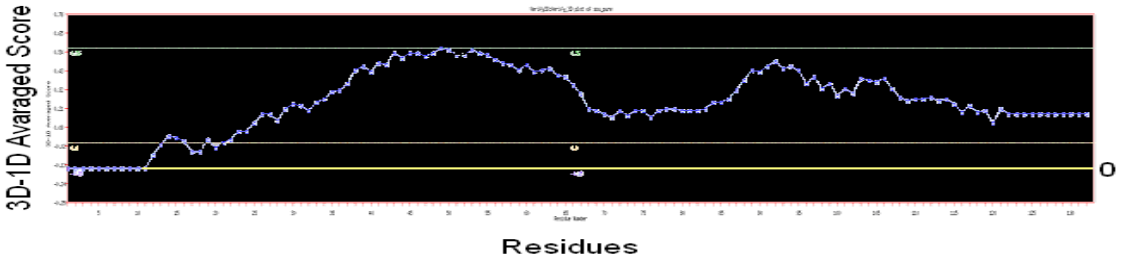


Fig 3: The 3D profiles verified results of 18kDa model, residues with positive compatibility score are reasonably folded.

Note that compatibility scores above Zero correspond to 'acceptable' side chain environments. From Fig.3, we can see that except four all residues are reasonable, which make us to believe that the structure of 18kDa protein is reliable. The φ and ψ distributions of the Ramachandran plots of non-glycine, non-proline residues are summarized in Fig. 4 and Tab. II.

Table I1: % of residue falling in the core region of the Ramachandran's plot

% of residue in most favored regions	77.0
% of residue in the additionally allowed zones	17.7
% of residue in the generously regions	2.7
% of residue in disallowed regions	2.6
% of non-glycine and non-proline residues	100.0

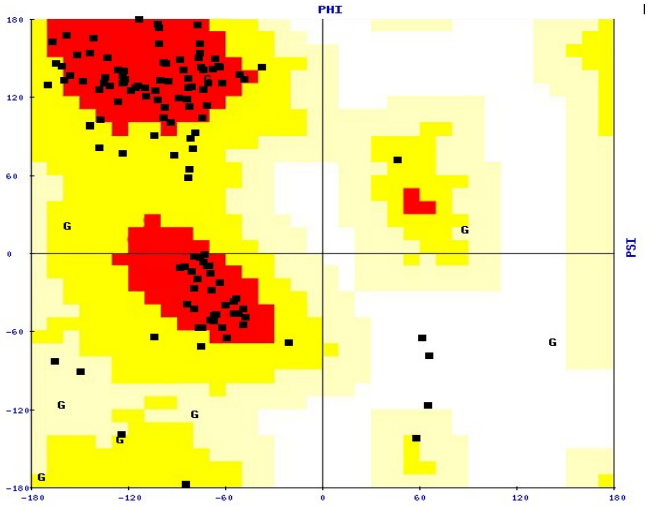


Fig 4: Ramachandran's map of 18kDa built using MODELLER software. The plot calculations on the 3D model of 18kDa were computed with the PROCHECK program

Altogether 77% of the residues of 18kDa antigen were in favored and allowed regions.
Figure 5 shows the structure alignment of Cα trace between 18kDa and 1GME. The root mean square deviation of the Cα atoms (Cα RMSD) between 18kDa and 1GME is 1.62 Å, which further indicates that the homology model is reliable.

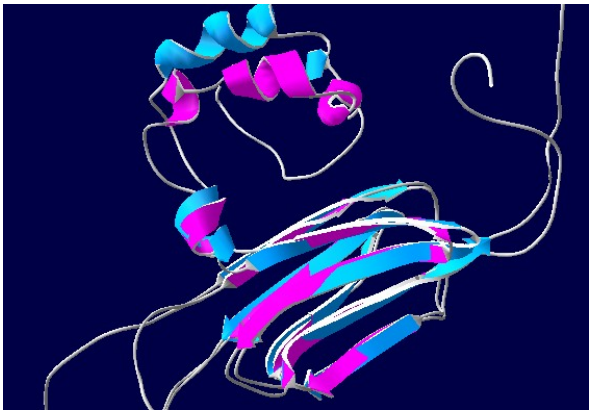


Fig 5: Superimposition of Cα trace of 18kDa (represented in pink color) and 1GME (represented in blue color).

Identification of binding region in 18kDa protein:
Active site is obtained using CASTp server, and the location of the site in the 3D structure of 18kDa protein is shown in Fig.6.

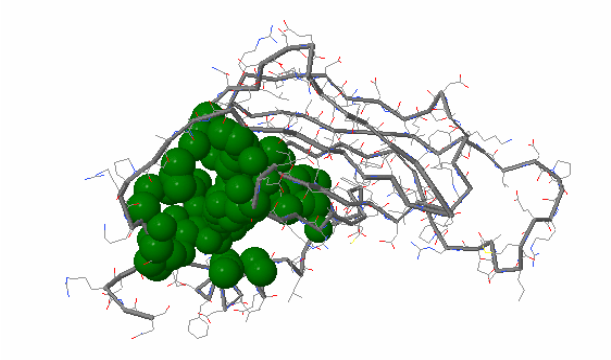
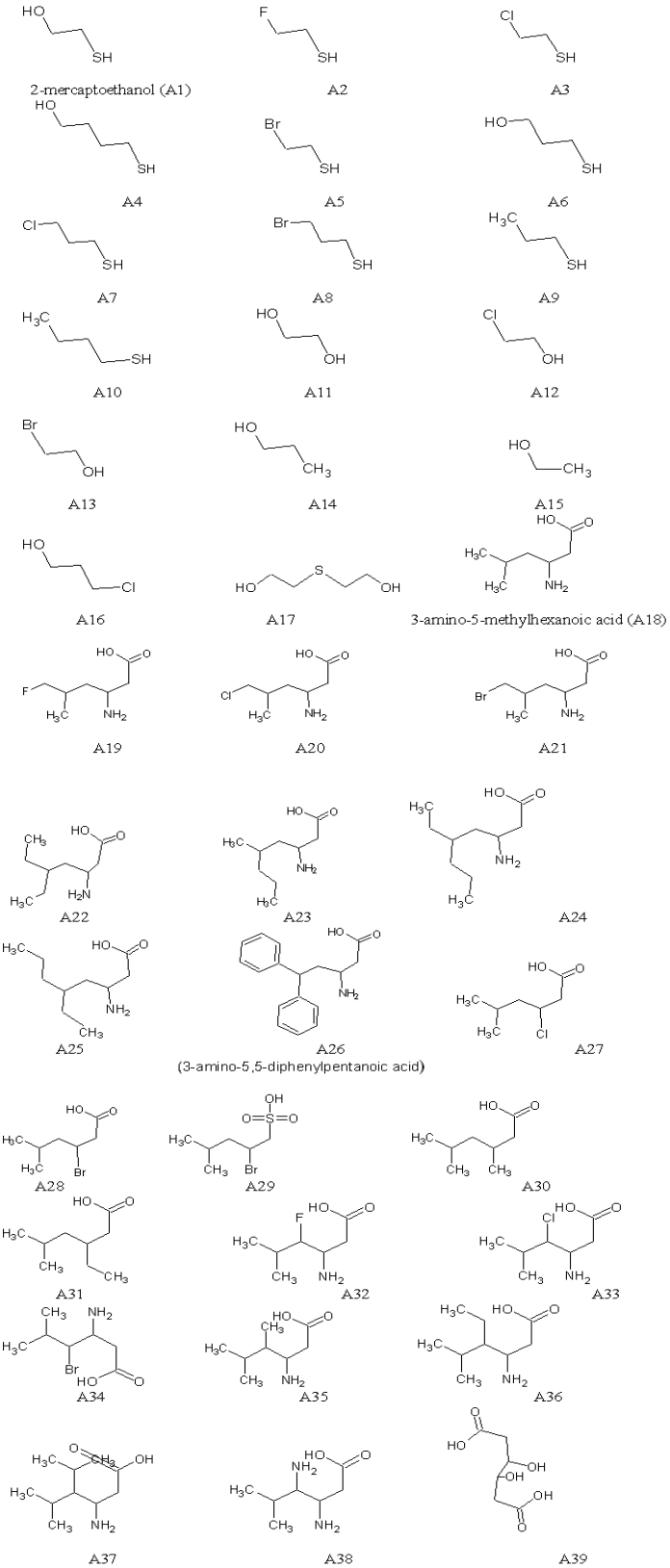


Fig. 6: Possible binding site of 18kDa

18kDa and 1GME are well conserved in both sequence and structure; their biological functions should be identical. In fact from the sequence alignment of 18kDa with 1GME, we know that the residues MET-03, ARG-04, ASP-31, ALA-32, TRP-33, ARG-34, GLU-35 ARG-89, GLN-90 LEU-91 and VAL-92 are conserved. By considering the experimental fact that the active site of 1GME includes all the residues mentioned above, and on other hand, the shape of the site in 18kDa is similar to that of 1GME binding site. Thus in this study the binding site is chosen to dock the inhibitors.

Docking study
All the reports suggest that inhibitors can bind to the antigen 18kDa. We are interested in testing the role of hydrogen bonding, in binding of 18kDa to inhibitors. In the following discussion, the interactions of the inhibitors with the receptor in modeled complexes are investigated, and we shall compare the inhibition ability of 18kDa by inhibitors. The molecular structure of inhibitors were built and optimized by Chemsketch program. The final structures are shown in Fig.7 (A1-A48).


2-mercaptoethanol (A1)
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
A13
A14
A15
A16
A17
3-amino-5-methylhexanoic acid (A18)
A19
A20
A21
A22
A23
A24
A25
A26
(3-amino-5,5-diphenylpentanoic acid)
A27
A28
A29
A30
A31
A32
A33
A34
A35
A36
A37
A38
A39

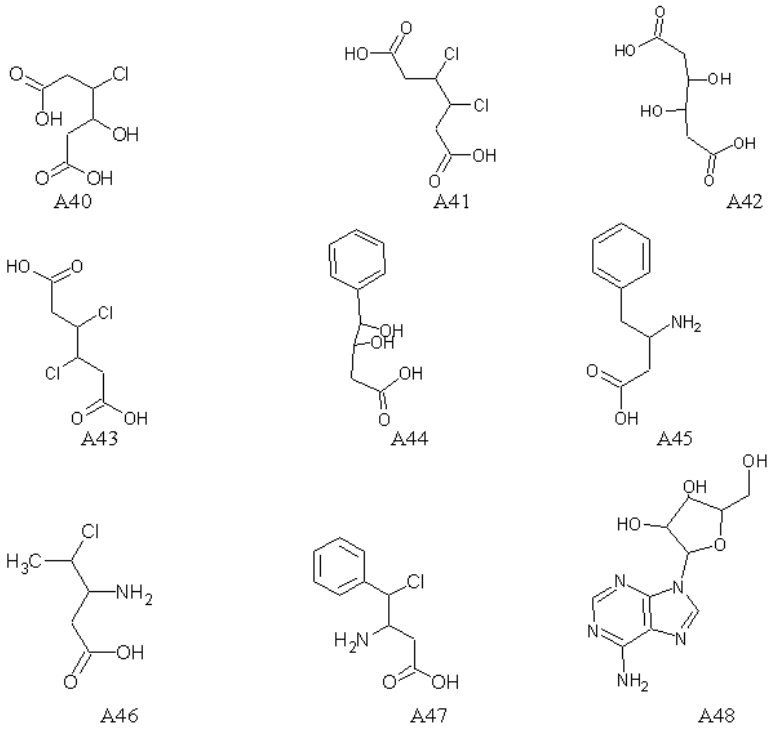


Fig. 7 (A1-A48): Structures of 2-mercaptoethanol and 3-amino-5-methylhexanoic acid inhibitors.

Docking of inhibitors with the active site of 18kDa:

To understand the interaction between 18kDa and 2-mercaptoethanol and 3-amino-5-methylhexanoic acid inhibitors, the inhibitor- 18kDa complex was generated using FRED 2.1, which is based on Rigid Body Shape-Fitting (Open Eye Scientific Software, Santa Fe, NM) and the binding 3D conformation complex of the best docked inhibitor. This figure shows that inhibitors are stabilized by hydrogen bonding. Hydrogen bonds play an important role for structure and function of biological molecules. Among the docked inhibitors, 3-amino-5,5-diphenylpentanoic acid is tightly bound by one hydrogen bond with the 18kDa (Fig.8).

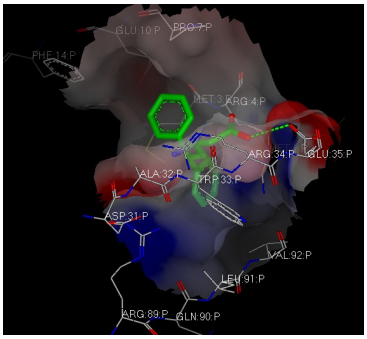


Fig.8: The hydrogen bonding interaction of complex 18kDa-3-amino-5,5-diphenylpentanoic acid.

The hydrogen of GLU-35 in 18kDa forms a hydrogen bond with the carbonyl O of 3-amino-5, 5-diphenylpentanoic acid. To determine the key residues that comprise the active site of the model, the interaction energies of the inhibitors with 18kDa were calculated. Tab.III gives deep interaction energies for all inhibitors.

Table.III: The total docking energies between 2-mercaptoethanol and 3-amino-5-methylhexanoic acid inhibitors and active site of 18kDa

Serial no	Chemscore	Screenscore	Chemgauss	Plp	Total energy
2-mercaptoethanol	-7.16	-42.30	-16.83	-17.89	-84.18
A2	-3.68	-31.83	-15.14	-18.92	-69.57
A3	-5.73	-28.33	-15.51	-16.80	-66.37
A 4	-3.17	-33.37	-16.62	-17.58	-70.74
A 5	-11.59	-39.86	-19.54	-18.49	-89.48
A 6	-10.74	-42.87	-23.52	-17.97	-95.1
A 7	-4.28	-38.56	-20.24	-21.19	-84.27
A 8	-3.74	-27.81	-20.32	-12.55	-64.42
A 9	-3.71	-35.51	-17.32	-18.91	-75.45
A 10	-4.80	-32.61	-19.76	-17.98	-75.09
A 11	-10.77	-32.39	-15.18	-10.04	-68.38
A 12	-8.22	-29.59	-15.44	-14.89	-68.14
A 13	-4.21	-32.06	-15.49	-11.17	-62.93
A 14	-8.35	-35.59	-16.51	-17.11	-77.56
A 15	-10.93	-28.66	-12.16	-10.88	-62.63
A 16	-10.13	-33.14	-19.10	-17.79	-80.16
A 17	-7.57	-31.50	-22.61	-14.20	-75.88
3-amino-5-methyl hexanoic acid	-4.48	-46.05	-30.29	-34.11	-114.93
A 19	-1.40	-44.46	-24.27	-23.61	-93.74
A 20	-3.65	-46.04	-24.96	-21.17	-95.82
A 21	-3.40	-52.38	-27.84	-22.88	-106.5
A 22	-3.24	-56.71	-26.50	-25.75	-112.2
A 23	-8.17	-51.47	-27.50	-24.63	-111.56
A 24	-8.82	-64.36	-27.29	-32.80	-133.27
A 25	-3.73	-50.07	-28.38	-22.85	-105.03
A 26(3-amino-5,5-diphenylpentanoic acid)	-13.04	-81.95	-37.09	-35.81	-167.89
A 27	-5.17	-59.53	-24.42	-23.91	-113.03
A 28	-5.91	-50.31	-25.53	-22.84	-104.59
A 29	-2.33	-45.29	-22.88	-21.35	-91.85
A 30	-1.54	-48.39	-21.20	-21.32	-71.13
A 31	-4.61	-54.96	-26.26	-24.58	-110.41
A 32	-7.29	-57.82	-25.13	-21.64	-111.88
A 33	-5.39	-49.66	-24.12	-25.31	-104.29
A 34	-6.58	-41.02	-28.33	-18.22	-105.81
A 35	-7.34	-56.10	-24.11	-26.49	-114.04
A 36	-6.96	-52.54	-28.09	-20.88	-108.09
A 37	-5.65	-49.47	-23.12	-22.81	-101.05
A 38	-7.18	-45.35	-24.60	-25.44	-102.57
A 39	-3.36	-46.29	-34.14	-16.96	-100.75
A 40	-5.17	-55.44	-27.45	-24.22	-112.28
A 41	-2.00	-54.24	-26.46	-23.79	-106.49
A 42	-3.36	-46.29	-34.14	-16.96	-100.75
A 43	-2.00	-54.24	-26.46	-23.79	-106.49
A 44	-4.71	-75.29	-29.45	-23.10	-132.55
A 45	-3.17	-67.28	-29.28	-26.10	-125.83
A 46	-2.18	-49.96	-23.91	-19.74	-95.79
A 47	-6.16	-69.73	-30.85	-31.81	-138.55
A 48	-9.76	-72.47	-40.15	-22.14	-144.52

From the Tab.III we can see that among the antigen-inhibitor complexes 3-amino-5, 5-diphenylpentanoic acid has a large favorable total energy of -167.89Kcal mol^{-1}. From these results we can suggest that MET-03, ARG-04, ASP-31, ALA-32, TRP-33, ARG-34, GLU-35 ARG-89, GLN-90 LEU-91 and VAL-92 are important residues in 18kDa and are the main contributors to the inhibitor interaction

MET-03, ARG-04, ASP-31, ALA-32, TRP-33, ARG-34, ARG-89, GLN-90 LEU-91 and VAL-92 are not involved in the bonding with 3-amino-5, 5-diphenylpentanoic acid. However, we think that these may be important residues because these form hydrogen bonds with other inhibitors. From the alignment result, we can see that GLU-35 is conserved, and this is important antigen activity.

In summary, the above results show that the total interaction energy between 18kDa and 3-amino-5, 5-diphenylpentanoic acid is higher than that between 18kDa and other inhibitors. The 18kDa and 3-amino-5, 5-diphenylpentanoic acid complex is more stable than that of the other complexes. Furthermore there are many common important residues in 18kDa binding to inhibitors. This indicates that there is a simple competitive inhibition between derivatives, 3-amino-5, 5-diphenylpentanoic acid is more preferred inhibitor. In our studies, GLU-35 is important for strong hydrogen bonding interaction. On the other hand the residues MET-03, ARG-04, ASP-31, ALA-32, TRP-33, ARG-34, ARG-89, GLN-90 LEU-91 and VAL-92 in 18kDa are the important residues in binding as they have strong bonds with the other inhibitors. As shown in Tab.III, these results can serve as a guide for the selection of candidate sites for further experimental studies of site-directed mutagenesis.

CONCLUSION

In this work, we have constructed a 3-D model of 18kDa domain using the MODELLER software. After energy minimization and molecular dynamics simulations, this refined model structure is obtained. The final refined model was further assessed by ERRAT and PROCHECK program, and the results show that this model is reliable. The stable structure is further used for docking of 2-mercaptoethanol and 3-amino-5-methylhexanoic acid inhibitors. Through the docking studies, the model structures of the ligand-receptor complex were obtained. The docking results indicate that conserved aminoacid residues in 18kDa play an important role in maintaining a functional conformation and are involved in donor substrate binding. The interactions of 18kDa and inhibitors in this study are useful for understanding the potential mechanisms of 18kDa and inhibitors. In particular, with the aid of some hydrogen bonds are formed in the docked complex. As is well known, hydrogen bonds play an important role for the structure and function of biological molecules. GLU-35 is important for strong hydrogen-bonding interactions with inhibitors. It is noticeable that 3-amino-5, 5-diphenylpentanoic acid is a more preferred inhibitor and that there is a simple competitive inhibition between inhibitors (Tab.III and Fig.8). On the other hand, the results reported here lead to the proposal of MET-03, ARG-04, ASP-31, ALA-32, TRP-33, ARG-34, ARG-89, GLN-90 LEU-91 and VAL-92 as key residues because they have strong hydrogen bonds with inhibitors. To the best of our knowledge GLU-35 is conserved in this domain and may be important for structural integrity or maintaining the hydrophobicity of the inhibitor-binding pocket. Among the inhibitors, 3-amino-5, 5-diphenylpentanoic acid showed best docking result with 18kDa. From the Tab.III we can see that among the inhibitor-18kDa complexes 3-amino-5, 5-diphenylpentanoic acid has a large favorable total energy of 167.89 Kcal mol^{-1}(Fig.12). In addition, as well as the others in Tab.III, these inhibitors are suggested as candidates for further experimental studies of structural-functional relationships.

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