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A N I N T E R N A T I O N A L J O U R N A L

In Silico Molecular Modeling and Docking Studies on the Leishmanial Tryparedoxin Peroxidase

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ABSTRACT

Leishmaniasis is one of the most common form of neglected parasitic disease that affects about 350 million people worldwide. Leishmanias *have a trypanothione mediated hydroperoxide metabolism to eliminate endogenous or exogenous oxidative agents. Both of 2-Cys peroxiredoxin (Prx) and glutathione peroxidase type tryparedoxin peroxidase (Px) are the terminal enzymes in the trypanothione dependent detoxification system. Therefore absence of trypanothione redox system in mammals and the sensitivity of trypanosomatids against oxidative stress, enzymes of this pathway are drug targets candidates. In this study, 3D structure of tryparedoxin peroxidase (2-Cys peroxiredoxin type) from* Leishmania donovani *(LdTXNPx) was described by homology modeling method based on the template of tryparedoxin peroxidase from* Crithidia fasciculata *and selected compounds were docked to the active site pocket. The quality of the 3D structure of the model was confirmed by various web based validation programs. When compared secondary and tertiary structure of the model, it showed a typical thioredoxin fold containing a central beta-sheet and three alpha-helices. Docking study showed that the selected compound 2 (CID 16073813) interacted with the active site amino acids and binding energy was -118.675 kcal/mol.*

Key words: Homology modeling, docking, *Leishmania donovani*, tryparedoxin peroxidase, hydroperoxide metabolism

INTRODUCTION

Leishmaniasis is caused by trypanosomatid protozoan parasites that belong to the genus *Leishmania* and it is one of the most neglected parasitic diseases that affect about 350 million people together with two million new cases yearly. Leishmaniasis is seen frequently in Southeast Asia, Africa, South America, including mostly Brazil and Mediterranean countries (WHO 2010). Leishmaniasis has three clinical forms seen in humans, which are cutanous leishmaniasis (*L. major, L. tropica* and *L. mexicana*); visceral leishmaniasis (*L. donovani* and *L. infantum*) that is responsible from thousands of deaths each year and mucocutaneous leishmaniasis (*L. braziliensis*). The disease is transmitted by infected female sandflies to the vertebrate hosts where the parasites infect and reproduce within their macrophages (Desjeux 2001, Pavli et al. 2010).

Parasites are exposed to various reactive oxygen species (superoxide anion radical, hydrogen peroxide, peroxynitrite and the hydroxyl radical) generated by the host defense system, or endogenously by parasite itself (Krauth-Siegel and Combos 1999, Müller et al. 2003, Krauth-Siegel and Comini 2008). However, they have adapted to survive and replicate inside the host macrophages, which produce ROS and NO derivatives for the destruction of microorganism normally (Assche et al. 2011). This is because of all the trypanosomatids have a trypanothione mediated

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hydroperoxide metabolism to eliminate endogenous, or exogenous oxidative agents. Detoxification of the hydroperoxidases comprises an electron transfer cascade that contains trypanothione, trypanothione reductase, tryparedoxin and tryparedoxin peroxidases (2-Cys peroxiredoxin and glutathione peroxidase type tryparedoxin peroxidases) enzymes (Müller et al. 2003). All the trypanosomatid protozoan parasites bear this thiol redox pathway and share same biochemical, morphological and molecular characteristics (Krauth-Siegel and Comini 2008). Due to lack of glutathione reductase, catalse, thioredoxin reductase and glutathione peroxidase mechanisms in the parasites, which are found in their mammalian hosts, trypanothione is the only system that protects parasites from the oxidant damage and also toxic effects of the heavy metals (Flohe et al. 1999, Krauth-Siegel and Comini 2008).

Both of 2-Cys peroxiredoxin (Prx) and glutathione peroxidase type tryparedoxin peroxidase (Px) are the terminal enzymes in the trypanothione dependent detoxification system in trypanosomatids. Electron transfer occurs between the trypanothione reductase and tryparedoxin mediated by trypanothione. Tryparedoxin peroxidase accepts an electron from the tryparedoxin for the reduction of the hydrogen peroxide and peroxynitrite (Irigoin et al. 2008). However, some other compounds could be as substrates for the 2-Cys peroxiredoxin (Prx) such as linoleic acid hydroperoxide (Nogoceke et al. 1997). Studies have indicated that the tryparedoxin peroxidases involved in the trypanothione dependent efflux system are essential, for the parasite survival and they have similar activities and cellular localizations (Dumas et al. 1997, Tovar et al. 1998, Wilkinson et al. 2000, Tetaud et al. 2001, Castro et al. 2002). RNA interference studies have shown that the inhibition of the enzymes cause impaired cell growth, enhanced sensitivity towards H_2O_2 and other oxidants and cell death (Wilkinson et al. 2003, Lin et al. 2005, Schlecker et al. 2005). However overexpression reveals enhanced resistance towards arsenite, hydroperoxide and NO (Lin et al. 2005, Castro et al. 2002, Pineyro et al. 2008). In addition, further proteomic analysis also reveals the expression of the eleveted levels of the trypanothione redox pathway proteins in both of trypanosomatid parasites *Trypanosoma* sp. and *Leishmania* sp. in different life stages (Daneshavar et al. 2012, Diaz et al. 2011). Some other findings also show that there is a positive correlation between the elevated levels of tryparedoxin peroxidase with the enhanced antioxidant defenses and resistance to conventional drugs (Wyllie et al. 2010).

Thus, existing treatments for the leishmaniasis are limited because they are based on a few drugs that have some side effects and increasing resistance to conventional drugs. This necessitates a continuous demand for novel drugs. In drug research process, selecting potential drug targets should play an essential role in the parasite survival and also should have a significant structural and functional difference from its mammalian counterparts (Sing et al. 2012). Together with the absence of trypanothione redox system in the mammals and the sensitivity of trypanosomatids against oxidative stress, enzymes of the pathway are attractive candidates for drug targets (Krauth-Siegel et al. 2005, Jaeger and Flohe 2006, Pineyro et al. 2008).

For computational drug design studies, 3D structure of the interest protein should be known. Crystal structure of the tryparedoxin peroxidase from *Trypanosoma cruzi* (*Tc*TXNPx) and *C. fasciculata* were dissolved by the method of X-ray diffraction (Alphey et al. 2000, Pineyro et al. 2005). However, currently there is no solved crystal structure for tryparedoxin peroxidase from the genus of *Leishmania.* Computational homology, based modeling methods are the best and fast techniques to predict the protein structure from a known 3D template when there is no solved structure (Hillisch et al. 2004, Werner et al. 2012). Templates for homology modeling should be selected from the evolutionary closest protein family and have a sequence identity higher than 50%, which is ideal for drug design (Hillisch et al. 2004, Bishop et al. 2008). Computer-aided drug discovery process comprises three main steps; prediction of the 3D structure of the protein, prediction of the possible ligands and docking procedures and biological testing of the drug candidates (Huang et al. 2010).

In this work, 3D structure of tryparedoxin peroxidase (2-Cys peroxiredoxin type) from *L. donovani* (*Ld*TXNPx) was described by homology modeling method based on the template of tryparedoxin peroxidase from *C. fasciculata* and docking studies was performed with selected five compounds. Homolog modeling study included template identification, model building, followed by the side chain refinement and validation of the resultant protein structure.

MATERIALS AND METHODS

Target sequence, template identification and sequence alignment

The amino acid sequence of the target tryparedoxin peroxidase for *L. donovani* was obtained from the (National Center for Biotechnology Information, NCBI) database with the accession number of AAK00633. For template identification, NCBI BLAST tool was used and two types of BLAST (Basic Local Alignment search Tool) protein-protein BLAST and PSI-BLAST (Position Specific Iterated-BLAST) was performed for template selection by searching against database Protein Data Bank proteins. At the end of comparative searching, one of the homolog structure that had the best score was selected as a template protein. Template protein PDB file and amino acid sequence in FASTA format was downloaded from the Protein Data Bank. Multiple sequence alignment of the template and target proteins were analyzed by ClustalW2 (Larkin et al. 2007) from the EBI (European Bioinformatics Institute) sequence analysis tools.

Homology model building and refinement

A homology model of the *Ld*TXNPx enzyme was constructed by using the software of Modeller v9.11 (Eswar et al. 2006). Model was built according to the target sequence, an alignment file and 3D structure of the template protein that was obtained from the Protein Data Bank (PDB). Refinement of the model was done by performing energy minimization by HyperChem software (Hypercube Inc. Gainesville, FL, USA). Secondary structure of the protein was estimated by web based program SOPMA (Geourjon and Deleage 1995) and NCBI CDD were used to determine the domains. The PDB file of the resultant model has been deposited in the Protein Model Data Base (PMDB) with ID number of PM0078249. All generated structures were visiulazed by MolSoft ICM-Browser (MolSoft LLC, La Jolla, CA, USA).

Model validation and bioinformatics

Stereochemistry of the model was checked by and RAMPAGE (Lovell et al. 2007). Structure quality of the protein was analyzed by ProSA (Wiederstein and Sippl 2007) and Protein Structure Validation Suite, PSVS 1.3. Statics of non-bonding interactions was performed by ERRAT (Colovos and Yeates 1993). Various physical and chemical parameters for the modeled protein included the molecular weight, theoretical pI, amino acid composition, extinction coefficient, aliphatic index and grand average of hydropathicity (GRAVY), which were computed by ProtParam tool from ExPASy.

Docking studies

In docking study, compounds were searched for the leishmanial activity (IC50, etc $\leq$ 1 nM) by using PubChem BioAssay database from NCBI. Three dimensional structures of the selected compounds were retrieved as SD file format. They were 3-naphthalen-2-yloxy-1,2-benzothiazole 1,1-dioxide (Compound ID: 790223), 3-methoxy-5-(3-methylsulfanyl-1,2,4-triazin-5-yl)-1,2,4-triazine (Compound ID: 2865728), 6,6-dimethyl-1-[3-[(3-propan-2-ylphenoxy)methyl]phenyl]-1,3,5-triazine-2,4-diamine (Compound ID: 16073813), 4-methyl-7-[(1-oxido-1-oxo-1,2-benzothiazol-3-yl)sulfanyl]chromen-2-one (Compound ID: 46912206) and N-(4,6-dimethylpyrimidin-2-yl)-4,5,7-trimethylquinazolin-2-amine (Compound ID: 53325662). Docking study was performed by using Molegro Virtual Docker software (Thomsen and Christensen 2006) and docking results were analyzed by Molegro Molecular Viewer 2.5.0 (Molegro ApS, Aarhus, Denmark).

RESULTS AND DISCUSSION

Template identification and sequence alignment

BLAST analysis was performed of tryparedoxin peroxidase from *L. donovani* (*Ld*TXNPx) against Protein Data Bank proteins (PDB). The three top results belonged to tryparedoxin peroxidase from *Crithidia fasciculata* (PDB ID: 1E2Y, Identities = 134/184 (73%), E-value 4e-101), tryparedoxin peroxidase (Txnpx) from *Trypanosoma cruzi* (PDB ID: 1UUL, Identities: 137/196 (70%), E-value: 3e-101) and peroxiredoxin I from *Schistosoma mansoni* (PDB ID: 3ZVJ, Identities = 117/181 (65%), E-value: 8e-86). Homology models having sequence identities between 30 and 50% could be used in structure-based target assessment and site-directed mutagenesis. Sequence identity above 50% was ideal for structure-based drug design and target assessment, site directed mutagenesis and assignment of protein function (Hillisch et al. 2004). The sequence identity between the selected template and target was 73%, hence models could be used directly in drug design studies. Distance tree of the BLAST results showed that these proteins were closely related. According to the BLAST search,

the closest enzyme was from *C. fasciculata* (PDB ID: 1E2Y, Alphey et al. 2000), which was selected as a template for homology modeling. Figure 1 shows multiple sequence alignments of the tryparedoxin peroxidases from *L. donovani*, *C. fasciculata* and *T. cruzi*.

Figure 1 - Multiple sequence alignment of the tryparedoxin peroxidases from *Crithidia fasciculata, Leishmania donovani* and *Trypanosoma cruzi*. Boxes show active site residues. (*) conserverd residues.

Structural features

All the peroxiredoxins shared strictly conserved cysteines at the position of 52 and 173 at the N- and C- terminal regions. They are often found in a -VCPT- and -VCP- motif (Alphey et al. 2000). These cysteine residues are involved in catalytic activity thus essential for activity (Flohe et al. 2002). As shown in the alignment, all four enzymes had these conserved regions. Because of having two Cys residues at the N- and C- terminal motif, they are called as 2-Cys peroxiredoxins. Secondary structure predictions showed that the enzyme had 61 residues in α-helix (30.65%), 43 residues in extended strands (21.61%), 18 in β–turn (9.05%) and others (77, 38.69%) in random coil form. Overall structure comprised four α–helices, seven β–strands and two 3_{10}- helix (θ1 and θ2). N terminus of the structure began with a β–hairpin (β1 and β2), followed by a section of 3_{10}-helix (θ1), two βαβ units (β3–α1–β4–α2–β5), 3_{10}-helix (θ2) found between (α2–β5), then α3, a β–hairpin (β6 and β7) and α4 before C- terminal. This secondary structure highly resembled to the secondary structure of the template enzyme, which had same folding features (Alphey et al. 2000). Like in *C. fasciciulata* TXNPx, Cys52 was found before α1 and Cys173 after that α4 helices.

The 3D structure of the model indicated that the enzyme comprised a typical thioredoxin fold that contained a central β–sheet (β7, β6, β3, β4) and three α helices (α1, α4 and α3) (Alphey et al. 2000) (Fig. 2). Hence, it has been classified as a member of the thioredoxin superfamily and glutathione peroxidase-like family according to the domain search and structural results. When compared 3D structures of *C. fasciculata* TXNPx and *L. donovani* TXNPx, they fit with a high similarity. Superposition of the C_α traces of the *Cf*TXNPx and *Ld*TXNPx had RMSD of 0.4223 Å and 179 atoms overlapping (Fig. 3).

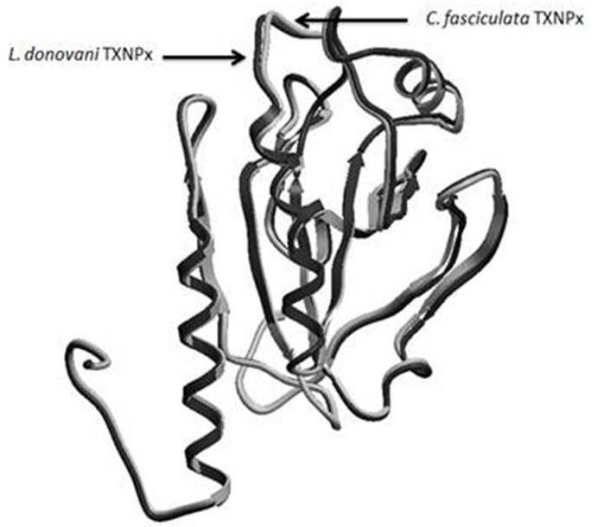


Figure 2 - Superposition of the template (*Crithidia fasciculata* tryparedoxin peroxidase, PDB ID 1E2Y) and the target (*Leishmania donovani* tryparedoxin peroxidase) proteins according to the C_α traces (RMSD = 0.4223 Å). Black color represents *L. donovani* TXNPx and white one is for *C. fasciculata* TXNPx.

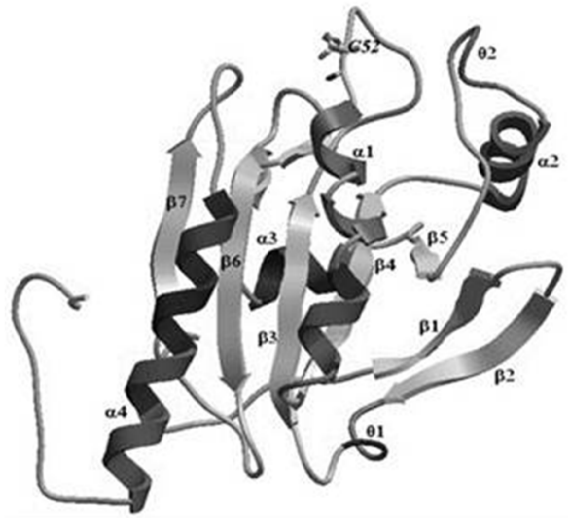


Figure 3 - 3D structural model of *Leishmania donovani* tryparedoxin peroxidase. Helix forms represent α–helices and large arrows for β–hairpin structures. N- terminus of the structure begins with a β–hairpin (β1 and β2) and terminates with α4 before C- terminal. Structure shows a typical thioredoxin fold contains a central β–sheet (β7, β6, β3, β4) and three α helices (α1, α4 and α3) around the sheet.

The peroxidase active site comprises Cys52 and Cys173, which are directly responsible for peroxidase activity (Hirotsu et al. 1999, Alphey et al. 2000, Schröder et al. 2000). In *C. fasciculata* TXNPx, Cys52 accepts a hydrogen bond from Arg128, donates to the carbonyl of Met147 and carboxylate of Glu55. Arg128 is thought to be stabilizing the ionized state of Cys52, lower its pK_a and increases the activity. Same active site residues are also found in *L. donovani* TXNPx at the same positions. *Ld*TXNPx comprises three different pockets. Two of them are very important because of having active site residues of Cys52 and Cys173. The first pocket is located between β3–α3 and α1 and interacted with some of the active site residues (Cys52, Met147 and Arg128) and also with others such as Tyr44, Leu46, Thr49, Ile56, Ala58, Pro148, Gly150 and Glu154 residues. The second one is found at the C-terminal and interacts with Arg158, Leu159, Ala162, Phe163, Val166, Cys173 and Trp177 residues.

Structure validation

Constructed new model, which was called as *Ld*TXNPx, was checked for stereochemistry, energy profiles, potential errors, non-bonding interactions and some physical and chemical properties. According to the ProtParam results, protein model is about 22.2 kDa, pI is 7.55, have 22 negatively and 23 positively charged residues, extinction coefficient was 25815, aliphatic index was 79.40 and grand average of hydropathicity was -0.108. According to the RAMPAGE (Lovell et al 2007), results 89.6% of residues (155) were in favored, 7.5% (13) in allowed and 2.9% (5) in outlier region in ramachandran plot that analyzed peptide dihedral angles. ERRAT was used to predict non-bonding interactions by examining the statistics of pair wise atomic interactions (CC, CN, CO, NN, NO, and OO). Program gave a result of 93.865 as overall quality factor, which meant only 10 residues showed incorrect determined regions (Colovos and Yeates 1993). ProSA calculated overall protein quality based on the scores of all experimentally determined protein chains available in PDB. The program gives z-score and a plot of the residue energies (Wiederstein and Sippl 2007). Z-score of *Ld*TXNPx was -6.46. It was within the range of scores typically found for the proteins of similar size and also close to the z-score value of the *Cf*TXNPx that was -7.4. The energy plot calculated the average energy over

each 40-residues fragment and overall negative values of the model indicated the structure is non-problematic. Protein Structure Validation Suite (PSVS 1.3) was also used to analyze overall model quality based on the global quality scores of four different structure validation softwares, which were Verify3D, ProsaII, Procheck and MolProbity (Table1). All quality check results showed that the model was close to the native form and the environment profile of the structure was good.

Table 1 - Protein structure validation suite (PSVS) results for the global quality of the protein model

Program	Verify 3D	ProsaII	Procheck**	Procheck (all)**	MolProbity Clashscore
Raw score	0.43	0.50	-0.66	-0.68	8.31
Z-score*	-0.48	-0.62	-2.28	-4.02	0.10

*With respect to mean and standard deviation for a set of 252 X-ray structures < 500 residues, of resolution <= 1.80 Å, R-factor <= 0.25 and R-free <= 0.28; a positive value indicates a 'better' score. **Selected residues: 1A-178A

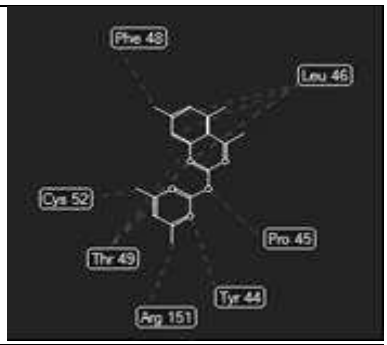
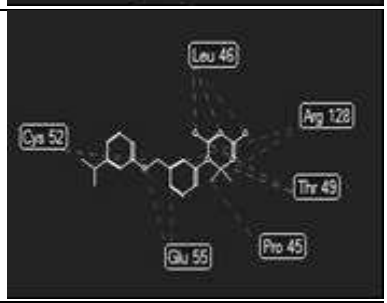
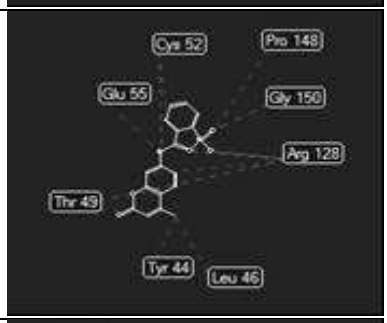
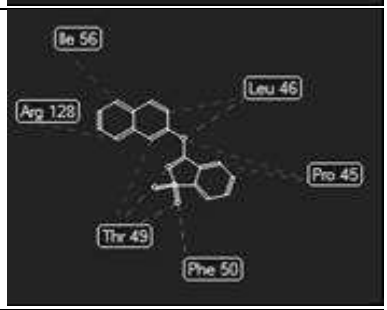
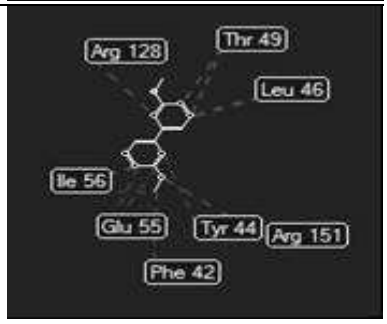
Docking studies

Molegro Virtual Docker uses a molecular docking algorithm called as MolDock which has higher docking accuracy than well known programs (GOLD, Surflex, FlexX and Glide). The scoring function, *E*score, is defined by the summation of *E*inter (ligand-protein interaction energy) and *E*intra (internal energy of the ligand) (Thomsen and Christensen 2006). Thus docking method use an energy-based scoring function, lower energy scores represent better protein-ligand bindings compared to higher energy values (Thomsen and Christensen 2006). Previous studies showed that the compound 2 (CID 16073813) inhibited the dihydrofolate reductase (DHFR) from *L. major* (K_i: 0.17378) (Booth, et al 1987), compound 1 (CID 53325662) had antiprotozoal activity against *L. donovani* (LC50: 32µM) and antitrypanosomal activity against *T. cruzi* (LC50: 8µM) by inhibiting trypanothione reductase enzyme (Holloway et al. 2009), compound 3 (CID 46912206) and 4 **(**CID 790223) had an inhibition on *L. mexicana* Pyruvate Kinase and Compound 5 (CID 2865728) had antitrypanosomal activity against bloodstream form of *T. brucei* (EC50: 5.7 µM) (Nhu et al. 2010), antimalarial activity against erythrocytic stage of *Plasmodium falciparum* 3D7 (EC50: 0.18 µM) (Nhu et al. 2010) and antiprotozoal activity against *L. donovani* MHOM/ET/67/L82 amastigotes (IC50: 8µM) (Holloway et al. 2009). According to docking results, all biological active compounds had interaction with the active site residues (Cys52, Arg128 and Glu55) (Hirotsu et al. 1999, Alphey et al. 2000, Schröder et al. 2000). Except compound 5, others had nearly same MolDock scores. Compound 2 had the highest MolDock score which was -118.675 kcal/mol. However there was no H bond interaction with catalytic residues. Compound **5** had the highest H bond score (-11.128 kcal/mol) and H bond interaction with important catalytic residue Arg128. Compound 3 and 5 had H bond interaction with Arg128 too (Table 2).

CONCLUSIONS

In *Leishmania* species, reductions of hyperoxidases are provided directly by the tryparedoxin peroxidase activities. Because of parasites are sensitive against to the oxidative stress, enzymes involved in this pathway are attractive targets for drugs. In the absence of experimental structure, *in silico* homology modeling of the proteins provides a fast and cost-effective method in the structural based drug discovery process. In this study, a homology model of tryparedoxin peroxidase from *L. donovani* (*Ld*TXNPx) was determined based on the template by the closely related trypanasomatid, *C. fasciculata*. When compared with the secondary and tertiary structure of the model, it showed a typical thioredoxin fold containing a central β–sheet (β7, β6, β3, β4) and three α helices (α1, α4 and α3). Docking study was indicated that the compound 2 had the highest docking score and could provide a scaffold for further drug design works.

Table 2 – Docking results of the selected compounds.

Compound No	Compound ID	MolDock Score (kcal/mol)	HBond Score (kcal/mol)	Target Residues	Dock View
1	53325662	-118.661	-5.358	Leu46[1] Thr49[1] Phe48[2] Cys52[2] Arg151[2] Tyr44[2] Pro45[2]	
2	16073813	-118.675	-6.233	Leu46[1] Thr49[1] Pro45[2] Cys52[2] Arg128[2] Glu55[2]	
3	46912206	-116.018	-9.168	Gly150[1] Arg128[1] Thr49[1] Glu55[2] Cys52[2] Pro148[2] Leu46[2] Tyr44[2]	
4	790223	-117.541	-10.000	Leu46[1] Thr49[1] Phe50[1] Pro45[2] Arg128[2] Thr49[2] Ile56[2]	
5	2865728	-97.929	-11.128	Leu46[1] Arg128[1] Thr49[1] Ile56[1] Arg151[1] Tyr44[2] Phe42[2] Glu55[2]	

[1]H bond interactions, [2]Steric interactions

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